**UNESP - Universidade Estadual Paulista**

**"Júlio de Mesquita Filho"**

**Faculdade de Odontologia de Araraquara**

**Maria Luísa de Alencar e Silva Leite**

# Síntese e avaliação de biomateriais associados a proteínas da matriz extracelular para a regeneração do tecido pulpar

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Tese apresentada à Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara, para obtenção do título de Doutor em Reabilitação Oral na Área de Prótese

**Orientador:** Prof. Dr. Carlos Alberto de Souza Costa

**Coorientadora:** Prof. Dra. Diana Gabriela Soares dos Passos

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**Maria Luísa de Alencar e Silva Leite**

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**RESUMO**

A regeneração tecidual guiada tem sido considerada uma estratégia promissora para substituir terapias endodônticas convencionais em casos de dentes com rizogênese incompleta. Portanto, no presente estudo, proteínas da matriz extracelular (fibronectina, laminina e colágeno tipo I) foram avaliadas a fim de selecionar um potente agente de sinalização sobre células da papila apical humana (hAPCs). Em seguida, com o objetivo de desenvolver biomateriais inovadores para regeneração pulpar de dentes com rizogênese incompleta, um scaffold tubular de nanofibras e um hidrogel de preenchimento foram sintetizados e associados à proteína da matriz extracelular com maior potencial bioativo. Assim, foi possível avaliar a influência dos biomateriais sobre a migração, adesão, proliferação e síntese de colágeno pelas hAPCs. No Artigo 1, diferentes concentrações (1, 5 e 10 µg/mL) de fibronectina (FN), laminina (LM) e colágeno tipo I (COL) foram aplicadas no fundo de compartimentos não tratados de placas de 96 compartimentos esterilizadas. Como controles negativo (CN) e positivo (CP), compartimentos não tratados e pré-tratados, respectivamente, foram usados. Após semear as hAPCs nos diferentes substratos, os seguintes parâmetros de bioatividade foram avaliados: adesão, viabilidade, espalhamento, síntese de colágeno total/colágeno tipo I e expressão gênica (ITGA5, ITGAV, COL1A1, COL3A1). Maior potencial de adesão foi observado para todas as concentrações de FN, sendo o efeito dose-dependente. Os melhores resultados de viabilidade e espalhamento celular, bem como síntese de colágeno ocorreram quando 5 e 10 µg/mL de FN foi utilizado. FN na concentração de 10 µg/mL aumentou a expressão de ITGA5, ITGAV e COL1A1 em comparação ao CP. Embora maiores valores de bioatividade tenham sido demostrados pela LM (5 e 10 µg/mL) em comparação ao CN, essas concentrações de LM exibiram menores efeitos bioativos em relação ao CP. As concentrações de COL avaliadas não mostraram bioatividade sobre as células em cultura. Portanto, concluiu-se que FN nas concentrações de 5 e 10 µg/mL exerceu efeitos bioativos mais intensos sobre as hAPCs. Então, no Artigo 2, a técnica de electrospinning foi empregada para obter, por meio do uso de poli-caprolactona (PCL), scaffolds de nanofibras aleatórias (NR) e alinhadas (NA), os quais tiveram suas propriedades biológicas avaliadas. As melhores formulações de NR e NA foram associadas a 0, 5 ou 10 µg/mL de FN sendo a bioatividade determinada. Finalmente, scaffolds tubulares de NR e NA revestidos com FN foram desenvolvidos e seu potencial quimiotático foi analisado usando um modelo in vitro para simular a regeneração pulpar de dentes com rizogênese incompleta. Foi demonstrado que todos os scaffolds testados são citocompatíveis. No entanto, NR e NA à base de 10% de PCL promoveram maior proliferação, adesão e espalhamento de hAPCs. Células poligonais e alongadas

foram observadas em NR e NA, respectivamente. Quanto maior a concentração de FN nos scaffolds, maior a migração, viabilidade, proliferação, adesão e espalhamento celular, bem como a síntese de colágeno e expressão gênica (ITGA5, ITGAV, COL1A1, COL3A1). Além disso, os scaffolds tubulares de NA associados com FN (10 µg/mL) mostraram maior potencial quimiotático sobre as APCs. Então, concluiu-se que os scaffolds tubulares de NA revestidos com FN são biomateriais promissores, os quais talvez possam ser usados, no futuro, para promover regeneração pulpar mediada por hAPCs em casos de dentes com rizogênese incompleta. No Artigo 3, hidrogéis com variada proporção de colágeno e gelatina (Col/Gel; v/v) foram inicialmente preparados e avaliados de acordo com os seguintes grupos: Colágeno (controle positivo); Col/Gel 4:6; Col/Gel 6:4; Col/Gel 8:2. A viabilidade, adesão/espalhamento das hAPCs semeadas nos hidrogéis foi determinada. Diferentes concentrações de FN (0, 5 ou 10 µg / mL) foram incorporadas à melhor formulação do hidrogel de colágeno/gelatina selecionada. Em seguida, as hAPCs semeadas nos biomateriais foram analisadas quanto a migração, viabilidade, adesão/espalhamento e expressão gênica de ITGA5, ITGAV, COL1A1 e COL3A1. As células do grupo Col/Gel 8:2 exibiram a maior viabilidade e adesão/espalhamento, semelhante ao observado no grupo controle. Ainda, as hDPCs exibiram maior migração, viabilidade, adesão/espalhamento e expressão gênica de marcadores envolvidos na regeneração pulpar quanto maior a concentração de FN incorporada ao hidrogel de colágeno/gelatina. Assim, concluiu-se que um hidrogel de colágeno/gelatina com 10 µg/mL de FN apresentou potente efeito bioativo e quimiotático sobre hAPCs em cultura.

**Palavras – chave:** Proteínas da matriz extracelular. Nanofibras. Hidrogéis.

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## ABSTRACT

Guided tissue regeneration has been considered a promising strategy to replace conventional endodontic therapy of teeth with incomplete root formation. Therefore, in the present study extracellular matrix proteins (fibronectin, laminin and type I collagen) were assessed to select a potent signaling agent on human apical papilla cells (hAPCs). Next, to develop interesting biomaterials for pulp regeneration of teeth with incomplete root formation, a tubular nanofiber scaffold and a filling hydrogel were synthesized and associated with the most bioactive extracellular matrix protein previously selected. The biomaterials were evaluated concerning their influence on migration, adhesion, proliferation and collagen synthesis by hAPCs. Then, in Paper 1, different concentrations (1, 5, and 10 µg/mL) of fibronectin (FN), laminin (LM), and type I collagen (COL) were applied to the bottom of non-treated wells of sterilized 96-well plates. As negative (NC) and positive (PC) controls, non-treated and pre-treated wells, respectively, were used. After seeding the hAPCs on the different substrates, the following bioactivity parameters were assessed: adhesion, viability, spreading, total collagen/type I collagen synthesis and gene expression of ITGA5, ITGAV, COL1A1, and COL3A1. The greatest cells attachment occurred for all concentrations of FN, and in a dose-dependent way. The highest cell viability, spreading and collagen synthesis was observed when 5 and 10 µg/mL of FN were used. FN at 10 µg/mL concentration increased the ITGA5, ITGAV, and COL1A1 expression in comparison with PC. Although the higher bioactivity values shown by LM at 5 and 10 µg/mL in comparison with NC, those concentrations of LM exhibited lower bioactive effects than PC. All concentrations of COL showed no bioactivity on cultured cells. Therefore, it was concluded that 5 and 10 µg/mL FN exerted the most intense bioactive effects on hAPCs. In Paper 2, random (NR) and aligned (NA) nanofiber scaffolds of poly-caprolactone (PCL) were obtained by electrospinning technique and their biological properties assessed. The best formulations of NR and NA were loaded with 0, 5 or 10 µg/mL of FN and their bioactivity evaluated. Finally, FN-loaded NR and NA tubular scaffolds were prepared, and their chemotactic potential was analyzed using an in vitro model to mimic the pulp regeneration of teeth with incomplete root formation. All scaffolds tested were cytocompatible. However, hDPCs seeded on 10% PCL-based NR and NA scaffolds presented the highest proliferation, adhesion and spreading. Polygonal and elongated cells were observed on NR and NA scaffolds, respectively. The higher the concentration of FN added to the scaffolds, greater the migration, viability, proliferation, adhesion/spreading, as well as collagen synthesis and gene expression (ITGA5, ITGAV, COL1A1, COL3A1) by hDPCs. In addition, tubular scaffolds with NA loaded with FN (10 µg/mL) showed the highest chemotactic potential on hAPCs. Then, it was concluded that FN-loaded NA scaffold

is an interesting biomaterial that may be used in a near future to promote hAPCs-mediated pulp regeneration of endodontically compromised teeth with incomplete root formation. In Paper 3, diverse concentrations of collagen and gelatin (Col/Gel; v/v) were used to prepare hydrogels, which were assessed according to the established groups: Collagen (positive control); Col/Gel 4:6; Col/Gel 6:4; Col/Gel 8:2. The viability, adhesion and spreading of cells seeded on the hydrogels were evaluated. Concentrations of 0, 5 or 10 µg/mL FN were incorporated into the best formulation of the collagen/gelatin hydrogel selected. Then, the hAPCs seeded on the biomaterials were assessed concerning their migration, viability, adhesion/spreading, and gene expression of ITGA5, ITGAV, COL1A1 and COL3A1. In Col/Gel 8:2 group, cells exhibited greater viability, adhesion and spreading in comparison with control. Higher values of hAPCs migration, viability, adhesion/spreading and gene expression of pulp regeneration markers were found, the higher the concentration was of FN incorporated into the collagen/gelatin hydrogel. Thus, one can conclude that the collagen/gelatin hydrogel with 10 µg/mL of FN had potent bioactive and chemiotactic effects on cultured hAPCs.

**Keywords**: Extracellular matrix proteins. Nanofibers. Hydrogels.

# SUMÁRIO

## 1 INTRODUÇÃO

Uma vez rompida a integridade do esmalte/dentina, o complexo dentina-polpa passa a ser exposto à diversas injúrias do meio bucal, que compreendem desde estímulos de origem microbiana, traumática e iatrogênica, até mesmo danos de origem química advindos de materiais dentários e seus componentes[1]. Quando a agressão é de grande intensidade, culminando em pulpite irreversível ou mesmo necrose pulpar, a manutenção do tecido infectado torna-se inviável. Assim, em dentes com rizogênese incompleta tem sido indicado a realização de tratamento endodôntico com intenção de promover a apicificação do dente comprometido. Por meio desse método, todo tecido pulpar é removido através do preparo químico-mecânico, seguido de preenchido do canal radicular com medicação intracanal. Esta medicação deve apresentar potencial de estimular o selamento biológico do ápice radicular por meio da formação de um tecido mineralizado[2]. Dessa forma, a continuidade da formação do ápice (apicigênese) é interrompida, impedindo que o canal radicular exiba morfologia normal e que a raiz apresente comprimento regular após a completa erupção do dente[3,4]. Raízes curtas podem comprometer a estabilidade do elemento dental e a resistência das paredes do canal radicular para suportar os esforços mastigatórios[5,6].

Diante desta problemática, os conceitos da engenharia tecidual têm sido cada vez mais aplicados com o objetivo de manter a vitalidade dos dentes ou estimular o reparo/regeneração de tecidos dentários danificados[3,7-11]. Este campo da ciência se fundamenta em três princípios básicos: 1) no uso de biomateriais que atuem como arcabouço temporário para a adesão, proliferação e diferenciação celular; 2) no estímulo de células a sintetizar e depositar um novo tecido similar ao de origem; e 3)

no emprego de agentes de sinalização com potencial para induzir a migração celular e potencializar a ação do biomaterial[12,13]. Assim, a substituição da terapia mecânica associada a aplicação de materiais cáusticos, a qual tem sido usada por décadas para induzir a apicificação, por estratégias que estimulem a regeneração tecidual, apresenta-se com uma alternativa viável e promissora. Tem sido demonstrado que técnicas biológicas usadas para tratar dentes com rizogênese incompleta podem recuperar as propriedades e características de um dente vital e assegurar a continuidade da formação radicular[3,9]. Para que este evento ocorra, o biomaterial (scaffold) a ser usado no procedimento deve exibir arquitetura similar àquela do microambiente in vivo, além de ter potencial bioativo capaz de acelerar o processo de regeneração tecidual[14,15].

Considerando as técnicas dentro do campo da engenharia tecidual direcionada a regeneração de tecidos lesados, duas estratégias biológicas surgiram como as abordagens mais promissoras: cell approach e cell-free approach[16-19]. A primeira, envolve a implantação de um biomaterial contendo células pré-cultivadas, na área do tecido danificado que se busca regenerar. Dentro deste contexto, além de apresentar estrutura porosa tri-dimensional e propriedades biológicas que permitam a acomodação das células, o biomaterial selecionado deve atuar como matriz extracelular temporária e regular o crescimento celular in vivo. A segunda estratégia (cell-free approach), envolve a utilização de biomateriais associados a potentes agentes de sinalização. Ao serem posicionados junto ao tecido previamente danificado, os biomateriais selecionados devem promover a liberação controlada dos agentes sinalizadores de tal maneira que esses consigam estimular a migração de células residentes em direção ao sítio de interesse, bem como promover a proliferação e diferenciação destas células, favorecendo a formação de

um novo tecido[16-19]. Esta modalidade de terapia parece ser a mais interessante para ser aplicada em casos de dentes com rizogênese incompleta. Isto porque num estudo histopatológico realizado em dentes de ratos com lesão periapical induzida, foi demonstrado que a papila apical se manteve viável mesmo após 90 dias de ter ocorrido a necrose pulpar[20]. Sabe-se que a papila apical é uma rica fonte de células tronco[16]. Assim, biomateriais associados a potentes agentes de sinalização poderiam ser introduzidos nos canais radiculares para induzir a migração de células presentes na papila apical, bem promover a como a adesão, proliferação e diferenciação dessas células ao longo de toda a extensão do canal, permitindo a síntese de um novo tecido no local[3].

Diante dessa perspectiva, Bottino et al.[21] demonstraram que o processo de adesão, proliferação e diferenciação de células-tronco mesenquimais é intensificado quando scaffolds com topografia de nanofibras são empregados em comparação com superfícies lisas. Os autores relataram que scaffolds de nanofibras podem ser obtidos pela técnica de electrospinning, a qual permite produção de um biomaterial que mimetiza a nano-morfologia estrutural e funcional da matriz extracelular. Por meio dessa técnica de electrospinning, nanofibras interconectadas com diâmetros variados e elevada área de superfície podem ser produzidas para favorecer a adesão celular, bem como a síntese de novo tecido semelhante ao de origem[12]. Essa técnica também permite obter nanofibras com disposição aleatória ou paralela, o que pode influenciar o potencial de migração e metabolismo celular[22-24]. Baseado nesses dados científicos prévios, foi possível estabelecer a hipótese de que a disposição paralela das nanofibras no biomaterial poderia favorecer e guiar a migração das células da papila apical para o interior do canal radicular, favorecendo a regeneração tecidual.

Por outro lado, diversos pesquisadores têm considerado os hidrogéis como opção interessante para uso na engenharia tecidual, particularmente devido ao seu fácil manuseio, bem como a importante capacidade deste produto se adaptar em sítios com anatomia irregular e consequentemente de difícil acesso[25-26]. A maioria dos hidrogéis apresenta característica muito similar à matriz extracelular do tecido conjuntivo, tais como alta umidade e propriedades reológicas que lhe confere plasticidade[27-29]. Uma vez aplicado sobre a loja cirúrgica, o hidrogel atua como material de preenchimento biocompatível. Então, ele é gradualmente degradado à medida que é substituído pelo novo tecido semelhante ao de origem, permitindo assim que o processo de regeneração ocorra no local[3,29].

A associação de biomateriais com agentes de sinalização tem sido demonstrada para induzir a regeneração tecidual guiada da polpa dental[19]. Dentre as substâncias que apresentam propriedades bioativas, diversas proteínas da matriz extracelular (PMEs), tais como fibronectina, laminina e colágeno I, têm sido propostas como elementos quimiotáticos e indutores, visto que participam de processos de reparação e remodelação tecidual[30,31]. Também já foi demonstrado que estas proteínas participam do processo de adesão e espalhamento celular[32-34], bem como podem induzir a migração, proliferação e diferenciação celular[31,35,36]. Um estudo recente, Bullard et al.[37] caracterizaram a composição do cordão umbilical humano liofilizado e avaliaram seus efeitos biológicos in vitro e in vivo. Os autores observaram que o cordão umbilical humano contém colágeno tipo I, ácido hialurônico, fibronectina e laminina, bem como fatores de crescimento, moduladores inflamatórios e outros reguladores de sinalização celular. Aumento da migração de fibroblastos e da proliferação de células indiferenciadas de origem mesenquimal e do tecido adiposo, associado a indução do potencial angiogênico, ocorreu quando as

células foram expostas a este biomaterial liofilizado. Esses efeitos in vitro foram concentração-dependente. Bullard et al.[37] também demonstraram que quando implantado em tecido conjuntivo subcutâneo de ratos, o biomaterial preparado com cordão umbilical liofilizado mostrou-se biocompatível e biodegradável.

A fibronectina tem se mostrado um potente agente de sinalização, capaz de mediar a adesão, migração, proliferação e diferenciação celular, apresentando assim, papel importante nos processos de formação, remodelação, reparação e regeneração tecidual[38-40]. A laminina tem sido descrita como uma macromolécula extracelular complexa que regula a migração, adesão e proliferação celular[41,42]. Especialmente na reepitelização e angiogênese, a laminina tem se mostrado essencial para manter as funções celulares[41]. O colágeno, por sua vez, é uma proteína abundante na matriz extracelular que caracteriza o microambiente localizado entre as células de todo o organismo. Sabe-se que mais de 90% do colágeno encontrado nos tecidos é do tipo I[39]. Esta proteína, a qual tem sido utilizada para mimetizar o microambiente in vivo, também tem potencial para promover a proliferação e diferenciação de células tronco[39,43].

Assim, com base nos importantes resultados de estudos disponibilizados na literatura e diante das limitações encontradas no tratamento convencional de dentes com rizogênese incompleta, torna-se necessário o desenvolvimento de promissores biomateriais associados a um potente agente de sinalização, de modo que estes possam atuar na terapia regenerativa endodôntica guiada por meio da indução de células da papila apical humana (hAPCs).

# 2 PROPOSIÇÃO

## 2.1 Objetivo Geral

O objetivo geral deste estudo foi desenvolver um scaffold tubular de nanofibras e um hidrogel de preenchimento, ambos contendo dosagens bioativas e quimiotáticas de uma PME, com capacidade de atuar positivamente em diversas funções e atividades de células da papila apical relacionadas com o processo de regeneração pulpar.

## 2.2 Objetivos Específicos

1. Avaliar o potencial bioativo de diferentes concentrações de PMEs (fibronectina, laminina e colágeno tipo I) sobre hAPCs, de acordo com os parâmetros de viabilidade, proliferação, adesão e espalhamento celular, bem como síntese de colágeno e expressão de genes relacionados à regeneração pulpar.
2. Desenvolver diferentes scaffolds a base de poli-caprolactona com nanofibras em disposição randômica (NR) e alinhada (NA) e selecionar as melhores formulações de acordo com a topografia de superfície e potencial na indução da proliferação, adesão e espalhamento de células hAPCS. A seguir, associar as melhores formulações de NR e NA a diferentes concentrações da PME com melhor potencial bioativo para avaliar seu potencial na migração, viabilidade, proliferação, adesão e espalhamento celular, bem como síntese de colágeno e expressão de genes relacionados à regeneração pulpar. Por fim, desenvolver scaffolds tubulares de NR e NA revestidos com a PME selecionada e analisar o

potencial quimiotático utilizando um modelo in vitro para simular a regeneração pulpar de dentes com rizogênese incompleta.

3. Desenvolver diferentes formulações de hidrogéis à base de colágeno/gelatina a fim de selecionar aquela com maior potencial para induzir a viabilidade, proliferação, adesão e espalhamento de células hAPCS. A seguir, associar a melhor formulação do hidrogel à PME com melhor potencial bioativo e avaliar seu potencial na migração, viabilidade, proliferação, adesão e espalhamento celular, bem como expressão de genes relacionados à regeneração pulpar.

# 3 PUBLICAÇÕES

## 3.1 Publicação 1*

### Bioactive effects of extracellular matrix proteins on apical papilla cells

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**Data Sharing and Data Accessibility**
The data that support the findings of this study are openly available in Mendeley Data at http://dx.doi.org/10.17632/knzz9hctcd.1.

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# Bioactive effects of extracellular matrix proteins on apical papilla cells


## Abstract

The use of a potent signaling agent is crucial to stimulate and guide the pulp tissue regeneration, especially in endodontic treatment of teeth with incomplete root formation. Objective: This study evaluated the bioactive properties of low concentrations of extracellular matrix proteins on human apical papilla cells (hAPCs). Methodology: Different concentrations (1, 5, and 10 µg/mL) of fibronectin (FN), laminin (LM), and type I collagen (COL) were applied to the bottom of non-treated wells of sterilized 96-well plates. As negative (NC) and positive (PC) controls, non-treated and pre-treated wells, respectively, were used. After seeding the hAPCs ($5x10^3$ cells/well) on the different substrates, the following bioactivity parameters were assessed: adhesion, proliferation, spreading, total collagen/type I collagen synthesis and gene expression (ITGA5, ITGAV, COL1A1, COL3A1) (ANOVA/Tukey; $\alpha=0.05$). Results: The greatest attachment potential was observed for all concentrations of FN, with the effect being dose-dependent. The highest cell proliferation, spreading and collagen synthesis values were observed when 5 and 10 µg/mL of FN were used. FN at 10 µg/mL concentration increased the ITGA5, ITGAV, and COL1A1 expression compared with PC. Although the higher bioactivity values shown by LM (5 and 10 µg/mL) in comparison with NC, these concentrations of LM exhibited lower bioactive effects than PC. All concentrations of COL showed no bioactivity on cultured hAPCs. Conclusion: It was concluded that FN at 10 µg/mL concentration exerted the most intense bioactive effects on hAPCs.


**Keywords**: Guided Tissue Regeneration; Dental pulp; Fibronectin; Laminin; Collagen.

## Introduction

During the last decade, the concepts of tissue engineering were introduced in Dentistry and since then special attention has been given to induction of pulp regeneration.[1-4] The following four factors has been considered as the basic principles of regenerative endodontics: 1. desinfection and detoxification of root canals; 2. use of a biomaterial that will act as a temporary scaffold for cell adhesion, proliferation, and differentiation; 3. presence of cells with potential to allow the synthesis of a new tissue similar to the original type; and 4. use of a signaling agents capable of inducing cells migration and enhancing the bioactive action of the biomaterial.[5,6]

In this context, two strategies have emerged as the most promising therapies for tissue regeneration: cell approaches and cell-free approaches.[7,8] The first involves implanting pre-cultured cells associated with a biomaterial at the site of injury. Thus, for tissue regeneration to occur, bioactive materials must provide a porous three-dimensional structure to accommodate cells, acting as a temporary extracellular matrix and regulating cell growth in vivo. The cell-free approaches involve the use of biomaterials associated with potent signaling agents. When introduced into the injury site, these agents release bioactive molecules to stimulate the migration of resident cells towards the site of interest, as well as their proliferation and differentiation, thereby favoring tissue healing.[7,8]

The cell-free therapy approach seems to be interesting for cases of teeth with incomplete root formation. A histopathological study using rat teeth with periapical lesion has demonstrated that a viable apical papilla was maintained even after 90 days of pulp necrosis.[9] The apical papilla is known to have a rich source of stem cells. Therefore, biomaterials associated with potent signaling agents could be used to attract these cells into the root canal. These biomaterials should also promote adhesion, proliferation and differentiation of the stem cells along the entire length of the root canal, allowing the synthesis of a new pulp-like tissue.[1]

Among several substances with bioactive properties, extracellular matrix proteins (ECMp), such as fibronectin (FN), laminin (LM) and type I collagen (COL), have been proposed as chemotactic and inducing agents, since the bioactive property of these proteins has previously been described in several biomedical fields.[10-15] Previous studies have shown that FN, either associated with other proteins, or not, was a potent signaling agent capable of mediating cell adhesion,

migration, proliferation, and differentiation; in addition to being involved in tissue formation, remodeling, repair and regeneration.[11,13,15] LM has been described as a complex extracellular macromolecule that also regulates cell migration, adhesion and proliferation.[10,12] Especially in the processes of reepithelization and angiogenesis, LM is essential for maintaining cellular functions.[10] COL is an abundant protein found in the extracellular matrix that surround cells throughout the body, with over 90% of this protein being type I. COL has been shown to be an excellent source for mimicking the microenvironment in vivo, and under specific conditions, it has proved to be effective in promoting stem cell differentiation and growth.[13,16]

One study characterized the dehydrated human umbilical cord (DHUc) in terms of tissue composition and evaluated its in vitro and in vivo effects.[14] The authors observed that COL, hyaluronic acid, FN and LM, among other proteins, which included growth factors, inflammatory modulators and cell signaling regulators, were present in the DHUc. Increased migration of fibroblasts, and higher level of mesenchymal stem cell proliferation, associated with the induction of angiogenic potential was observed in vitro in a concentration-dependent way when the cells were treated with DHUc. When implanted into subcutaneous tissue of rats, this biomaterial proved to be biocompatible and biodegradable.[14] Researchers have reported that decellularized human dental pulp, which maintains several proteins proper of this specialized connective tissue, like COL, FN and LN, is a suitable scaffold to mimic the complexity of the dental pulp extracellular matrix.[17,18] Decellularized biological scaffolds allow the attachment and proliferation of stem cells from apical papilla[17] as well as human dental pulp stem cells[18] that are also stimulated to differentiate into odontoblast-like cells close to the dentin substrate.[18]

Despite the extracellular matrix proteins play a fundamental role on pulp regeneration and formation of a new pulp-like tissue, only scarce data have been provided concerning the influence of FN, LN and COL on the metabolism and function of human apical papilla cells (hAPCs), especially when young teeth with incomplete root formation have lost their vitality. Thus, the effects of FN, LM and COL on the adhesion, proliferation and spreading of hAPCs as well as on the potential of these cells to synthesize collagen and express genes related with pulp regeneration were assessed in the present in vitro study.

**Methodology**

Firstly, approval of the research was obtained from the Research Ethics Committee of Araraquara School of Dentistry/ UNESP, São Paulo, Brazil (protocol Nº. 80806617.3.0000.5416) as well as signed terms of informed consent from patients and their guardians, according to the Declaration of Helsinki. Subsequently the hAPCs were obtained from the apical papilla of four healthy third molars with incomplete root formation. These teeth were provided by volunteers aged 16 to 18 years old, of both sexes, who were patients attended at the surgery clinic. The teeth were extracted by orthodontic reasons. To obtain the primary culture of hAPCs, the extracted teeth were immediately immersed in Minimum Essential Medium Eagle Apha-culture medium (α-MEM; GIBCO, Carlsbad, CA, USA) supplemented with antibiotic and antifungal (100 U/mL penicillin, 100 g/mL streptomycin, and 0.25 g/mL amphotericin; GIBCO) at 4ºC, for 1 hour. Then, in a procedure performed in a biosafety cabinet (Bio Protector 12 Plus; VECO, Campinas, SP, Brazil), the papilla was sectioned with a sterile scalpel blade, at the apical limit of the root. This tissue was transferred to a 1.5 mL tube containing phosphate buffered saline (PBS 1X; GIBCO), followed by mechanical disintegration with sterile surgical scissors. Then, the apical papilla fragments were incubated in α-MEM containing 3 mg/mL type I collagenase (Sigma-Aldrich, Saint Louis, MO, USA) at 37°C and 5% $CO_2$ for 2 h. After this period, the cells were centrifuged and washed with PBS 1X, seeded in wells of a 6-well plate (Corning, Tewksbury, MA, USA), using α-MEM supplemented with 10% fetal bovine serum - FBS, 100 U/mL penicillin, 100 g/mL streptomycin, and 0.25 g/mL amphotericin; GIBCO) for this purpose. After 3 h incubation, the supernatant was discarded and the cells that remained attached to the bottom of the wells were sub-cultured in 100x15 mm petri dishes (Corning). Cells at passage 3 to 5 were used in the experiments.

Fibronectin (2.0 mg/mL, bovine plasma; Sigma-Aldrich), laminin (1 mg/mL, Engelbreth-Holm-Swarm lathrytic mouse tumor; Santa Cruz Biotechnology), and type I collagen (3.67 mg/mL, rat trial; Corning, Bedford, MA, USA) were used in this study. These ECMps were diluted in PBS 1X to obtain concentrations of 1, 5 and 10 µg/mL that were applied (50 µL) to the bottom of wells without pretreatment of sterilized polystyrene 96-well plates (Corning; Product Number: 3370). The 96-well plates were then centrifuged at 1500 rpm at 4ºC for 10 min. After keeping the plates at 4ºC for 18

h, the remaining material was aspirated. These plates were incubated with 0.5% bovine serum albumin (BSA; Santa Cruz Biotecnology) for 10 min to block the uncoated surface,[19,20] followed by washing with PBS 1X at 37ºC. All these steps were performed for both negative (NC) and positive (PC) control groups. In the NC, PBS solution without ECMp was applied to the bottom of non-pretreated wells of sterilized polystyrene 96-well plates (Corning; Product Number: 3370). In the PC, APBS without ECMp solution was applied to the bottom of wells of polystyrene 96-well plates that were pre-treated by the manufacturer for cell culture purpose (Corning; Product Number: 3395). Table 1 shows all groups established according to the polystyrene 96-well plates used, as well as the type and concentration of ECMp.

After performing the treatments of wells with ECMp, the hAPCs ($5x10^3$ cells/well) were seeded in the respective wells and incubated at 37ºC and 5% $CO_2$ for up to 5 days, with the culture medium being changed every 48 h. Then, the bioactivity parameters were evaluated. All assays were performed twice to ensure the data reproducibility.

Cell adhesion: Since the non-treated 96-well plates did not allow cell attachment on the bottom of the wells, the attachment potential of the microplate treated with different concentrations of ECMp was assessed. For this purpose, protocols of dynamic systems, in which microchannels are treated with proteins to induce cell chemotaxis,[19-21] were adapted to this study. After 24 h of applying cell suspensions to the plate wells (n=6), the hAPCs capable of migrating and attaching to the bottom of wells were evaluated under a light microscope. For this purpose, the cells were fixed with 2.5% glutaraldehyde for 2 h, washed with distilled water and stained with 0.1% crystal violet for 15 min. For quantitative analysis, photographs (1 field of the central region of each well of the microplate; 10x magnification) were obtained using a camera (Olympus C5060, Miami, FL, USA) coupled to a microscope (Olympus BX51, Miami, FL, USA). The images were evaluated using Image J 1.45S software (Wayne Rasband, National Institutes of Health, USA) for cell counting.

Cell proliferation: This analysis was performed by the MTT assay (n=6). After time intervals of 1, 3 and 5 days in complete $\alpha$-MEM, cells were incubated for 4 h in α-MEM supplemented with 10% MTT solution (5 mg/mL; Sigma-Aldrich).[22] Then, the formazan crystals produced were dissolved in acidified isopraponol and absorbance of the resulting solution was measured at 570 nm (Synergy H1, Biotek, Winooski, VT, USA). The average absorbance value obtained in the negative control group over the

1-day period was considered 100% cell proliferation. This parameter was used to determine the percentage of viability for the other groups.

Cell spreading: For this analysis (n=4), after time intervals of 1, 3 and 5 days in complete α-MEM, the cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) and incubated with Actin Red 555 probe (Life Technologies; Grand Island, NY, USA) in 2% BSA (1:20) to detect the actin filaments. After washing the cells with PBS, they were incubated with Hoescht (1:5000; Invitrogen, Carlsbad, CA, USA) for 15 min for nuclear counter-staining.[23] The F-actin assay was then analyzed using a fluorescence microscope (Leica DM 5500B).

Total collagen synthesis: The cells were cultured for 5 days in α-MEM without FBS, and the culture medium was collected and replaced every 48 h. The pool of collected culture medium of each sample was stored at -20ºC until the Sirius Red assay was performed (n=6). For this purpose, the culture was transferred to 1.5 mL tubes containing Direct Red solution in saturated picric acid (0.1%), and then incubated for 1 hour, under agitation at 400 rpm, in a dry bath at 25°C. The tubes were centrifuged, the supernatant was discarded, and 0.01 M hydrochloric acid was added. The tubes were centrifuged again, the supernatant was discarded, followed by the addition of 0.5 M sodium hydroxide to solubilize the precipitated material.[24] The resulting solution from each sample was subjected to absorbance analysis in a spectrophotometer at 555 nm (Synergy H1).[24] The percentage of total collagen synthesis for each sample was calculated based on the mean absorbance values of the negative control group.

Type I collagen synthesis: The medium pool collected in the previous analysis (n=6) was also used to evaluate the type I collagen synthesis (COL-I), which was detected by enzyme-linked immunosorbent (ELISA) assay using a standardized kit (Duoset human COL-I - R&D Systems, Minneapolis, MN, USA), according to manufacturer's instructions. For this purpose, 96-well plates for ELISA (Corning) were treated with 4 µg/mL of Capture Antibody and incubated overnight. After this period, the wells were incubated with Reagent Diluent 1X for 1 hour. Then, standardized aliquots of the samples and standard curve points were applied and then incubated for 2 h. After this, the wells were maintained with 100 ng/mL Detection Solution for 2 h, followed by incubation in dark room, with 2.5% Streptavidin-HRP, for 20 min. The wells were washed with Wash Solution 1X between the all steps described. Finally, Substrate Solution (color reagent A + color regent B; 1:1) was

applied and after 20 minutes, the Stop Solution was added. The absorbance reading was performed at a wavelength of 450 nm in a spectrophotometer (Synergy H1). The concentration of type I collagen for each sample was determined according to the standard curve. The percentage of type I collagen synthesis for each sample was calculated based on the average concentration values, in ng/mL of the positive control group.

Reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR): After incubating the cells in complete $\alpha$-MEM for 5 days (n=4), the RNA was isolated using the RNAqueous kit (Ambion, Grand Island, NY, USA) in accordance with the manufacturer's instructions. To obtain sufficient RNA for the reaction, a pool of cells obtained from two wells was used for each group. Then, the cDNA synthesis (n=4) was determined by using 500 ng of total RNA associated with random hexamer primers and Moloney leukemia virus reverse transcriptase, according to the instructions of the High Capacity RT kit supplier (Applied Biosystems, Foster City, CA, USA). The gene expression of ITGA5, ITGAV, COL1A1 and COL3A1 (Table 2) by qPCR was performed, using pre-designed sets of primers and probes (Gene expression assays, Applied Biosystems) to detect target genes using the TaqMan system (TaqMan Universal PCR Master Mix, Applied Biosystems) with the StepOne Plus equipment (Applied Biosystems). In addition, the cycling conditions optimized by the manufacturer were used, and the cycle threshold (CT) values for each sample were calculated using the thermal cycler software, and analyzed using the $2^{\Delta\Delta CT}$ method. The results were normalized according to the constitutive gene expression (GAPDH) and expressed as a fold change in relation to the positive control.

The data from cell adhesion, viability, total protein synthesis, type I collagen synthesis, and RT-qPCR assays were evaluated for adherence to the normal curve (Shapiro-Wilk test, $p>0.05$) and homoscedasticity (Levene test, $p>0.05$). Then, the data were submitted to the one-way or two-way ANOVA test, followed by the Tukey post-hoc test by means of SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). All statistical inferences were based on the 5% level of significance. The statistical power of the samples was calculated according to Kim[25] (2016) using the DSS Research calculator, which showed a power level > 95.0% for each analysis. The cell spreading images were analyzed descriptively.

**Results**

Cell adhesion: As demonstrated in Figure 1A and B, the highest attachment potential occurred in Group FN10, followed by Groups FN5, FN1, PC, LM10, and LM5 ($p<0.05$). There was no significant difference between Groups LM1, COL1, COL5, and COL10 in comparison with Group NC ($p>0.05$).

Cell proliferation: As shown in Figure 2, increased cell proliferation occurred over time-points for all groups ($p<0.05$), except for those treated with type I collagen and Group NC ($p>0.05$). The best cell viability result was observed in the groups treated with 5 or 10 µg/mL of FN after time intervals of 1, 3 and 5 days ($p<0.05$). At the last time-point, this increase was 30x higher when compared with Group NC, 1.7x higher than in Group PC, and 2.7x higher than the group treated with 10 µg/mL LM. In addition, the group FN1 showed higher cell viability values than the group LM10 in all time-points ($p<0.05$).

Cell spreading: In Figure 3 it can be observed that cells seeded on the bottom of the wells treated with 5 or 10 µg/mL of FN showed better spreading on the substrate, as well a higher rate of proliferation over the time-points. In the groups treated with COL and in Group NC, lower numbers of cells organized in clusters that exhibited contracted cytoskeletons remained attached to the wells bottom.

Total collagen synthesis: As shown in Figure 4A, increase in the total collagen synthesis occurred in Groups FN10 and FN5, followed by Groups PC and LM10 in comparison with Group NC ($p<0.05$).

Type I collagen synthesis: Figure 4B demonstrates that higher synthesis of type I collagen occurred in Group FN10 ($p<0.05$), followed by Groups FN5 and PC ($p<0.05$), which showed no significant difference between them ($p>0.05$). Groups LM5 and LM10 showed lower values of type I collagen synthesis in comparison with Group PC ($p<0.05$), but values were higher than those observed for Group NC ($p<0.05$).

RT-qPCR: As shown in Figure 5A-D, higher levels of ITGA5, ITGAV, and COL1A1 gene expression occurred in Group FN10 in comparison with Group PC ($p<0.05$). However, there was not significant difference between FN10 and PC groups to COL3A1 gene expression ($p>0.05$). Group FN5 showed no statistical difference when compared with Group PC for all evaluated genes ($p>0.05$). However, Groups LM5 and LM10 demonstrated lower values of gene expression than Group PC ($p<0.05$).

## Discussion

The application of tissue engineering concepts for the purpose of pulp tissue regeneration has been relevant, especially in endodontic treatment of teeth with incomplete root formation. The thin and fragile walls of the root canal, associated with the short length of the root make these teeth susceptible to fracture, in view of the forces to which they are submitted.[7,26] In this context, conservative treatments that aim to stimulate the migration of cells from apical papilla into the root canal and allow the synthesis of a new pulp-like tissue seem to be an interesting alternative that would allow the return of tooth vitality and continuity of root formation. For this purpose, the use of a potent signaling agent is crucial to stimulate and guide tissue regeneration.[5,6] Studies have shown that extracellular matrix proteins in concentrations between 0.5 to 100 µg/mL can act as a bioactive agent on cells from different sources.[27-30] Therefore, in the present study, the biological activities of low concentrations of ECMp (fibronectin, laminin and type I collagen) on hAPCs were assessed.

Type I collagen can regulate the cell behavior by specific signals and trigger several biological activities that are essential for tissue development and homeostasis.[11] However, the different concentrations of COL tested in this investigation showed no bioactive potential on the hAPCs. As observed in the negative control, only a few cells remained attached to the COL over the time-points. These cells, which exhibited cytoplasm contraction, were organized in clusters. Parisi et al.[15] (2020) reported that adhesion not only represents the interaction between cells and the substrate on which they were seeded, but also the interaction and communication among surrounding cells, which activate the subsequent mechanisms of proliferation and differentiation. These authors showed that lack of cell adhesion to the substrate and interaction with surrounding cells resulted in apoptosis.[15] Therefore, according to the data obtained in the present study, and given the relevant role of collagen in different cell functions,[4,11,31-33] we suggest that the concentrations of type I collagen tested on the hAPCs were insufficient to stimulate cell adhesion, proliferation and spreading. As the aim of the present study was to evaluate and compare the bioactive effects of low dosages of ECMp, the same concentrations of FN, LM and COL were evaluated on hAPCs. However, based upon the negative data

obtained for COL, we recommend the assessment of higher concentrations of this type of ECMp on hAPCs in future studies.

Although the cell attachment of LM was lower in comparison with the positive control, a dose-response effect on hAPCs was observed when different concentrations of this protein were used. Thus, greater cell adhesion, proliferation and spreading was determined for 5 and 10 µg/mL of LM in comparison with the negative control. A recent study showed that LM expression in blood vessels regulated the viability and migration of oligodendrocyte precursor cells via β1 integrin-focal adhesion kinase (FAK).[30] In addition, Liu et al.[34] (2017) demonstrated in vivo that 316L stainless steel treated with stromal cell-derived factor-1α (SDF-1α)/laminin had increased biocompatibility and improved the healing of vascular lesions. Enhanced adhesion, migration and proliferation of human limbal epithelial stem cells was also demonstrated when specific isoforms of LM incorporated into a fibrin-based hydrogel were tested.[12] These positive effects of LM have been shown on Schwann cells[33] as well as on hepatocytes and endothelial cells.[11,13] Therefore, the results of the present study, in which LM stimulated the adhesion, proliferation and spreading on hAPCs, corroborate with these data previously reported by several researchers.[11,12,13,33]

It was also shown in the present laboratory study that the bioactive potential of 1, 5 and 10 µg/mL of FN was concentration-dependent and promoted strong adhesion on hAPCs compared with the positive control and other experimental groups. Additionally, concentrations of 5 and 10 µg/mL of FN stimulated the highest proliferation and spreading of hAPCs at all time-points. These data are in line with a previous investigation in which the authors demonstrated that an experimental hydrogel containing FN exerted higher chemotactic effect on NIH 3T3 fibroblasts than the same hydrogel containing LM.[32] The role of FN in inducing cell migration and differentiation on dental pulp cells is already known.[36,37] Chang et al.[38] (2016) evaluated the effect of titanium surfaces treated with glow discharge plasma followed by FN adsorption on MG-63 osteoblast-like cells. The authors observed that the modified titanium surfaces enhanced cell adhesion, migration and proliferation. Matsui et al.[28] (2015) also reported an increased cell adhesion in situ when FN-coated hydroxyapatite was assessed under a defined experimental condition. Amplified cell migration, adhesion, spreading and proliferation occurred when periodontal ligament cells were treated with 5 or 10 µg/mL of fibronectin.[27] More

recently, Parisi et al.[15] (2020) demonstrated that high concentrations of fluid containing FN allowed rapid deposition of this protein on biomaterials, what improved the adhesion and proliferation of cells on them.

Taking into consideration all the interesting data concerning the positive effects of FN and LM on different cell lines and based upon the preliminary results obtained in this in vitro study, the two highest concentrations of FN and LM were selected for the subsequent experiments. In the present investigation, only the use of 10 µg/mL of FN significantly increased the synthesis of total collagen, as well as gene expression and synthesis of type I collagen in comparison with the positive control. Despite the tendency towards increase in the expression of type III collagen, there was no significant difference between FN10 and the positive control. Collagen is known to be an abundant protein in the extracellular matrix of pulp tissue, with type I and type III collagen representing about 56% and 41%, respectively, of the organic content.[31] According to previous studies, the presence of FN in tissue engineering strategies has been described as being an approach to induce collagen synthesis and stimulate tissue regeneration.[33,39] Collagen and other proteins present in the extracellular matrix have interaction sites with different specificities for cell membrane receptors, turning the cells capable of binding and triggering distinct biological activities related to development, homeostasis and formation of a new tissue.[11]

To better understand the mechanism of action of FN and LM on hAPCs, gene expression of α5 integrin (ITGA5) and αv integrin (ITGAV) was also evaluated; these genes are related to cell migration and adhesion.[25,40-42] The interaction of α5β1 and αvβ3 integrins and the role of these membrane receptors in the detection of extra-cellular matrix proteins have been demonstrated.[42] Although these integrins have distinct biological intracellular mechanisms, both act in the detection processes of specific ligands that trigger particular cell signaling related to the projection mechanisms and directional migration of the cytoskeleton. The joint action of integrins allows cells to adapt to the microenvironment and perform their functions in tissue remodeling and regeneration.[45] In the present study, higher gene expression of ITGA5 and ITGAV was observed only for FN10 in comparison with the positive control ($p<0.05$). In a study conducted with a parental fibroblast lineage, Spoerri et al.[43] (2020) observed that when the α5β1 and αVβ3 integrins were activated by PAR1 and PAR3 proteases, via Gβγ and PI3K signaling, they changed their conformation, thereby enhancing their affinity to FN. Thus, the cell adhesion established was

strengthened by means of specific biological signals mediated by PARs via Gα13, Gαi, ROCK and Src. Also, the authors reported that after triggering cell adhesion, the cell migration and proliferation were accelerated.[33] Furthermore, the effect of proteins that contain a functional domain of FN and LM was also observed in a primary culture of human keratinocytes.[44] When these cells were subjected to GTPase inhibitors in the presence of FN and LM-mimic surfaces, the modulation of cell adhesion and migration was dependent on the Rac1 and Rho pathways.[44] Nevertheless, in the presence of the α5β1 integrin receptor, FN-mimic was capable of inducing cell migration and increasing keratinocyte adhesion, whereas under these conditions, LM-mimic was unable to stimulate cell motility.[44] Therefore, we could suggest that the type and concentration of the ECMp influence the cell adhesion and migration, and at least partly explain the strong bioactive potential of 10 µg/mL of FN on the HAPCs observed in the present study.

Overall, the need to replace the traditional endodontic treatment of teeth with incomplete root formation (which aims to induce apexification and later fill the root canal with definitive materials), has led to the hypothesis that associating the bioactive properties of FN with those of an interesting biomaterial can regenerate the lost pulp tissue. Previous studies have shown that the association of extracellular matrix proteins with scaffolds approximated the conditions of the in vivo environment.[13,29,33] Thus, they mediated the mechanical-chemical signals in the processes of adhesion, migration, proliferation and differentiation of various cell types, and in the synthesis of a new matrix.[13,29,33] The results obtained in the present study cannot be directly extrapolated to clinical situations. However, in spite of the limitations of this laboratory investigation, it seems appropriate to conduct further studies to assess techniques using the cell-free approaches proposed by Galler et al.[7] (2020). In these, the root canals may be filled with biomaterials containing dosages of FN fibronectin capable of stimulating the migration of cells from apical papilla and allowing new pulp-like tissue formation.

## Conclusions

According to the methodologies used in the present study, it was concluded that 10 µg/mL of fibronectin could act as a potent bioactive agent for human apical papilla cells by inducing cell adhesion, proliferation, spreading and collagen synthesis.

**Acknowledgements**

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## Tables

**Table 1.** Experimental and control groups according to type of polystyrene 96-well plate and type and concentration of extracellular matrix protein (ECMp).

| Groups | Pre-treated wells | ECMp | Concentration (µg/mL) |
|---|---|---|---|
| NC (negative control) | - | - | 0.0 |
| PC (positive control) | + | - | 0.0 |
| FN1 | - | Fibronectin | 1.0 |
| FN5 | - | Fibronectin | 5.0 |
| FN10 | - | Fibronectin | 10.0 |
| LM1 | - | Laminin | 1.0 |
| LM5 | - | Laminin | 5.0 |
| LM10 | - | Laminin | 10.0 |
| COL1 | - | Type I collagen | 1.0 |
| COL5 | - | Type I collagen | 5.0 |
| COL10 | - | Type I collagen | 10.0 |

**Table 2.** TaqMan assays used to analyze gene expression.

| Gene | Assay ID | Encoded protein |
|---|---|---|
| *COL1A1* | Hs01076756_g1 | Type I collagen |
| *COL3A1* | Hs00943809_m1 | Type III collagen |
| *ITGA5* | Hs01547673_m1 | Integrin α5 |
| *ITGAV* | Hs00233808_m1 | Integrin αV |
| *GAPDH* | Hs02786624_g1 | Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH; constitutive gene) |

## Figures

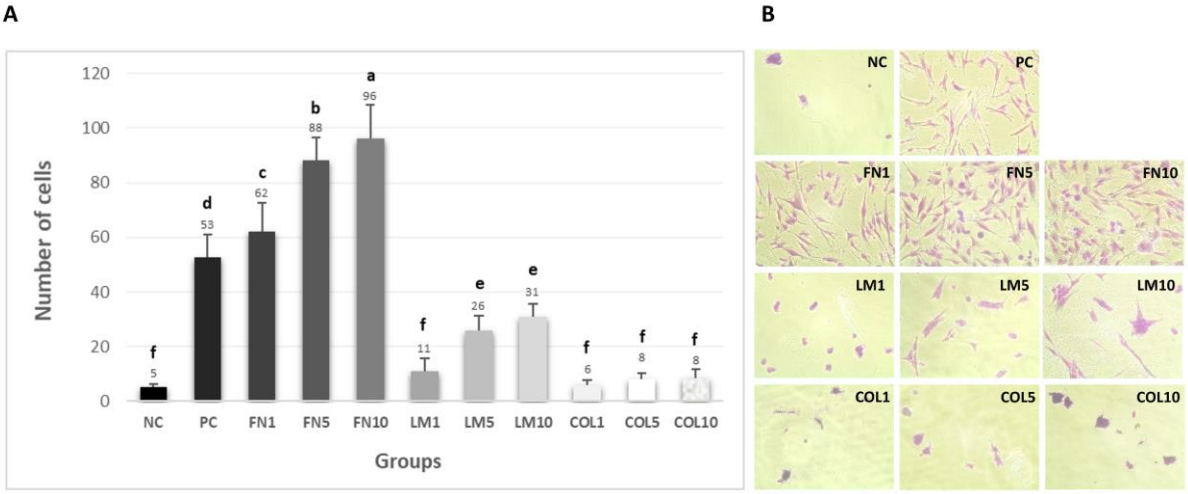


**Figure 1.** Cell adhesion assay. (A) Mean and standard deviation of the number of cells adhered to the bottom plate treated according to the group. Different letters demonstrate a significant difference between groups (n=6; one-way ANOVA/Tukey tests; α=0.05). (B) Representative images of the cell adhesion.

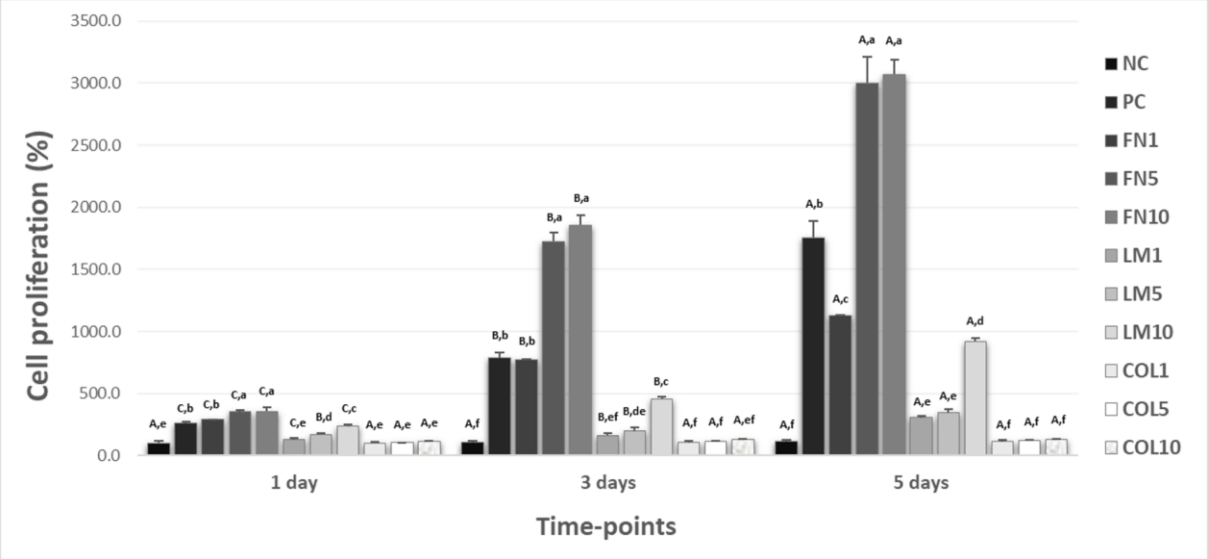


**Figure 2.** Mean and standard deviation of cell proliferation values (n=6; two-way ANOVA/Tukey tests; α=0.05). Different capital letters indicate a significant difference between the time-points for each group. Different lowercase letters demonstrate a significant difference between groups in each time-point.

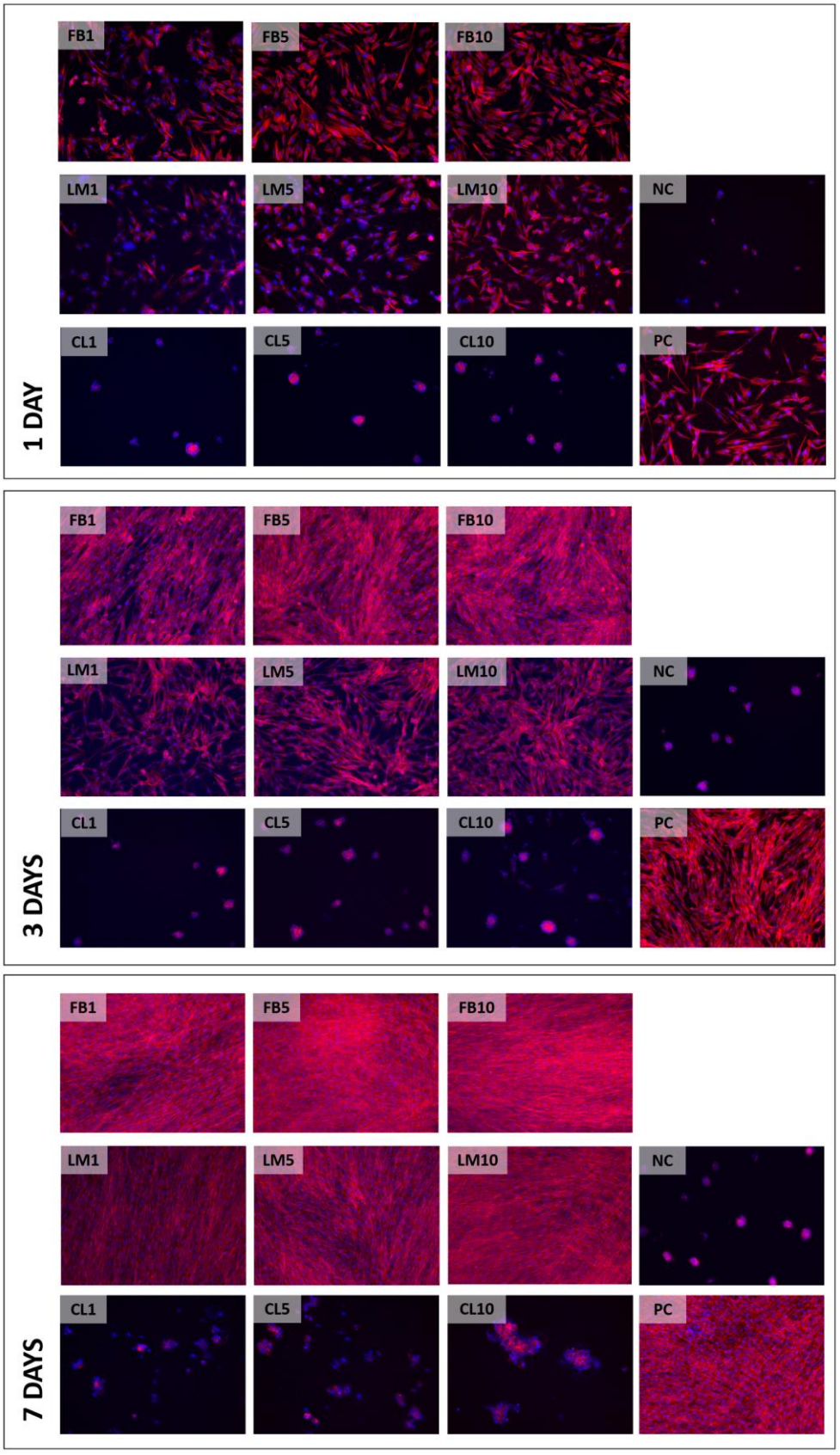


**Figure 3.** Representative images of F-actin assay (10x). Red fluorescence indicates the actin filaments. Blue fluorescence indicates the cell nucleus.

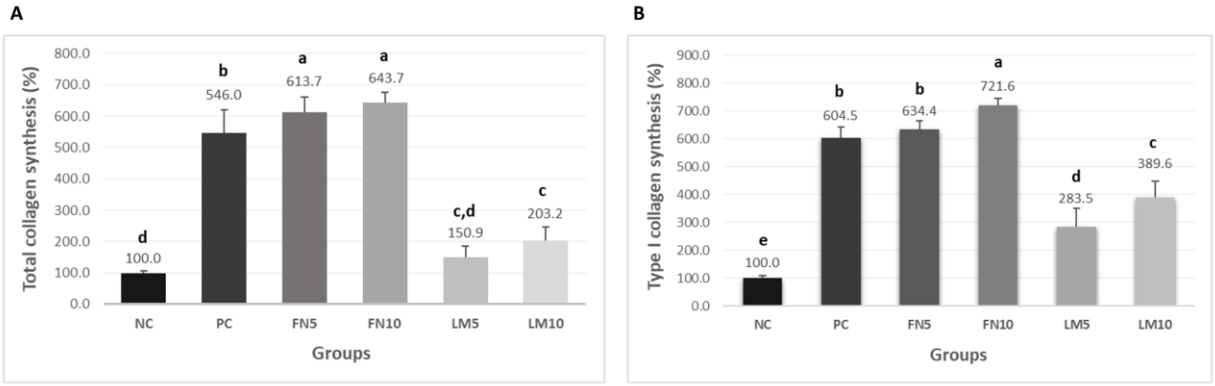


**Figure 4.** Mean and standard deviation of total collagen (A) and type I collagen (B) synthesis values (n=6; one-way ANOVA/Tukey tests; α=0.05). Different letters demonstrate a significant difference between groups.

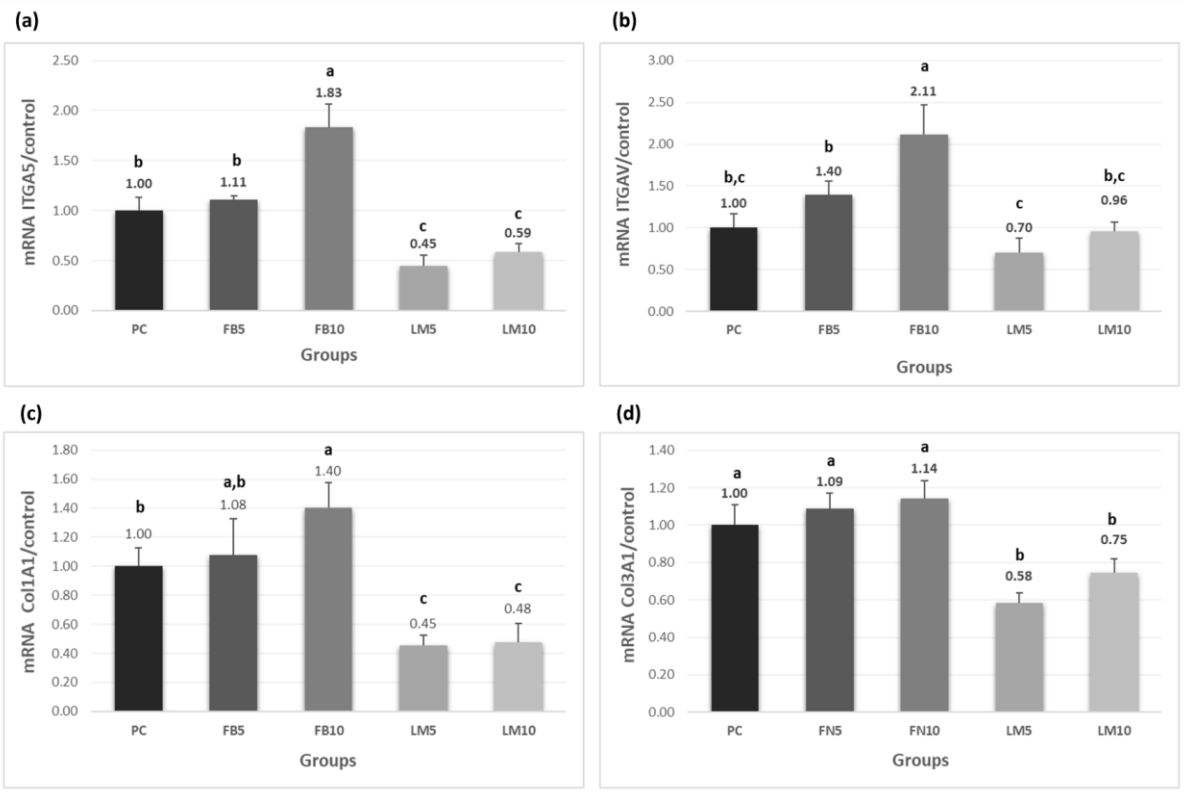


**Figure 5.** Mean and standard deviation of mRNA gene expression values of the ITGA5 (A), ITGAV (B), COL1A1 (C) and COL3A1 (D) markers (n=4; one-way ANOVA/Tukey tests; α=0.05). Different letters demonstrate a significant difference between groups.

### 3.2 Publicação 2*

# Development of fibronectin-loaded nanofiber scaffolds for guided pulp tissue regeneration

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**Running Heads:** Fibronectin-loaded nanofiber scaffolds for pulp regeneration

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**Abstract**

Fibronectin-loaded nanofiber scaffolds were developed and assessed concerning their bioactive potential on human apical papilla cells (hAPCs). Firstly, random (NR) and aligned (NA) nanofiber scaffolds of poly-caprolactone (PCL) were obtained by electrospinning technique and their biological properties were evaluated. The best formulations of NR and NA were loaded with 0, 5 or 10 μg/mL of fibronectin (FN) and their bioactivity was assessed. Finally, FN-loaded NR and NA tubular scaffolds were prepared and their chemotactic potential was analyzed using an in vitro model to mimic the pulp regeneration of teeth with incomplete root formation. All scaffolds tested were cytocompatible. However, NR and NA based on 10% PCL promoted the highest hAPCs proliferation, adhesion and spreading. Polygonal and elongated cells were observed on NR and NA, respectively. The higher the concentration of FN added to the scaffolds, greater cell migration, viability, proliferation, adhesion and spreading, as well as collagen synthesis and gene expression (ITGA5, ITGAV, COL1A1, COL3A1). In addition, tubular scaffolds with NA loaded with FN (10 μg/mL) showed the highest chemotactic potential on hAPCs. It was concluded that FN-loaded NA scaffolds may be an interesting biomaterial to promote hAPCs-mediated pulp regeneration of endodontically compromised teeth with incomplete root formation.

**Keywords:** Apical papilla cells, Fibronectin, Nanofibers, Pulp regeneration, Tissue engineering.

## Introduction

The integrity of dental enamel and dentin is important for maintaining both the function and esthetic appearance of teeth, and once this is damaged, the dentin-pulp complex is exposed to several injuries. These may be caused by microbial, traumatic and iatrogenic stimuli as well as the toxic effects caused by dental materials and their components (de Souza Costa et al., 2014). When teeth with incomplete root formation are subjected to intense pulp damage capable of causing irreversible pulpitis or even necrosis, specific endodontic therapy is necessary to promote physiological apexification. In this clinical situation, all pulp tissue should be mechanically/chemically removed and the root canal filled with an intracanal medication that stimulates biological sealing of the root apex through mineralized tissue deposition (Silujjai & Linsuwanont, 2017). This procedure interrupts the physiological apexification, which may compromise the stability of teeth and root wall resistance to masticatory efforts. Thus, different tissue regeneration strategies have appeared as promising alternatives to ensure the biological continuity of apical root formation (Nagy et al., 2014; Bottino, Pankajakshan, & Nör, 2017).

Cell-free therapy, which consist of using biomaterials associated with potent signaling agents for guided tissue regeneration, has been widely evaluated in the last decades. When introduced into the damaged tissue area, one may expect that such biomaterials can induce migration of resident cells as well as stimulate the adhesion, proliferation and differentiation of cells capable of synthesizing a new local tissue (Galler, Eidt & Schmalz, 2014). This modality of therapy appears to be very promising for clinical situations of endodontically compromised teeth with incomplete root formation, since the healthy apical papilla is a stem cells-rich connective tissue (Duarte et al., 2014; Nagy et al., 2014).

Scaffolds with similar architecture to the in vivo microenvironment in which they will be inserted, and that exhibit potential to stimulate regeneration of the lost tissues have been

developed (Gupte & Ma, 2012; Chen et al., 2018). From this perspective, studies have shown that the processes of adhesion, proliferation, and differentiation of mesenchymal stem cells are intensified when scaffolds with nanofiber topography were used regarding to smooth surfaces (Bottino et al., 2015). Nanofiber scaffolds can be obtained by the electrospinning technique, which allows the production of a biomaterial that mimics the structural and functional nano-morphology of the extracellular matrix (Bottino et al., 2015). This technique generates interconnected nanofibers with varying diameters and a high surface area that enhance cell adhesion and synthesis of a new tissue (Albuquerque et al., 2014). Furthermore, nanofibers can be obtained with different arrangements relative to each other, such as with random or aligned disposition, which may influence cell migration and metabolism (Gupta et al., 2009; Kai et al., 2011; Teh, Toh & Goh, 2011).

Association of biomaterials with potent chemotactic agents is extremely relevant within the strategy of guided pulp tissue regeneration (Albuquerque et al., 2014). Fibronectin (FN) has been proposed as an agent for inducing chemotaxis and increasing cell migration, adhesion, proliferation and differentiation (Guetta-Terrier et al., 2015; Grigoriou et al., 2017; Jacobsen et al., 2017; Chen et al., 2017; Parisi et al., 2020). The root pulp tissue of mammalian teeth exhibits dense collagen fibers disposed in parallel (Ricucci et al., 2017). Therefore, one may consider that scaffolds with nanofibers organized in a parallel arrangement, associated with chemotactic and bio-inductor agents, such as FN, would be interesting to use for filling the root canal of endodontically-compromised teeth. Thus, the aim of the present study was to develop FN-loaded nanofiber scaffolds with variable morphology, and use a specific in vitro model to assess their bioactive and chemotactic potential on human apical papilla cells (hAPCs).

## 1. Materials and Methods

### 2.1. Establishment of hAPCs

The apical papilla of four third molars with incomplete root formation, which was obtained from different donors of both sexes (16 to 18 years old), were submitted to the enzymatic digestion technique (Soares et al., 2017) to establish a primary culture of hAPCs. The patients and their guardians signed the term of free and informed consent that was approved by the Institutional Research Ethics Committee (Araraquara School of Dentistry, UNESP, São Paulo, Brazil; No. 80806617.3.0000.5416). Then, a pool of hAPCs was prepared by mixing the cells from all apical papilla after passage 1. This pool of hAPCs was expanded until the passage 3 in culture. For this purpose, it was used complete alpha minimum essential medium (α-MEM; GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (GIBCO), and antibiotic and antifungal agents (100 U/mL penicillin, 100 g/mL streptomycin, and 0.25 g/mL amphotericin; GIBCO). To determine the presence of a sub-population of stem cells from apical papilla (SCAPs) in this primary culture, the cells were characterized for the expression of multipotent stem cell markers (Nanog, Oct-3/4, CD-146, STRO-1 and CD-44) by immunofluorescence analysis, according to the protocol described by Soares et al., (2017). Cells at passage 3 to 6 were used to perform all laboratory experiments.

### 2.2. Synthesis, characterization and biological evaluation of experimental nanofiber scaffolds

Solutions of 10, 12.5 and 15% poly-caprolactone (w/v; PCL; Sigma-Aldrich, Saint Louis, MO, USA) were prepared in chloroform/dimethylformamide (8:2; v/v) under magnetic stirring for 24 h. After this, the experimental nanofiber scaffolds were synthesized by the electrospinning technique. For this purpose, the solutions were transferred to 5 mL syringes with needles (18 gauge) which were coupled to an automatic injection pump (KDScientific, Holliston, MA, USA) adjusted to a speed of 1 mL/h. To create the electric field, the positive pole of the high voltage power supply (12-13 kW) was fixed to the needle tip and the negative

pole was fixed to the aluminum metal collector. The distance between the needle tip and the collector was 18 cm. To obtain the random nanofiber scaffolds (NR), round glass coverslips (13 mm in diameter; Perfecta, São Paulo, SP, Brazil) were placed in a flat static collector. The aligned nanofiber scaffolds (NA) were obtained after positioning glass coverslips in a cylindrical rotary collector (4 cm in diameter). Afterwards, the scaffolds were kept in a desiccator at room temperature for 72 h. The following groups were established: NR-10%PCL, NA-10%PCL, NR-12.5% PCL, NA-12.5%PCL, NR-15%PCL and NA-15%PCL.

Morphological characterization of the experimental scaffolds was performed by placing them on metal stubs that were gold-sputtered and analyzed by Scanning Electron Microscopy (10-15kV; JEOL-JMS-6610V Scanning Microscope, Tokyo, Japan). The nanofiber diameters and the percentage of the interfibrillar spaces were assessed. For this purpose, 5 images of samples from each group were obtained, then 50 nanofibers were randomly selected in each image to evaluate the mean diameter on a nanoscale by means of Image J 1.45S software (Wayne Rasband, National Institutes of Health, USA). With the use of the same 5 images initially obtained from each group, a specific tool of the Image J 1.45S software was used to determine the percentage of interfibrillar spaces present in the nanofiber scaffolds (Soares et al., 2020).

For biological evaluation of the scaffolds, they were placed on glass coverslips that were individually placed on the bottom of wells of 24-well plates. Sterilized metal rings with an internal diameter of 8 mm were placed over the scaffolds to stabilize the samples in the wells and delimit the area to be analyzed. These scaffolds were subjected to a disinfection/washing protocol (Stefani, Cooper et al., 2016) with 50% alcohol (10 min - 1x), 70% alcohol (20 min - 3x) and phosphate buffered saline (10 min - 2x, PBS; GIBCO) and then kept in α-MEM overnight. After this, the culture medium was aspirated and the hAPCs ($5x10^4$ cells) were seeded on the scaffolds in 10 μL of complete α-MEM. After 30 min

incubation, 1 ml of complete α-MEM was added to each well and the scaffold/cell sets were submitted to additional incubation for up to 7 days, with the culture medium being changed every 2 days. In the control group, the cells were seeded on sterilized round glass coverslips, under the same experimental conditions. After time intervals of 1, 3 and 7 days of incubation, the viability (Live/Dead assay, n=4), proliferation (Alamar Blue assay, n=6), adhesion and spreading (F-actin assay, n=4) of the hAPCs were analyzed (Soares et al., 2020).

2.3 Evaluation of the bioactive potential of nanofiber scaffolds associated with fibronectin (FN)

Based on the biological results obtained in the first part of this study, the groups NR-10% PCL and NA-10% PCL were selected for association with different concentrations of FN. For this purpose, PBS solutions supplemented with 0, 5 or 10 µg/mL of FN (bovine plasma; Sigma-Aldrich) were applied on the nanofiber scaffolds placed on the bottom of wells of 24-well plates, to form the following groups: SC-NR, SC-NA, SC-NR+5FN, SC-NA+5FN, SC-NR+10FN and SC-NA+10FN. After this, the plates were centrifuged at 1500 rpm for 10 min and maintained at 4ºC for 18 h. The remaining solution was aspirated, followed by washing with PBS at 37ºC. To confirm the adsorption of FN into the nanofiber scaffolds, an immunofluorescence assay was performed after incubating the nanofiber scaffolds for 7 days at 37ºC. For this assay, the scaffolds (n=4) either associated with FN, or not, were fixed in 4% paraformaldehyde, washed with PBS, followed by blocking with 5% BSA and incubation with anti-fibronectin antibody (monoclonal mouse IgG1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% BSA (1:50) for 1 h. The scaffolds were rinsed with PBS and then incubated with Fluorescein (FITC) AffiniPure Goat Anti-Mouse IgG in 1% BSA (1:50; Santa Cruz Biotechnology) for 1 h in a dark room. Finally, the scaffolds were rinsed again with PBS, placed on new round glass coverslips and assessed under a fluorescence microscope (Leica DM 5500B, Nussloch GmbH, Nussloch, Germany). After this, the bioactive potential

of the scaffolds - loaded with FN, or not - was evaluated as described below. For cell migration analysis (n=4), scaffolds were individually placed on the bottom of wells of 24-well plates. After applying culture medium in the wells, the scaffolds floated. In the meantime, hAPCs (3x104) were seeded on the upper surface of transwell inserts (8 μm pores; FluoroBlok cell culture inserts, Corning, Tewksbury, MA, USA) and incubated at 37°C and 5% $CO_2$ for 2 h. Then, the inserts with the hAPCs attached on them were carefully positioned in each well, in such way that the top of the scaffolds remained in direct contact with the lower surface of the transwell inserts. After incubation for 24 h, the upper and lower surfaces of the transwell inserts and the scaffolds were fixed in 4% paraformaldehyde at 4ºC for 15 min. Subsequently, the cell membranes were sealed with 0.1% Triton X-100, and the cells were incubated for 30 min with a specific fluorescent probe in 2% BSA (1:20; Actin Red 555, Life Technologies) for cytoskeleton detection. Afterwards, the cells were rinsed with PBS and incubated with Hoescht (1:5000; Invitrogen, Eugene, Oregon, USA) for 15 min, for nuclear counter-fluorescence. Microscopic fluorescence images of the transversal section of the scaffolds were obtained (Leica DM 5500B) for qualitative analysis of the migratory capacity of the cells to the scaffolds. For the quantitative analysis, 4 images were obtained of different fields of the upper surface of each insert, and the cell's nucleus were counted using the ImageJ software (Soares et al., 2017). The arithmetic mean of the values obtained was determined for each sample, and this data was considered relative to an experimental unit (n=4/group).

Subsequently, the hAPCs ($3x10^4$) in 10 μL of complete α-MEM were again seeded on the nanofiber scaffolds, either loaded with FN, or not, which were placed on the bottom of wells, as described above. After 30 min, α-MEM (1 mL) was added and the scaffold/cell sets, which were incubated for up to 7 days, with the culture medium being replaced every 2 days. After time intervals of 1, 3 and 7 days, the viability (Live/Dead assay, n=4), proliferation

(Alamar Blue assay, n=6), adhesion and spreading (F-actitn assay; n=4) of cells were assessed (Soares et al., 2020). The total collagen (Sirius red assay, n=6) and type I collagen (ELISA assay, n=6; Duoset human COL-I - R&D Systems, Minneapolis, MN, USA) synthesis were evaluated after 7 days (Basso et al., 2016). At the same time-point, the gene expression of GAPDH (constitutive gene), ITGA5, ITGAV, COL1A1 and COL3A1 (Real-time Polymerase Chain Reaction Assay, n=4) was determined (Soares et al., 2020).

2.4 Development and analysis of the chemotactic potential of FN-loaded nanofiber tubular scaffolds

According to the electrospinning parameters described above, random (nanoR) and aligned (nanoA) nanofibers strips based on 10% PCL were obtained (8 mm x 100 mm) and hand-spiraled using a sterilized device capable of producing tubular scaffolds (SC-TB) with 3 mm in diameter and 8 mm long. After disinfecting and washing the SC-TBs, PBS solution supplemented with 0 or 10 µg/mL of FN was applied on the SC-TB placed in wells of 96-well plates. These plates were centrifuged at 1500 rpm for 10 min and maintained at 4ºC for 18 h. The remaining solution was aspirated, followed by washing with PBS at 37ºC. Then, the following groups were established (n=4): SC-$TB_{nanoR}$, SC-$TB_{nanoA}$, SC-$TB_{nanoR}$ + 10FN, SC-$TB_{nanoA}$ + 10FN. To mimic the in vivo regenerative therapy of teeth with incomplete root formation, the SC-TB were inserted into sterile cylindrical devices (Figure 1; PYREX™ Cloning Cylinder; Corning) individually placed in wells of 48-well plates. After this, the hAPCs ($3x10^5$ cells) were seeded in 5 µL of complete α-MEM on the upper surface of the SC-TB and incubated for 30 min to allow the initial cell adhesion. Subsequently, 500 µL of complete α-MEM was placed in each well and the plates were kept in incubator (Thermo Fisher Scientific, Asheville, NC, USA) at 37ºC and 5% $CO_2$ for 28 days, with the culture medium being replaced every day. After this period, cell migration into the SC-TBs was assessed. For this purpose, the SC-TBs were fixed with 4% paraformaldehyde and frozen in

Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) at -80ºC for 24 h. Then, using a cryostat (Leica CM 1820 UV, Buffalo Grove, IL, USA), serial longitudinal sections, 5 µm thick, were obtained and placed on clean histological glass slides. Four sections of each group were permeabilized with 0.1% Triton X-100 and incubated with Actin Red 555 probe (Life Technologies) in 2% BSA (1:20) to show evidence of the actin filaments (Soares et al., 2020). Afterwards, these sections were incubated with Hoescht (1:5000; Invitrogen, Eugene, Oregon, USA) for nuclear counter-staining. After washing with PBS, the specimens were transferred to other glass slides to be analyzed under a fluorescence microscope (Leica DM 5500B). For the quantitative analysis, 4 images of each group were selected to measure the distance (µm) covered by the cells inside the SC-TB. Three standardized fields were selected from each image and a specific tool of the ImageJ software was used to measure the hAPCs migration. The values obtained were considered as experimental unit (n=12/group).

### 1.5 Statistical analysis

All assays were performed at least twice to ensure the reproducibility of data. The data normality and homoscedasticity were analyzed, and then the data were submitted to one or two-way ANOVA, followed by the Tukey post-test ($\alpha$=0.05). The statistical power of the samples showed > 95.0% power level for each analysis using the DSS Research calculator.

## 2. Results

### 3.1. Establishment of hAPCs

The immunofluorescence analysis preliminarily performed in this study demonstrated that a number of primary cells obtained from apical papilla was positively stained for Nanog, Oct-3/4, CD-146, STRO-1 and CD-44 (Figure 2). Therefore, one may consider that a sub-

population of SCAPs was present in the primary culture employed in all following experiments carried out.

3.2. Synthesis, characterization and biological evaluation of experimental nanofiber scaffolds

The use of a flat static collector resulted in scaffolds with random nanofibers (Figure 3) that gave rise to the Groups NR-10%PCL, NR-12.5%PCL and NR-15%PCL. The cylindrical rotary collector allowed us to obtain scaffolds with aligned nanofibers, which stablished the Groups NA-10%PCL, NA-12.5%PCL and NA-15%PCL. Thicker nanofibers and smaller interfibrillar spaces were observed as higher was the concentration of PCL used to prepare the scaffolds (Table 1, Figure 3).

The images from Live/Dead assay (Figure 4) demonstrated that all nanofiber scaffold formulations were citocompatible, especially NR-10%PCL and NA-10%PCL Groups in which enhanced cell viability was observed at 3 and 7-day periods ($p<0.05$; Figure 5). Enhanced cell proliferation occurred for all groups over the time-points ($p<0.05$). Despite the increased cell viability observed for all nanofiber scaffolds formulations in comparison to control at 1 and 3-days periods ($p<0.05$), only the NR-10%PCL, NA-10%PCL and NA-12.5%PCL Groups maintained this difference at 7 days ($p<0.05$). Cells seeded on random or aligned 10% PCL nanofiber scaffolds presented higher adhesion and spreading in all time-points (Figure 6). The architecture of the nanofibers seemed to influence the cells morphology. Then, cells with more elongated morphology were observed on aligned nanofiber scaffolds and cells with polygonal morphology were attached to random nanofiber scaffolds.

3.3. Evaluation of the bioactive potential of nanofiber scaffolds associated with FN on hAPCs

The immunofluorescence images presented in Figure 7 demonstrated that the adsorption of FN on the nanofibers was concentration-dependent. This difference in the FN adsorption pattern influenced the bioactive potential of the nanofiber scaffolds, with the highest

chemotactic potential observed in SC-NR+10FN and SC-NA+10FN Groups ($p < 0.05$; Figure 8a). These results were corroborated by the images from the Transwell assay (Figure 8b), in which cells seeded on the upper surface of the insert migrated to its lower surface. These cells reached the surface of the subjacent FN-loaded scaffolds. The increased incorporation of FB into the nanofiber scaffolds significantly improved the cell viability (Figure 9), proliferation ($p<0.05$; Figure 10), adhesion and spreading (Figure 11), as well as total collagen ($p<0.05$; Figure 12a) and type I collagen ($p<0.05$; Figure 12b) synthesis. In these tests, the topography of the nanofibers influenced only the morphological pattern of the hAPCs, with polygonal and elongated cells being observed in the random and aligned nanofiber scaffolds, respectively. Higher expression of ITGA5, ITGAV, COL1A1 and COL3A1 was observed in the SC-NR+10FN and SC-NA+10FN Groups in comparison with control ($p<0.05$; Figure 13a-d).

3.4. Development and analysis of the chemotactic potential of FN-loaded nanofiber tubular scaffolds

Overall, the 10 µg/ml FN-loaded random or aligned SC-TB stimulated grater cell migration and proliferation in comparison with the SC-TB without FN in their composition ($p<0.05$), such as shown in the Figure 14. However, the aligned arrangement of the nanofibers associated with this potent signaling agent allowed the cells to reach the lower region of the apical third of the SC-TB ($p<0.05$).

## 4. Discussion

In this in vitro study FN-loaded nanofiber scaffolds were developed, and their chemotactic and bioactive potential were evaluated on hAPCs, aiming their future application to pulp regeneration of teeth with incomplete root formation. For this purpose, a synthetic polymer (PCL) approved by the Food and Drug Administration, which is biocompatible, biodegradable, and has low cost (Kai et al., 2018; Oliveira et al., 2019) was first used to

synthesize the nanofiber scaffolds. The strong resistance of PCL to hydrolytic degradation (Stefani et al., 2016) could maintain the integrity of this biomaterial inside the root canal over the course of time, which is important to allow the migration and proliferation of apical papilla cells along this tubular structure. Therefore, while the nanofiber scaffold is slowly degraded, a new tissue may be synthesized, providing structural and functional support for guided pulp tissue regeneration (Albuquerque et al., 2014; Bottino, Pankajakshan & Nör, 2017). In the present study, with the purpose of developing scaffolds with a specific topography capable of favoring the proliferation, adhesion and spreading of the hAPCs, variable concentrations of PCL solutions (10, 12.5 and 15%) were tested and two different types of electrospinning techniques (flat or rotatory collectors) were used to obtain random and aligned nanofiber biomaterials.

According to the scanning electron microscopy analysis, the higher the PCL concentration in the scaffold was, the larger was the diameter of the nanofibers, and the smaller were the interfibrillar spaces. Previous studies have shown that the increase in polymeric concentration used for synthesizing scaffolds by the electrospinning technique, resulted in thicker fibers, which changed the production of fibers from nanometric to micrometric scale (Yang et al., 2005; Teh, Toh & Goh, 2011). Yang et al. (2005) demonstrated that the high speed of the rotary collector produces a tangential force during ejection of the polymer to stretch the fibers favoring the formation of nanofiber. These findings were corroborated by the data obtained in the present study, in which the same concentration of PCL applied on a cylindrical rotary collector gave rise to fibers that were thinner than those obtained with a flat static collector. In contrast, the porosity of the scaffolds was influenced by the arrangement and diameter of the fibers, with the higher percentage of interfibrillar spaces being observed in NR-10% PCL and NA-10% Groups. Previous studies have shown that biomaterials with nanofibrillar topography, large surface area, and porosity

could improve cell proliferation and differentiation (Oliveira et al. 2019; Ranjan et al., 2020). In the present study, the highest rates of viability, proliferation, adhesion and spreading of hAPCs occurred when these cells were seeded on NR-10%PCL and NA-10%PCL scaffolds.

In addition, the different nanofiber arrangements influenced the morphology of hAPCs. At 1 and 3-day cell culture periods, polygonal-shaped hAPCs were observed on the random nanofiber scaffolds. On the other hand, elongated cells were seen on aligned nanofiber scaffolds. Several researchers have shown that cells obtained from nerves, muscles and periodontal tissue follow the direction of aligned nanofibers when seeded on them, resulting in elongated cytoplasmic cell projections and greater phenotypic expression in comparison with random nanofibers (Gupta et al., 2009; Kai et al., 2011; Teh, Toh & Goh, 2011). Despite the different morphology of hAPCs in the present study, both topographies of the nanofiber scaffolds based on 10% PCL increased the cell proliferation without difference between them. Since the role of the scaffold is to mimic the extracellular matrix of the dental pulp and this tissue has a diversity of proteins that can be dispersed or organized in parallel (Ricucci et al., 2017), the hAPCs were able to proliferate in both scaffolds topographies tested in this investigation. Therefore, as the nanofibers arrangement can also influence other cell parameters, FN-loaded scaffolds with random and aligned nanofibers based on 10% PCL were tested regarding their bioactivity.

FN at concentrations of 0, 5 and 10 µg/mL were added to random and aligned nanofiber scaffolds. The higher the concentration of FN incorporated into the scaffold was, the higher were the rates of migration, viability, proliferation, adhesion and spreading of hAPCs, and levels of total collagen and type I collagen synthesis that occurred. Our results were corroborated by the findings of Grigoriou et al. (2017), who showed that FN-treated poly(ethyl acrylate) and poly(methyl acrylate) surfaces stimulated the adhesion, migration and proliferation of human fibroblasts, as well as the local secretion and reorganization of new

matrix. Enhanced rates of viability, migration and spreading of fibroblasts, endothelial and vascular smooth muscle cells were also reported by Jacobsen et al. (2017) who exposed these cells to FN-loaded silk fibroin fibers. To gain better understanding of the mechanism of action of FN, the gene expression of α5 integrin (ITGA5) and αV integrin (ITGAV) was assessed in the present study. The α5 and αv integrins are membrane receptors and play an important role in several essential biochemical and biomechanical signals capable of inducing cell adhesion, migration and proliferation (Kawamura et al., 2019; Diaz et al., 2020). In the present study, higher levels of expression of ITGA5 and ITGAV were observed in SC-NR+10FN and SC-NA+10FN Groups. Chen et al. (2017) showed that the interaction of α5β1 integrin present in the membrane of human keratinocytes with the surface containing the functional domain of FN improved cell adhesion and migration. The authors also reported that when these cells were seeded on FN-mimic surfaces in the presence of GTPase inhibitors, the cell chemotaxis was mediated by the Rac1 and Rho pathways. Whereas it is known that the cell cycle progression is positively regulated by a family of protein kinases referred to as the cyclin-dependent kinases (CDKs) (Parisi et al., 2020). During the transition from phase G1 to phase S, this cycle is up-regulated by CDK-2 and CDK-4, which induce cyclins E and D1, respectively, via MAPK/ERK pathway. When activated by specific signaling agents, the role of integrin is associated with phosphorylation of ERK pathway and induction of cyclin D1 (Parisi et al., 2020). Since FN has a functional domain to bind to integrin, it can stimulate cell cycle progression (Parisi et al., 2020). Therefore, this may - even partially - explain the increased cell proliferation observed in the present study when FN was added to the nanofiber scaffolds.

To determine the possible influence of FN on the synthesis of a new matrix, the gene expression of type I collagen (COL1A1) and type III collagen (COL3A1) was assessed in the present in vitro investigation. The extracellular matrix (ECM) is known to be an important

tissue component that provides not only structural support, but also a way to transmit biological signals responsible for tissue morphogenesis and homeostasis (Theocharis et al., 2016; Parisi et al., 2020). Goldberg et al. (2008) reported that type I and type III collagen were the main components of the pulp tissue matrix. Other authors have shown that FN is a potent signaling agent capable of inducing collagen synthesis, contributing to the renovation and maintenance of connective tissue functions (Grigoriou et al., 2017; Ramaswamy, Vorp & Weinbaum, 2019). In the present study, a significant increase in the expression of COL1A1 and COL3A1 was observed in the SC-NR+10FN and SC-NA+10FN Groups, confirming the strong bioactivity of these biomaterials. Therefore, these encouraging in vitro data indicated that the potential of these products for promoting pulp tissue regeneration should be further assessed in vivo.

In this laboratory study, hAPCs seeded on different topographies of nanofiber scaffolds associated with 0, 5 or 10 μg/mL of FN presented similar gene expression. Some cell types, such as those from nerves, muscles, and periodontal tissue exhibit elongated morphology (Gupta et al., 2009; Kai et al., 2011; Teh, Toh & Goh, 2011). Since the topography of biomaterials exert influence on cells morphology, one may suggest that scaffolds with aligned nanofibers favor the phenotypic expression of these cells (Gupta et al., 2009; Kai et al., 2011; Teh, Toh & Goh, 2011). However, cells from apical papilla and pulp tissue have variable morphology that depends on the region in which they are located (Ricucci et al., 2017). These data demonstrate the plasticity of such cells, which seems to explain, at least partially, the maintenance of the phenotypic expression of hAPCs seeded on substrates with diverse topographies. In this way, we can justify the results obtained in the present study, in which no significant difference in the gene expression of specific markers was observed when hAPCs were cultured on random or aligned nanofiber scaffolds.

To gain better understanding of the influence of random and aligned topography of nanofibers associated with a chemotactic dosage of FN, an in vitro model was developed in the present study, to mimic the pulp regeneration of teeth with incomplete root formation. Cell migration is known to be a crucial mechanism for allowing cell proliferation in three-dimensional structures, which depends on cell adhesion and spreading (Kawamura et al., 2019; Parisi et al., 2020). Guetta-Terrier et al., (2015) demonstrated that 3T3 fibroblasts seeded on aligned fibers coated with FN adhered to this substrate and assumed a robust spindle-shaped morphology. Whereas cells seeded on FN-unloaded fibers were weakly adhered to the biomaterial. The authors also showed that fibroblasts along the FN-loaded fibers exhibited several fin-like protrusions that were hundreds of micrometers long, which played a role in cells migration. These data were corroborated by the results of the present laboratory study, in which the aligned arrangement of nanofibers present in the FN-loaded scaffolds enhanced the migration of cells into the tubular biomaterial.

Taking into consideration the limitations of data obtained from in vitro studies, the promising results found in this investigation determined that scaffolds with aligned nanofibers coated with a chemiotactic and bioactive dose of FN (10 µg/mL) may be an interesting option of biomaterial to promote pulp regeneration of endodontically compromised teeth with incomplete root formation. As preliminarily shown in this study, it could be expected that when clinically inserted into the root canal of the above-mentioned teeth, the new biomaterial would stimulate the migration of apical papilla cells, and support their adhesion, proliferation, and spreading along the entire length of the root canal. Thus, while the biomaterial is slowly being degraded, a new pulp-like tissue would fill the root canal. Once the tooth vitality is restored, the root formation could be completed. Despite the encouraging scientific data shown in the present investigation, in vivo studies are needed to assess the behavior of this innovative biomaterial.

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**Data Availability Statement**

The data that support the findings of this study are available.

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## Tables

**Table 1**. Mean values of nanofiber diameters and interfibrillar spaces

| Groups | Mean of nanofiber diameters (nm) | Mean of interfibrillar spaces (%) |
|---|---|---|
| NR-10%PCL | 708.8 | 33.3 |
| NA-10%PCL | 600.8 | 24.8 |
| NR-12.5%PCL | 1246.7 | 15.4 |
| NA-12.5%PCL | 743.2 | 21.2 |
| NR-15%PCL | 1701.1 | 9.8 |
| NA-15%PCL | 932.8 | 15.3 |

**Figures**

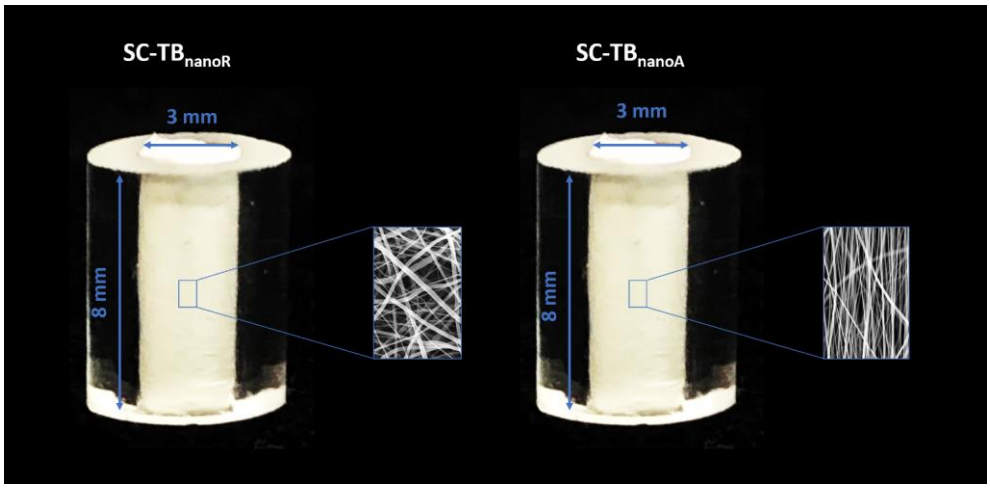


**Figure 1.** Representative images of SC-TB$_{nanoR}$ and SC-TB$_{nanoA}$ inserted in cylindrical device that simulated in vitro regenerative therapy of teeth with incomplete root formation.

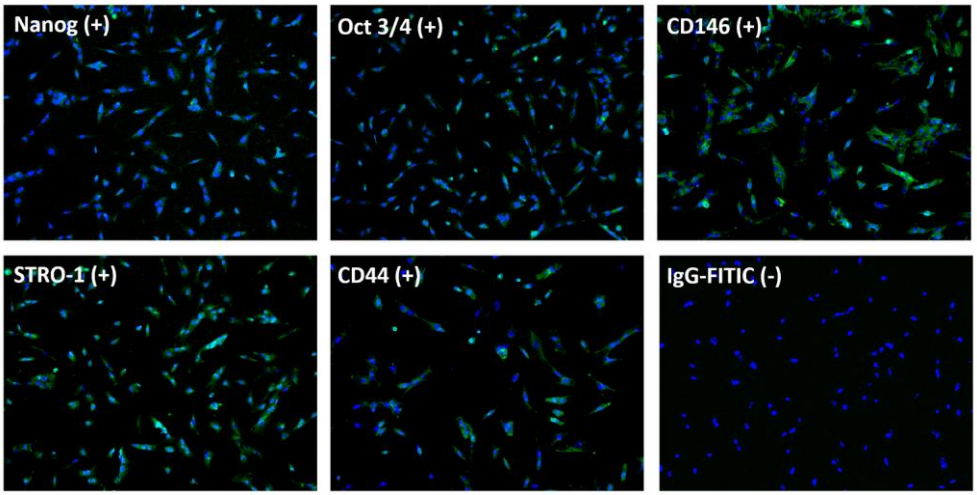


**Figure 2.** Immunofluorescence staining for multipotent stem cells markers (10x magnification). Green fluorescence (FITC) indicated positive staining for Nanog, Oct-3/4, CD-146, STRO-1 and CD-44. Blue fluorescence indicated cell nucleus (Hoescht). Cells incubated only with IgG- FITC represented negative control of this analysis.

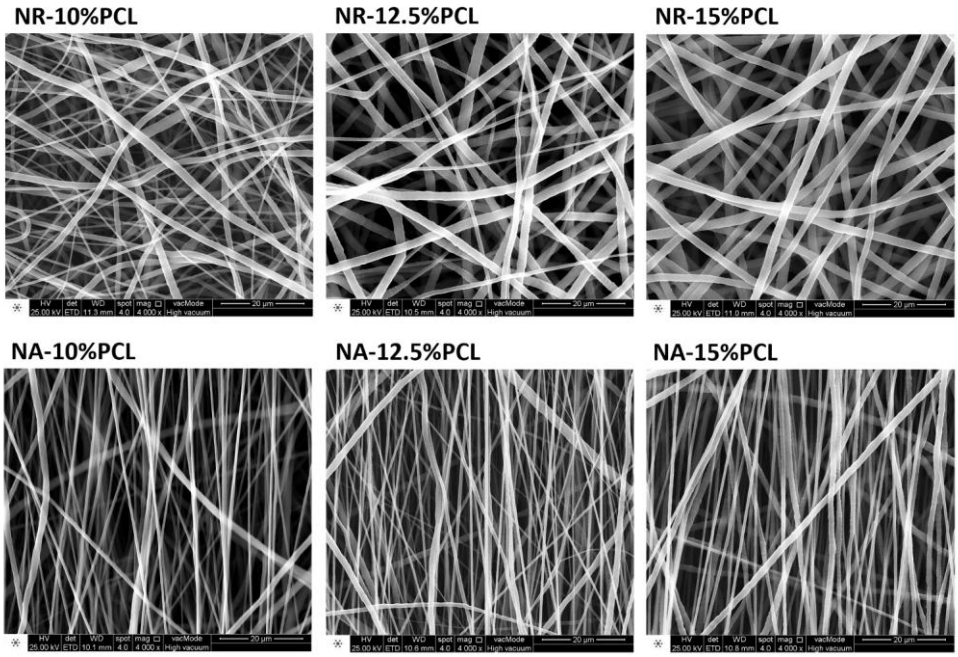


**Figure 3.** Scanning electron microscopy images of nanofiber scaffolds according to parameters tested (4000x magnification).

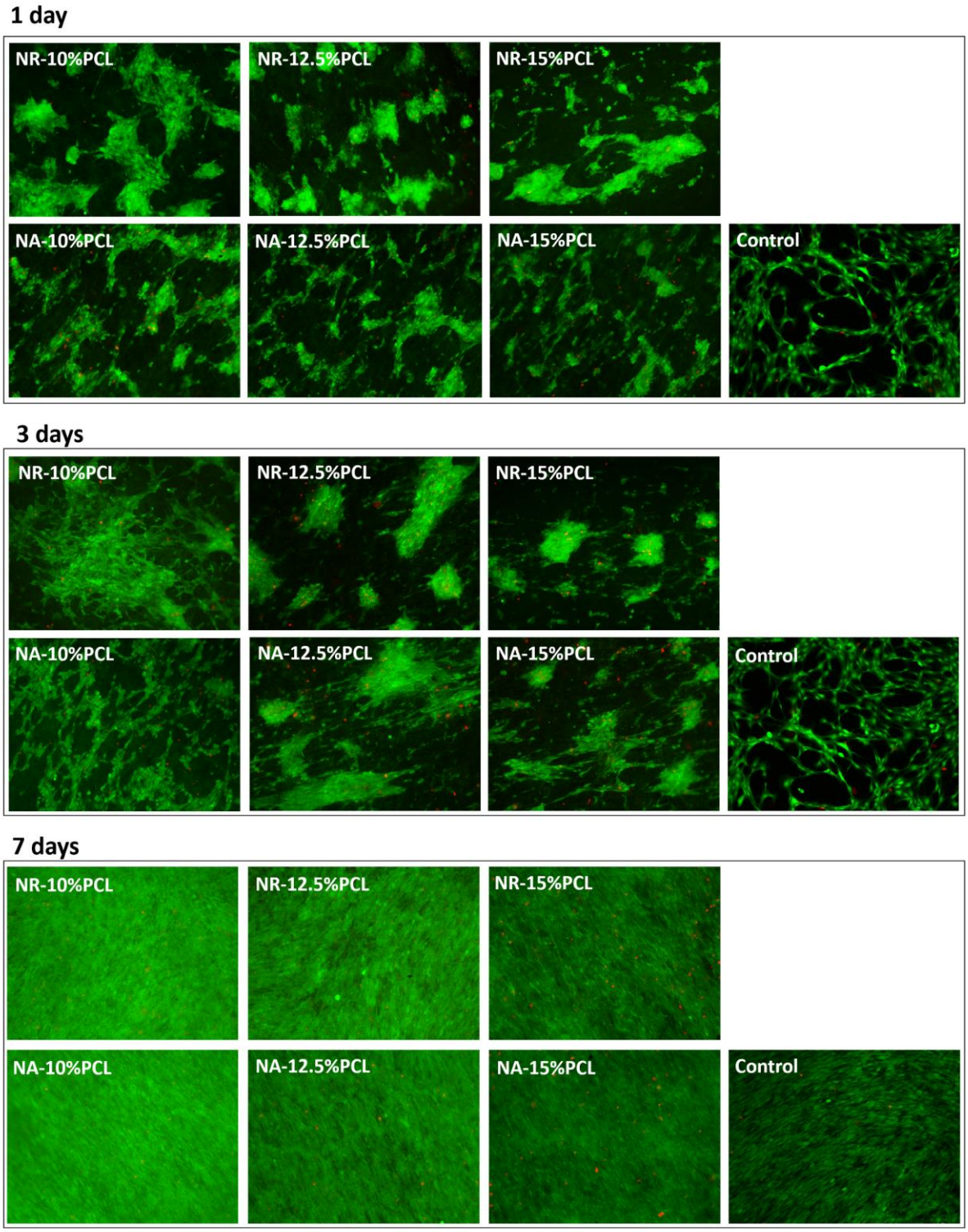


**Figure 4.** Cell viability on different formulations of nanofiber scaffolds (10x magnification). Green fluorescence indicated live cells. Red fluorescence indicated dead cells.

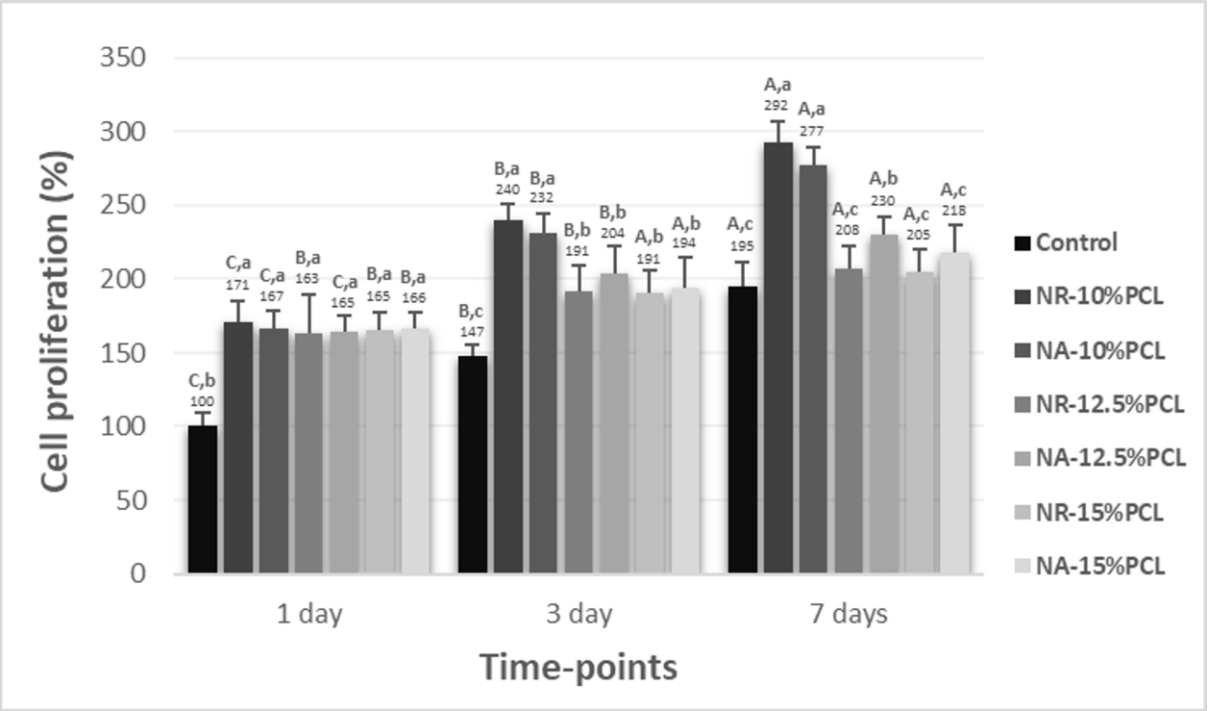


**Figure 5.** Mean and standard deviation of cell proliferation values on different formulations of nanofiber scaffolds. (n=6; two-way ANOVA/Tukey tests; α=0.05). Different capital letters indicated significant difference between time-points for each group. Different lowercase letters demonstrated significant difference between groups in each time-point. The mean fluorescence value of the control group at 1 day was considered as 100%.

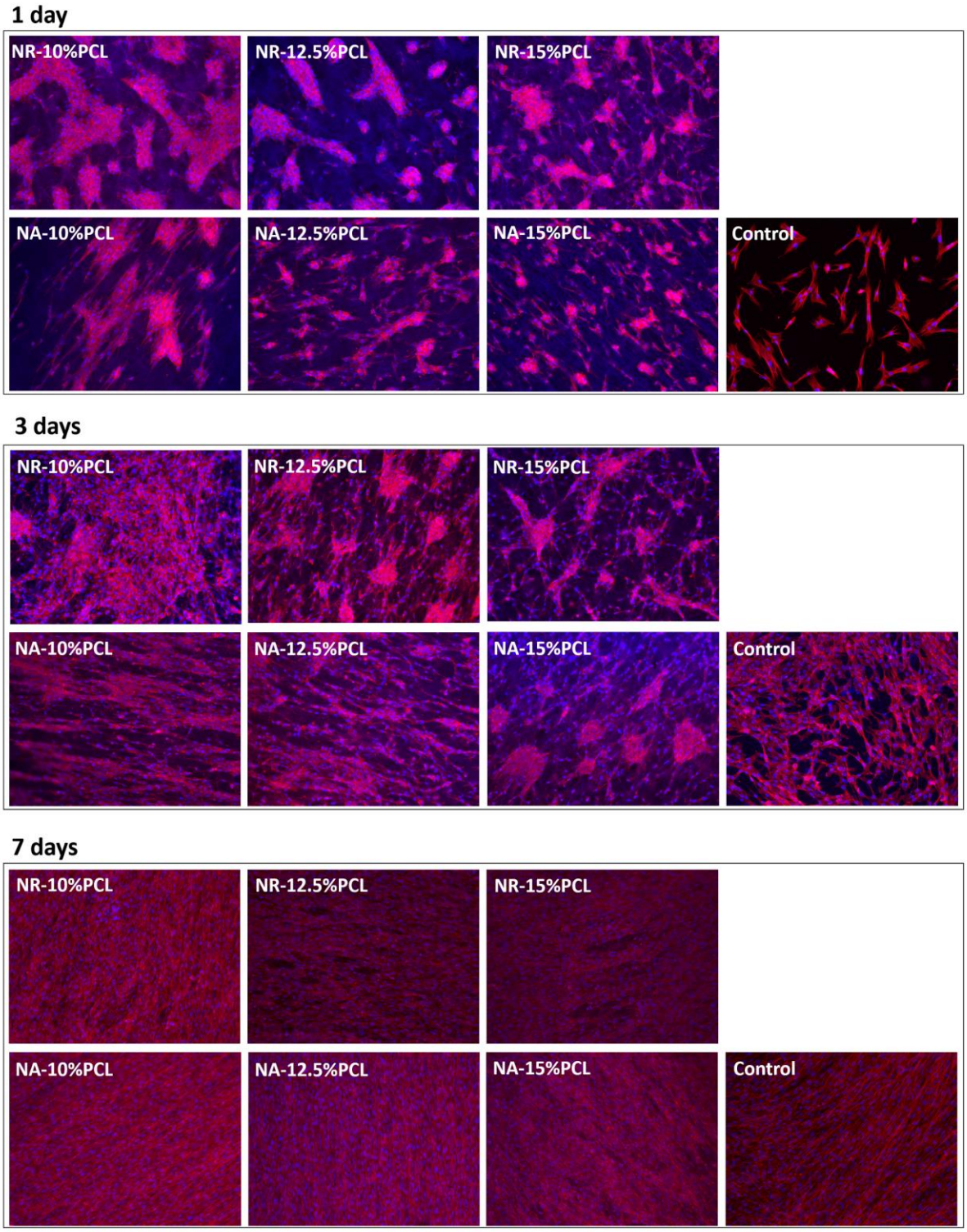


**Figure 6.** Cell adhesion and spreading on different formulations of nanofiber scaffolds (10x magnification). Red and blue fluorescence indicated actin filaments and cell nucleus, respectively.

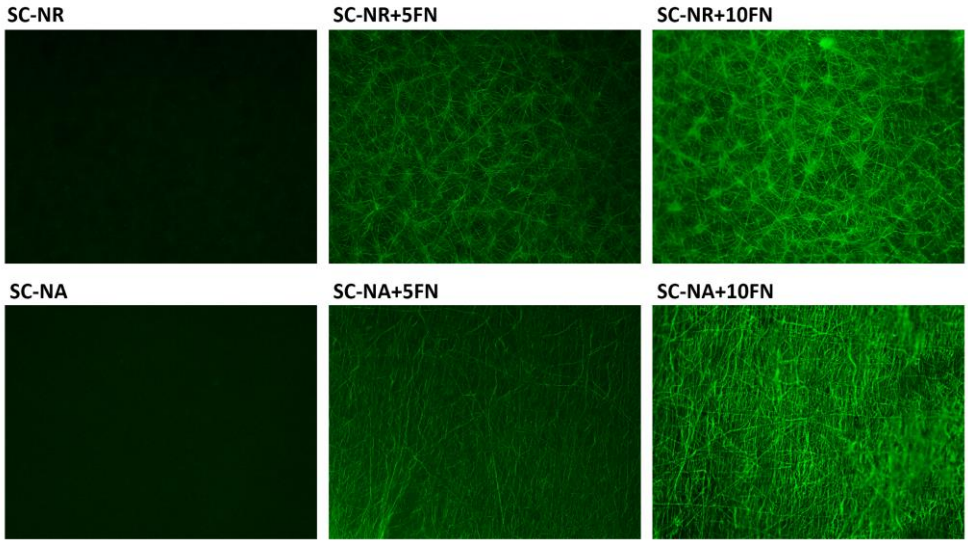


**Figure 7.** Immunofluorescence assay to evaluation of fibronectin adsorption on nanofiber scaffolds. Green fluorescence indicated presence of fibronectin.

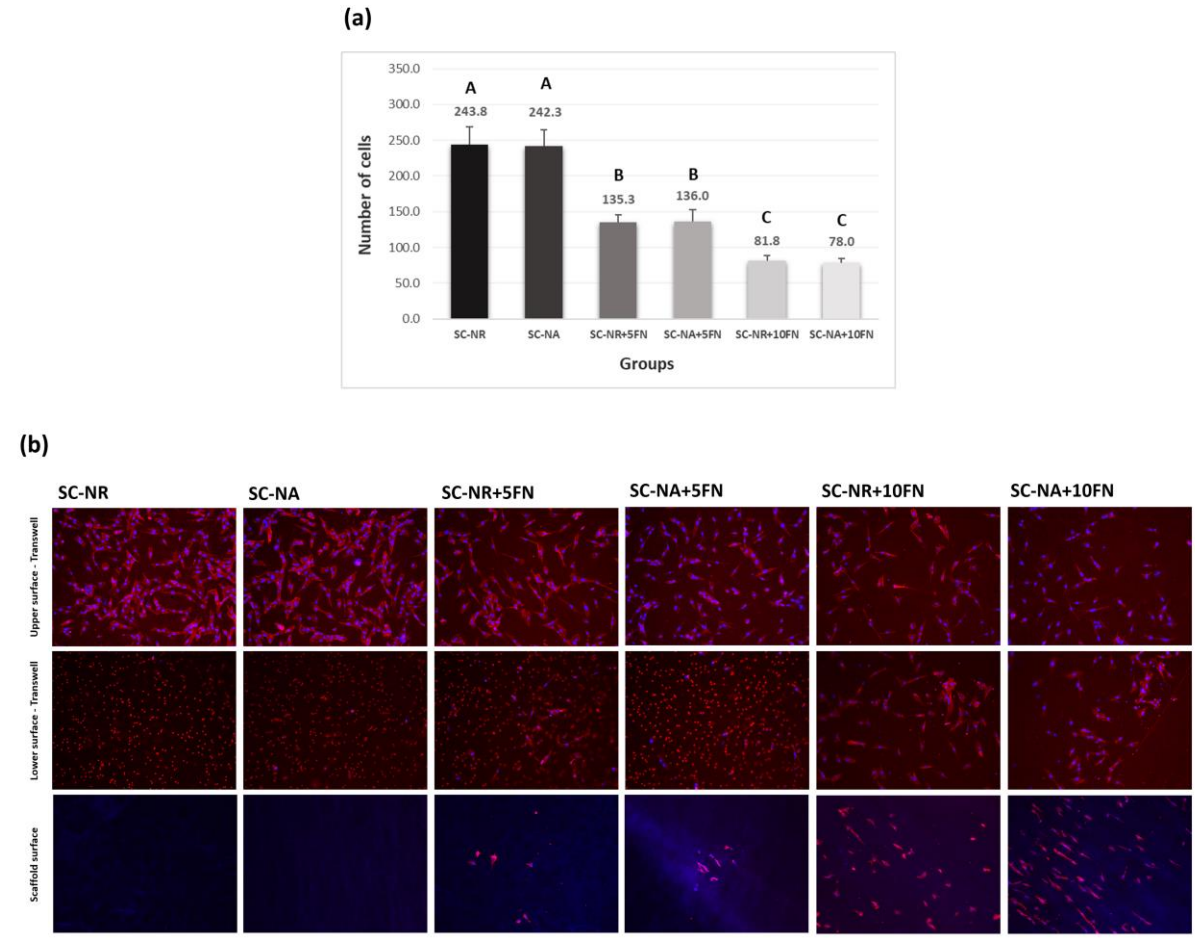


**Figure 8.** Active cell migration assay. (a) Mean and standard deviation of number of cells on upper surface of transwell inserts according to group (n=4; one-way ANOVA/Tukey tests; $\alpha$=0.05). Different capital letters demonstrated significant difference between groups. (b) Images of upper and lower surface of transwell inserts and scaffold surface of each group (10x magnification). Red fluorescence indicated cytoskeleton of cells. Blue fluorescence indicated cell nucleus.

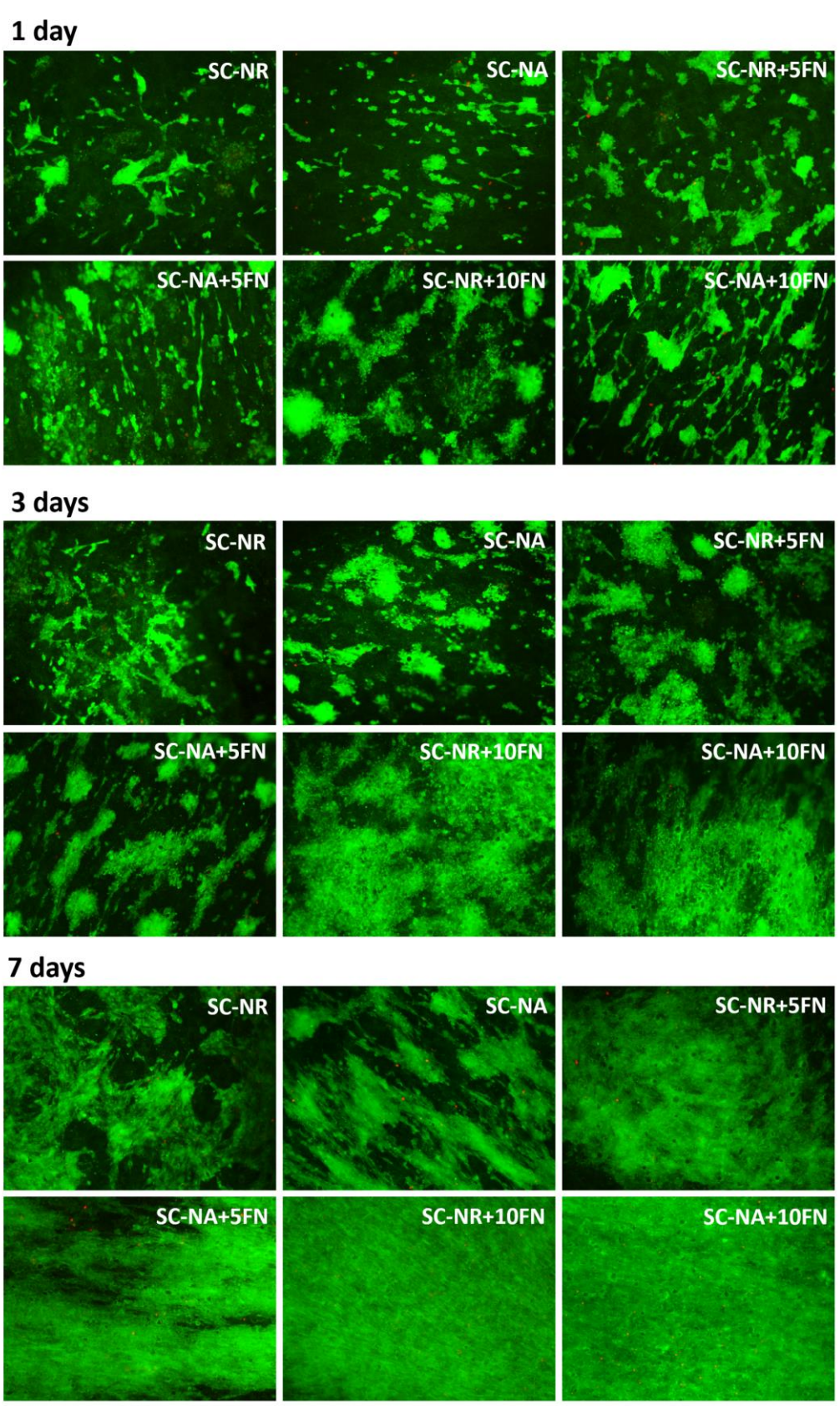


**Figure 9.** Cell viability on nanofiber scaffolds either associated with fibronectin, or not (10x magnification). Green and red fluorescence indicated live and dead cells, respectively.

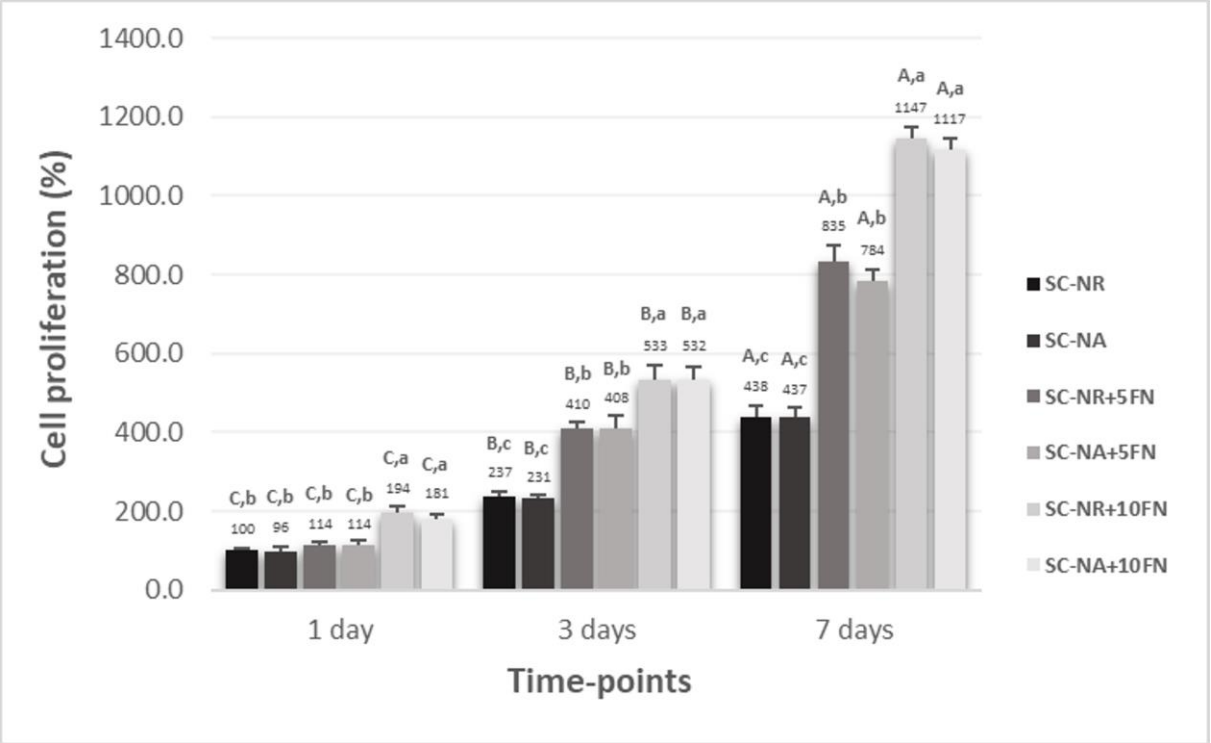


**Figure 10.** Mean and standard deviation of cell proliferation values on nanofiber scaffolds either associated with fibronectin, or not (n=6; two-way ANOVA/Tukey tests; α=0.05). Different capital letters indicated significant difference between time-points for each group. Different lowercase letters demonstrated significant difference between groups in each time-point. The mean fluorescence value of the SC-NR group at 1 day was considered as 100%.

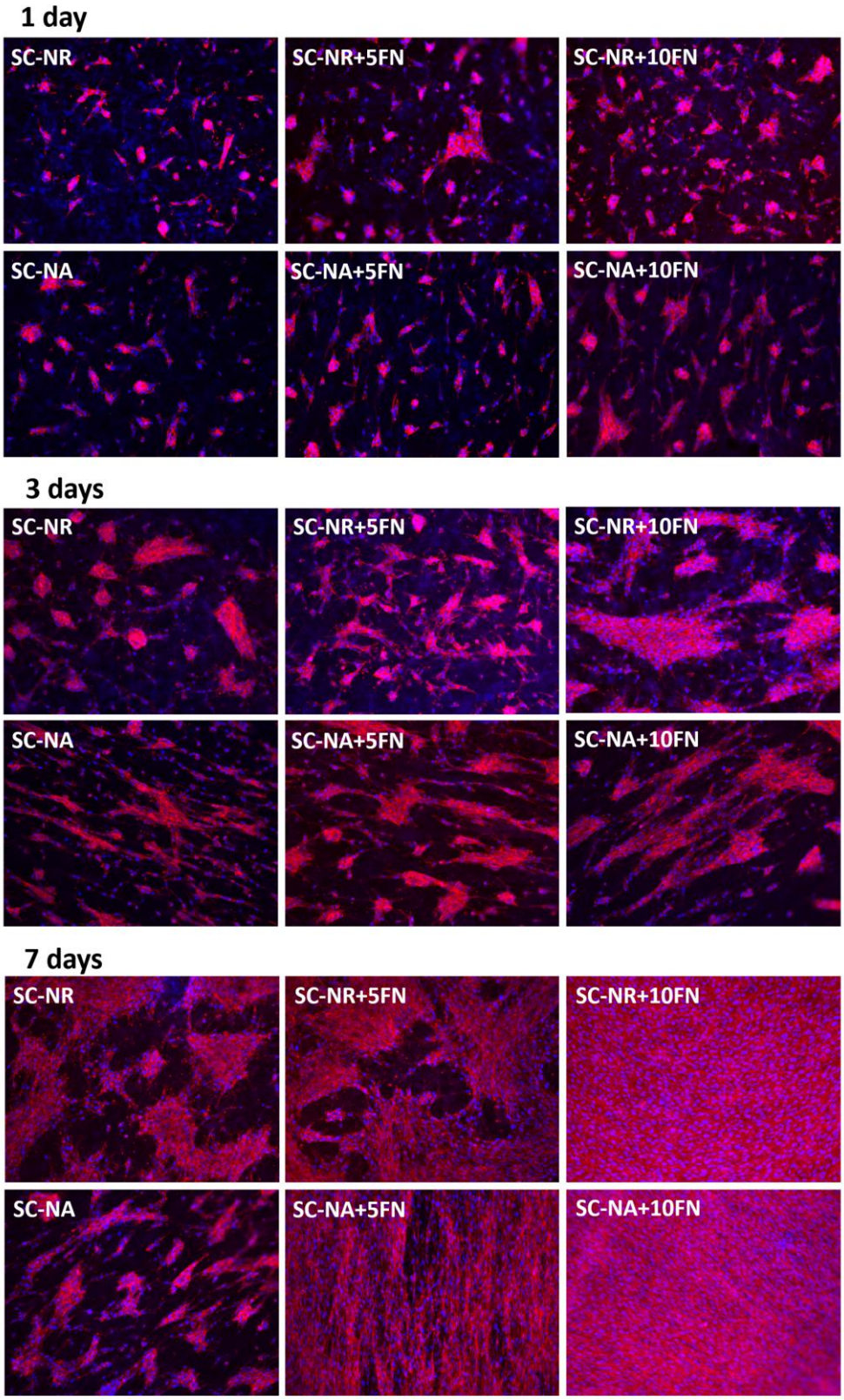


**Figure 11.** Cell adhesion and spreading on nanofiber scaffolds either associated with fibronectin, or not (10x magnification). Red and blue fluorescence indicated actin filaments and cell nucleus, respectively.

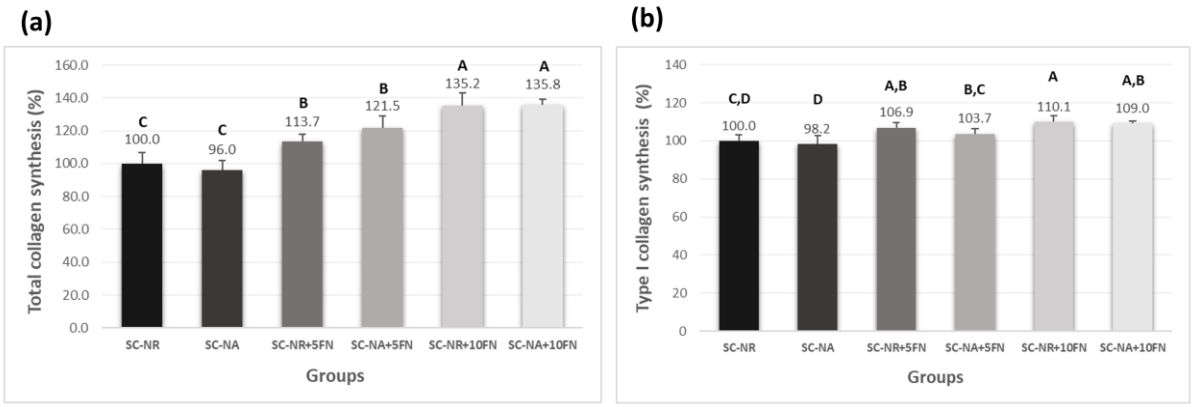


**Figure 12.** Mean and standard deviation of total collagen (a) and type I collagen (b) synthesis values (n=6; one-way ANOVA/Tukey tests; α=0.05). Different capital letters demonstrated significant difference between groups. The mean absorbance value of the SC-NR group was considered as 100%.

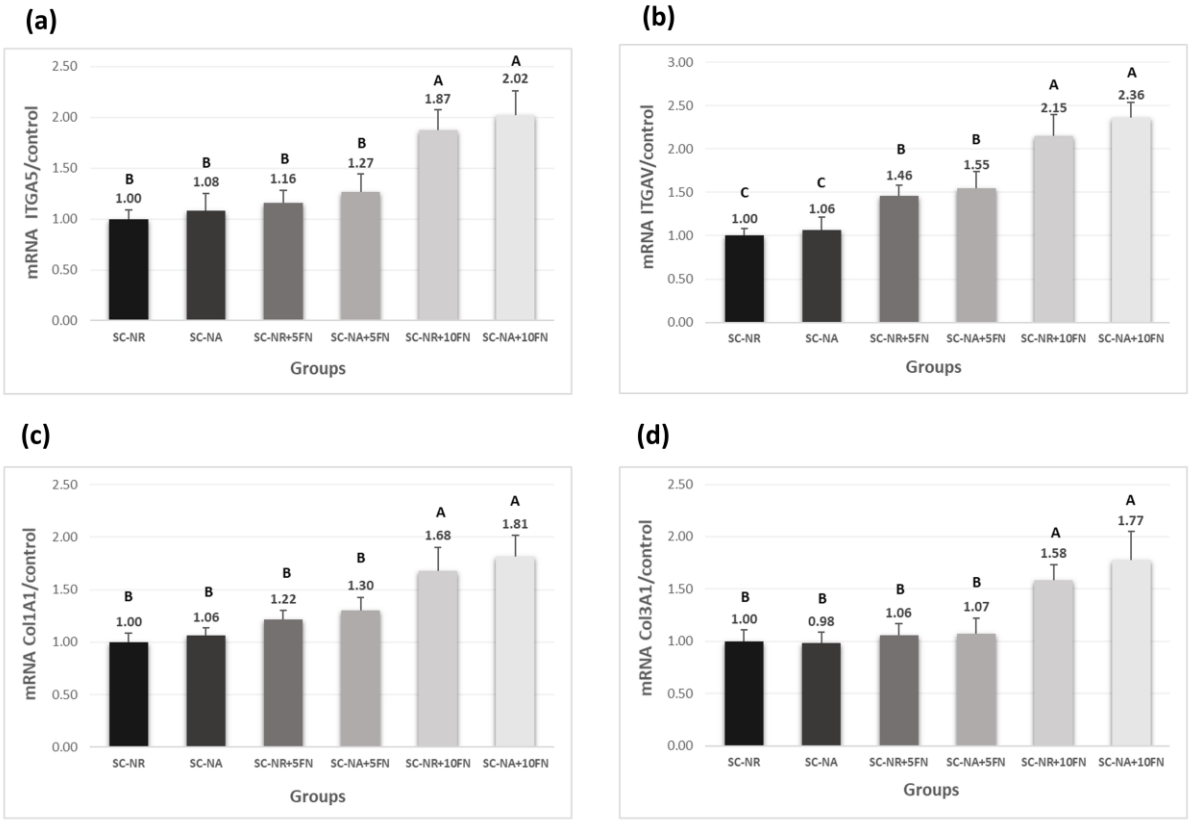


**Figure 13.** Mean and standard deviation of mRNA gene expression values of ITGA5 (a), ITGAV (b), COL1A1 (c) and COL3A1 (d) markers (n=4; one-way ANOVA/Tukey tests; $\alpha$=0.05). Different capital letters demonstrated significant difference between groups. The mean value of the SC-NR group was considered as 100%.

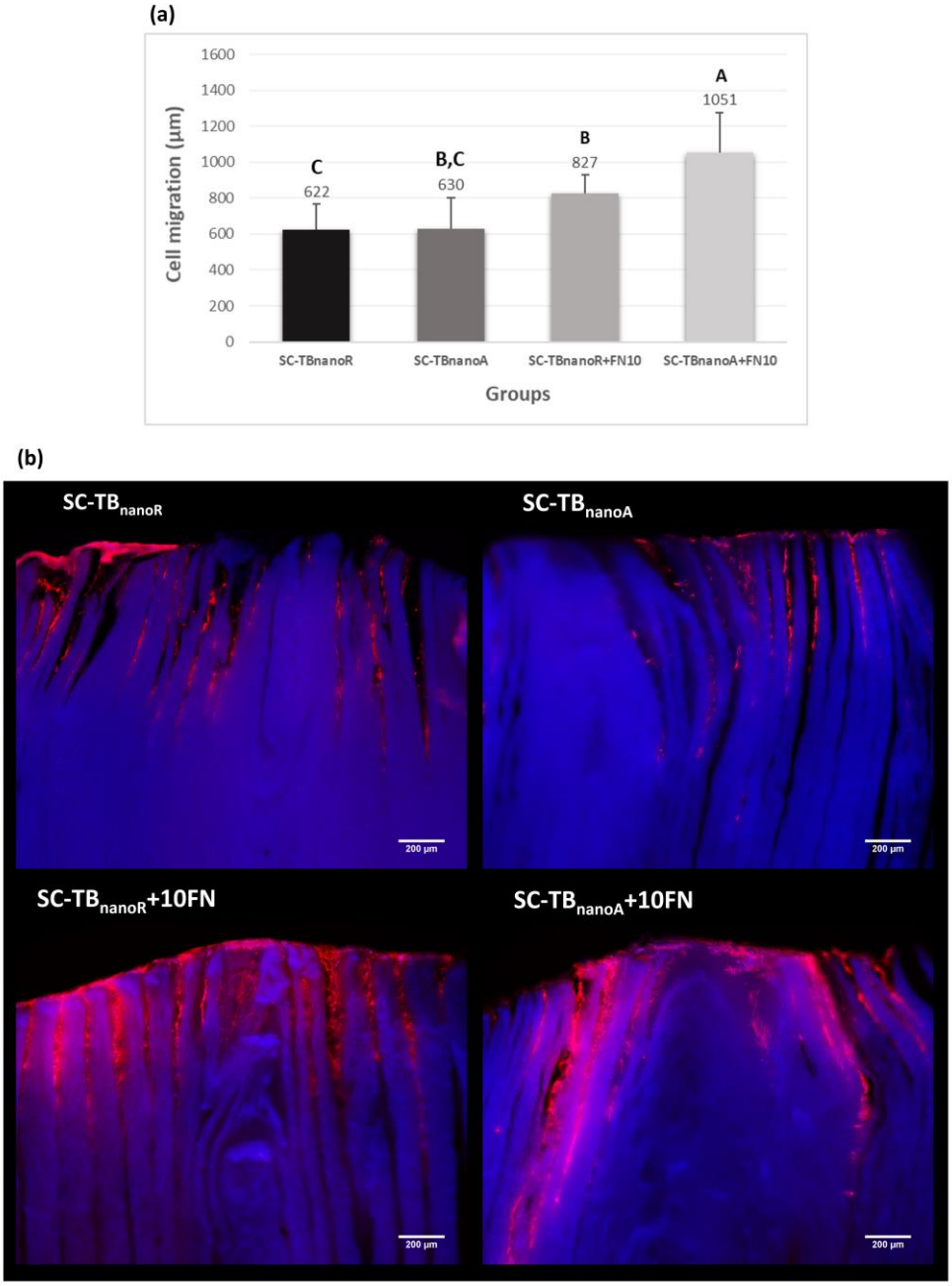


**Figure 14.** Cell migration assay on tubular nanofiber scaffolds. (a) Mean and standard deviation of distance (µm) covered by the cells inside the scaffolds (n=12; one-way ANOVA/Tukey tests; α=0.05). Different capital letters demonstrate significant difference among groups. (b) Representative images of the hAPCs migration (5x magnification). Actin filaments of the cells are shown in red fluorescence.

## 3.1 Publicação 3*

# Fibronectin-loaded Collagen/Gelatin Hydrogel is a Potent Signaling Biomaterial for Dental Pulp Regeneration

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**Declarations of interest:** none.

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**Fibronectin-loaded Collagen/Gelatin Hydrogel is a Potent Signaling Biomaterial for Dental Pulp Regeneration**

**Abstract**

**Introduction:** Guided tissue regeneration has been considered a promising biological strategy to replace conventional endodontic therapies of teeth with incomplete root formation. Therefore, in the present study, a collagen/gelatin hydrogel either containing dosages of fibronenctin (FN), or not, were developed and assessed concerning their bioactive and chemotactic potential on human apical papilla cells (hAPCs). **Methods**: Hydrogels were prepared by varying the ratio of collagen and gelatin (Col/Gel; v/v), and used to establish the following groups: Collagen (positive control); Col/Gel 4:6; Col/Gel 6:4; Col/Gel 8:2. The viability, adhesion and spreading of cells seeded on the hydrogels were evaluated. Different concentrations of FN (0, 5 or 10 µg/mL) were incorporated into the best formulation of the collagen/gelatin hydrogel selected. Then, the hAPCs seeded on the biomaterials were assessed concerning the cell migration, viability, adhesion and spreading, and gene expression of ITGA5, ITGAV, COL1A1 and COL3A1. **Results**: Col/Gel 8:2 group exhibited better cell viability, adhesion and spreading in comparison with Control. Higher values of hAPCs migration, viability, adhesion, spreading and gene expression of pulp regeneration markers were found, the higher the concentration was of FN incorporated into the collagen/gelatin hydrogel. **Conclusion**: Collagen/gelatin hydrogel with 10 µg/mL of FN had potent bioactive and chemotactic effects on cultured hAPCs.

**Keywords**: Hydrogel, Collagen, Gelatin, Fibronectin, Pulp regeneration.

## Introduction

Various protocols for guided regeneration of damaged tissue have been extensively assessed and successfully applied in diverse fields of health sciences, especially in medicine and dentistry. Overall, this innovative strategy associates biomaterials with potent signaling agents capable of stimulating the migration of cells to the implantation site (1,2). Furthermore, by means of specific biological mechanisms, an adequate biomaterial for guided tissue regeneration should be able to improve cell adhesion, migration, proliferation and differentiation, as well as stimulate the local synthesis and deposition of a new functional tissue (3,4).

Considering all scientific data available regarding cell induction therapies, one may suggest that this type of biological strategy could also be used for regenerating the pulp tissue of young teeth with incomplete root formation. For this purpose, a bioactive material should be inserted into the root canal to stimulate the inward migration and proliferation of apical papilla cells, in order to replace the lost connective tissue with a new pulp-like tissue (1,5). Duarte et al (6) demonstrated that 60 days after inducing pulp necrosis, a specific biomaterial used to fill the root canals induced the formation of vital pulp-like tissue in the apical third of roots. Several in vivo studies have also shown pulp-like tissue formation inside root canals that were filled with scaffolds containing cells, and then implanted into subcutaneous connective tissue of rats (7-10).

Researchers have reported that hydrogels are interesting alternatives to develop biomaterials for pulp regeneration because of their easy application technique and adequate adaptation in sites with irregular anatomy (11,12). In addition, hydrogels are very similar to extracellular matrix of connective tissues since they have high humidity and rheological properties that provide them with plasticity (13-15). Within tissue engineering strategies, collagen and gelatin have been considered as interesting natural sources to mimic the connective tissue (1,2,5). Since biocompatible hydrogels inserted into damaged tissues are gradually degraded and replaced by the newly regenerated tissue (5,15), the association of these biomaterials with potent signaling agents could be used for guided pulp tissue regeneration (1). Fibronectin (FN) is a high-molecular weight glycoprotein capable of improving cell adhesion, proliferation and differentiation when combined with scaffolds (16-19). Besides mediating a variety of cell interactions, FN also has strong chemotactic potential, inducing rapid migration of regional stem cells to the site of interest (16,19-21). Thus, clinical strategies, based on the concepts of bioengineering and pulp biology, in which hydrogels and FN are combined to develop a new biomaterial, seem to be an interesting alternative for the treatment of teeth with incomplete root formation. By these methods, pulp tissue can be regenerated, thus restoring the tooth vitality and ensuring continuity of the apical root formation (3,5,22). Therefore, the aim of this study was to develop a new biomaterial, by incorporating different

dosages of FN into a collagen/gelatin hydrogel formulation, and assessing their bioactive effects on human apical papilla cells (hAPCs).

## Materials and Methods

***Obtaining human apical papilla cells (hAPCs)***

This study was conducted after being approved by the Research Ethics Committee (Protocol Nº. 80806617.3.0000.5416; Araraquara School of Dentistry/UNESP, Brazil) and in compliance with the Declaration of Helsinki. Thus, patients of both sexes (16-18 years old) were asked to consent to donating four healthy third molars with incomplete root formation (Fig.1A), from which the hAPCs were obtained by using the enzymatic disaggregation technique (23). These cells were grown in α-MEM (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (GIBCO), antibiotic and antifungal (1% penicillin/streptomycin, 0.1% amphotericin; GIBCO) at 37°C and 5% $CO_2$. Cells from passage 3 to 5 were used in the experiments. Immunofluorescence multipotent stem cell markers were used to demonstrate the presence of a sub-population of apical papilla stem cells (APSCs) in the primary culture used in this study (Fig. 1B). Detailed methodology has been provided in the Supplementary Material.

***Biological evaluation of experimental hydrogels in hAPCs***

Type I collagen solution was set (3.67 mg/mL, rat trial; Corning, Bedford, MA, USA) and maintained at 4°C. Then, 5% gelatin solution (w/v; Sigma-Aldrich, Saint Louis, MO, USA) at 25ºC was prepared under magnetic stirring overnight. Collagen and gelatin (Col/Gel) solutions in the ratios (v/v) of 10:0, 4:6, 6:4 and 8:2 were obtained, establishing the following groups: Collagen (10:0 - positive control); Col/Gel 4:6; Col/Gel 6:4; Col/Gel 8:2. These solutions were dissolved in α-MEM 10x (3:1; v/v), followed by pH neutralization (5 M NaOH) at 4ºC. The hydrogels were applied on the bottom of wells of 96-well plates and incubated at 37ºC for 30 min. The hAPCs seeded on the gelled hydrogels ($2x10^4$ cells/well) were assessed relative to their viability (Alamar Blue; Live Dead), adhesion and spreading (F-actin) (24). Detailed methodology has been provided in the Supplementary Material.

***Chemotactic and bioactive potential of collagen/gelatin hydrogel associated with FN***

After adding different concentrations (0, 5 or 10 µg/mL) of FN (bovine plasma; Sigma-Aldrich) to collagen/gelatin hydrogel (8:2; v/v), the biomaterials were applied on the bottom of wells of 24 or 48-well plates and incubated at 37ºC for 1 h. The following groups were established: Collagen; Col/Gel; Col/Gel+5FN;

Col/Gel+10FN. As positive control, type I collagen-based hydrogel was used. The chemotactic potential of these biomaterials was assessed by active cell migration assay. The hAPCs seeded on the biomaterials ($1x10^5$ cells/well) were also evaluated relative to their viability, adhesion, spreading, and gene expression of ITGA5, ITGAV, COL1A1 and COL3A1 (Real-time Polymerase Chain Reaction) (24). Detailed methodology has been provided in the Supplementary Material.

***Statistical analysis***

After analyzing the normality and homoscedasticity, the data were submitted to one-way ANOVA and Tukey tests. *P*-values <0.05 were considered statistically significant. The data were expressed as the mean and standard deviation (SD) of three independent experiments (**P*<0.05, ***P*<0.01). The statistical power of the samples was calculated for each analysis using the DSS Research calculator (> 95.0%). The images of cell viability, adhesion and spreading were analyzed descriptively.

## Results

***Biological evaluation of experimental hydrogels in hAPCs***

Cell viability in Col/Gel 8:2 and Collagen (Control) groups was similar at 1, 3 and 7-day time intervals (*P*<0.05; Fig. 2A). The increase in gelatin ratio in the hydrogel formulations reduced the viability of hAPCs in comparison with the control (*P*<0.05). The Live/Dead assay images showed the cytocompatibility of all hydrogel formulations tested, and that cell proliferation occurred in Col/Gel 6:4, Col/Gel 8:2 and Control groups over time (Fig. 2B). However, only Col/Gel 8:2 group showed cell adhesion and spreading similar to those of control (Fig. 2C).

***Chemotactic and bioactive potential of collagen/gelatin hydrogel associated with FN***

Overall, cells seeded on the upper surface of the insert were capable of migrating to achieve the lower surface of this device. However, the hAPCs only reached the surfaces of FN-loaded biomaterials (Fig. 3A). Although the number of cells was higher in Col/Gel and Control groups than in the Col/Gel+FN5 and Col/Gel+FN10 groups (*P*<0.05; Fig. 3B), the highest number of cells capable of migrating through the FluoroBlok membrane of the inserts to reach the biomaterial surface was observed in Col/Gel+FN10 group (Fig. 3A). The Alamar Blue assay demonstrated no difference in cell viability between Collagen (control) and Col/Gel groups (*P*>0.05); however, the incorporation of FN into the hydrogels increased the cell viability in all time

intervals ($P<0.05$; Fig. 3C). These results were confirmed by the Live/Dead and F-actin assays, in which the highest values of cell viability/proliferation and adhesion/spreading, respectively, occurred in Col/Gel+FN10 group (Figs. 3D/E). The high amount of hAPCs observed on all hydrogel formulations, determined the cytocompatibility of these biomaterials. Furthermore, the incorporation of 10 µg/mL of FN into the hydrogel (Col/Gel+FN10 group) enhanced the gene expression of ITGA5, ITGAV, COL1A1 and COL3A1 in comparison with the Control group ($P<0.05$; Fig. 4A-D).

## Discussion

In clinical situations of irreversible pulpitis or pulp necrosis of teeth with incomplete root formation, preliminary therapies have been recommended to induce fast apexification. After this, the conventional endodontic treatment is performed, by filling the root canals with synthetic materials (5). However, these procedures interrupt the continuity of root formation, compromising not only the capacity of the root wall to support masticatory force, but the tooth stability as well (22). Based on these facts, strategies for pulp regeneration using concepts of tissue engineering and pulp biology have been introduced as interesting alternatives to recover the tooth vitality and allow the continuity of root formation (3,5,12,22). Therefore, in the present study, collagen/gelatin hydrogels either associated with FN, or not, were developed and then assessed relative to their bioactive and chemotactic effects on hAPCs.

Firstly, the hydrogels were prepared using two natural polymers: type I collagen and gelatin. Based on the Live/Dead assay employed in this in vitro study, in which a high number of viable cells remained attached to all hydrogels formulations tested, one can consider that both proteins have cytocompatibility with the hAPCs. Type I collagen is a great source for biomaterial development since it can mimic the fibrous environment of the extracellular matrix, and represents 90% of collagenous proteins in live organisms (25). Considering that collagenous biomaterials can improve the adhesion, spreading, migration, proliferation and differentiation of cells (4,11,23,26), collagen has been regarded as a bioactive agent that plays a role in tissue repair and remodeling tissue processes (20,27,28). However, the relatively high cost of this natural polymer has increased the demand for other natural sources for application in tissue regeneration strategies (29). Gelatin has a wide range of bioavailability from a wide range of animal tissues, low cost, and has the properties of biocompatibility and biodegradability, which make it useful for development of new biomaterials (30,31). Therefore, formulations of hydrogel containing different ratios of collagen and gelatin (4:6, 6:4, 8:2; v/v) were initially tested in the present study, in order to select the formulation with the most interesting biological properties as regards effect

on hAPCs. Since the optimal formulation should have characteristics similar to type I collagen-based hydrogel, this biomaterial was used as positive control.

In addition to the positive influences on angiogenesis and tissue development/neoformation, collagen provides mechanical resistance/strength to connective tissue (17,28). Therefore, low concentrations of collagen incorporated into a hydrogel may result in fast degradation of the biomaterial, compromising its biological properties (32). Despite the increased cell proliferation observed in this study for Groups Col/Gel 6:4 and Col/Gel 8:2 over the course of the time intervals, only in the Col/Gel 8:2 group the hAPCs exhibited viability, adhesion and spreading comparable with the values found for the Control group (Collagen). Therefore, the higher the ratio of gelatin incorporated into the hydrogel, the lower was the capacity of this biomaterial to act as a temporary scaffold for cell proliferation, adhesion and spreading. This result could, at least partially, be explained by the low resistance to hydrolytic degradation attributable to gelatin, which is a product of partial collagen hydrolysis (33). Therefore, since Col/Gel 8:2 group exhibited the best biological properties on hAPCs comparable to Control group (Collagen), this collagen/gelatin hydrogel formulation was selected for incorporation of different concentrations of FN.

Previous studies have shown that FN modulates the migration of NIH 3T3 fibroblasts (20) and induces myofibroblast proliferation, migration and differentiation (16). FN is known to interact synergistically as an anchoring point for cells and as a signaling agent, because when it is detected by membrane receptors (integrins), it activates specific transduction pathways to control cell adhesion, migration, proliferation and differentiation (21). These data may explain the results of the present study, in which the higher levels of migration, proliferation, adhesion and spreading of cells occurred the higher was the concentration of FN incorporated into the collagen/gelatin hydrogel. In a recent study, Bullard et al (17) assessed the bioactivity of dehydrated human umbilical cord (dHUC), which has type I collagen and FN in its composition. The authors showed that dHUC induced the angiogenesis, and the migration and proliferation of undifferentiated mesenchymal cells. This biomaterial also exhibited biocompatibility and biodegradability when implanted into connective subcutaneous tissue of rats, determining the fundamental role of collagen and FN in the regeneration and remodeling of connective tissue.

For improved understanding of the mechanism of action of the experimental FN-loaded biomaterials developed in the present study, the gene expression of α5 integrin (ITGA5), αV integrin (ITGV), type I collagen (COL1A1) and type III collagen (COL3A1) was assessed. The best results were observed for Group Col/Gel+10FN that showed the highest values for all genes tested. The interaction of α5β1 and αvβ3 integrins in

the detection of FN has previously been demonstrated. Although these membrane receptors have distinct intra-cellular biological mechanisms, both act in the process of detection of specific ligands that trigger specific cell signals related to cell migration (19). Furthermore, the joint action of integrins allows the cells to adapt to the substrate coated with FN, favoring cell adhesion (18,19). Moreover, Spoerri et al (34) observed that when α5β1 and αVβ3 integrins were activated by protease-activated receptors through different signaling pathways, they changed their conformation to allow their high affinity for FN, thereby accelerating cell adhesion, spreading and migration. In addition to mediating the deposition of collagen fibers in the extracellular matrix during tissue repair (27,28), this protein, added to a chitosan-based hydrogel, enhanced the expression and synthesis of type I and type III collagen by dental pulp-mesenchymal stem/stromal cells (35).

In summary, the incorporation of FN (10 µg/mL) into the collagen/gelatin hydrogel formulation resulted in a biomaterial with potent chemotactic and bioactive action on hAPCs. In view of the promising scientific data obtained in this study, this new biomaterial, applied inside the root canal would be expected to stimulate the migration of hAPCs and to act as a temporary scaffold for new local pulp-like connective tissue formation. Thus, this biological strategy might allow the physiologic continuity of root formation. However, given the limitations of this study, and based on the fact that data from laboratory investigations cannot be directly extrapolated to clinical conditions, further in vitro and in vivo studies are needed to assess the possibility of using this biomaterial for guided pulp regeneration of endodontically compromised teeth with incomplete root formation.

**Acknowledgments**

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The authors deny any conflicts of interest related to this study.

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**Figures**

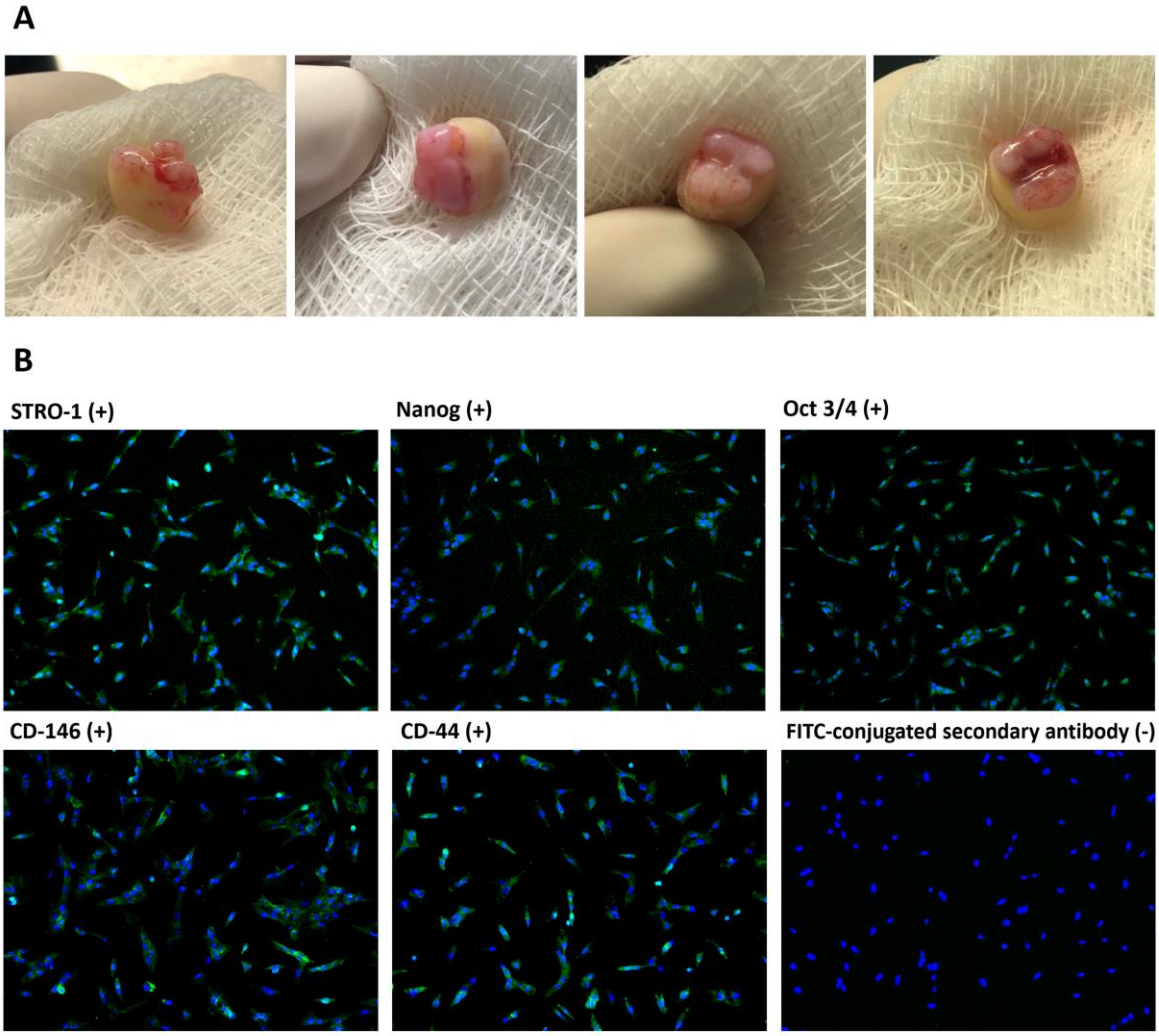


**Figure 1.** (A) Third human molars with incomplete root formation used to obtain the hAPCs. (B) Immunofluorescence assay for multipotent stem cell markers (STRO-1, Nanog, Oct-3/4, CD-146 and CD-44). Green fluorescence (FITIC) indicates positive staining for stem cells. Blue fluorescence indicates cell nucleus. hAPCs incubated only with FITC-conjugated secondary antibody (-) represent negative control.

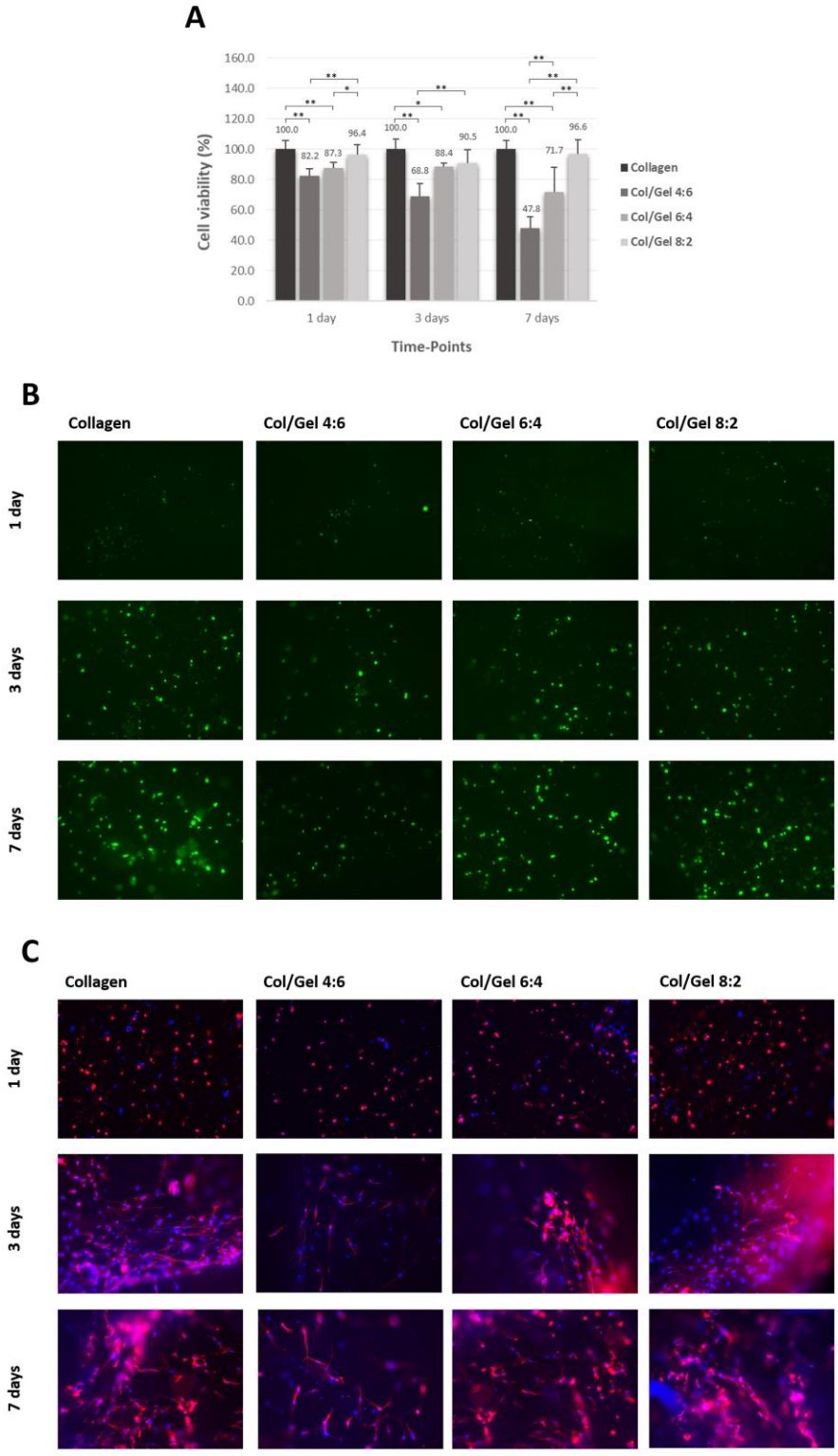


**Figure 2.** (A) Alamar Blue assay. Mean and SD of the cell viability values (%) of groups in time intervals (one-way ANOVA/Tukey tests, α=0.05; n=6). ⌐¬ indicates a significant difference between groups at each time-point (*P<0.05, **P<0.01). (B) Live/Dead assay. Green fluorescence indicates live cells and red fluorescence indicates dead cells (10x magnification; n=4). (C) Cell adhesion and spreading. Red fluorescence indicates actin filaments and blue fluorescence indicates cell nucleus (10x magnification; n=4).

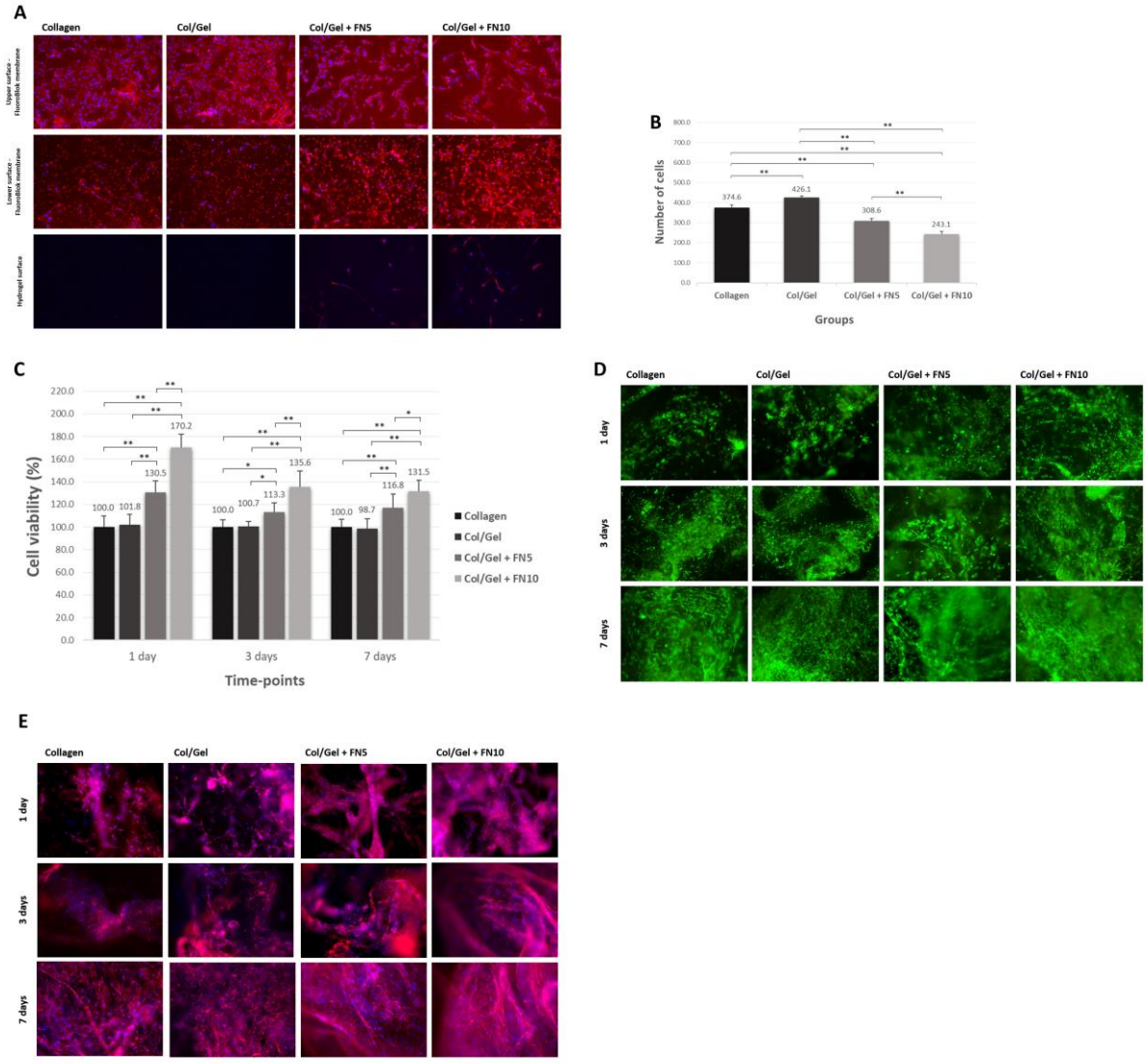


**Figure 3.** (A) Active cell migration assay. Images of upper and lower surfaces of FluoroBlok membrane, and biomaterials containing FN or without FN. Red fluorescence indicates the cytoskeleton of cells and blue fluorescence indicates cell nucleus (10x magnification). (B) Mean and SD of number of cells on upper surface of FluoroBlock inserts according to the group (one-way ANOVA/Tukey tests, α=0.05; n=4) (C) Alamar Blue assay. Mean and SD of cell viability values (%) of groups in time intervals (one-way ANOVA/Tukey tests, α=0.05; n=8). ⌐¬ indicates significant difference between groups (*P<0.05, **P<0.01). (D) Live/Dead assay. Green fluorescence indicates live cells and red fluorescence indicates dead cells (10x magnification; n=4). (E) Cell adhesion and spreading. Red fluorescence indicates actin filaments and blue fluorescence indicates cell nucleus (10x magnification; n=4).

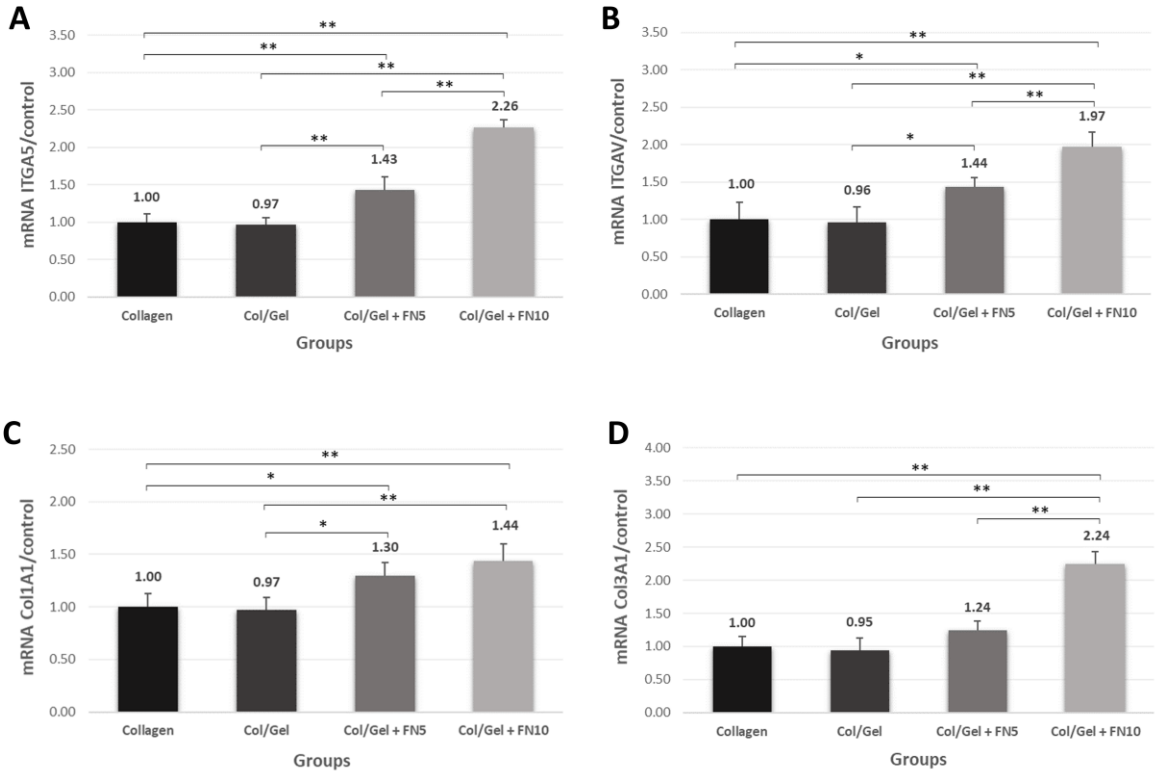


**Figure 4.** ITGA5 (A), ITGAV (B), COL1A1 (C) and COL3A1 (D) mRNA gene expression. Mean and SD of values of mRNA/control (one-way ANOVA/Tukey tests, α=0.05; n=4). ⌐¬ indicates significant difference between groups (**P*<0.05, ***P*<0.01).

## Supplementary Material

### Materials and Methods

**Immunofluorescence Assay:** After obtaining the hAPCs, the cells (passage #3) were characterized for the expression of multipotent stem cell markers (STRO-1, Nanog, Oct-3/4, CD-146 and CD-44). For this purpose, hAPCs ($6x10^4$/coverslips) were seeded on round glass coverslips (13 mm; Perfecta, São Paulo, SP, Brazil) placed in 24-well plates (Corning, Tewksbury, MA, USA) and incubated at 37°C and 5% $CO_2$ for 24 h. Then, these cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), either permeabilized or not with 0.1% Triton X-100 (Sigma-Aldrich), followed by block of non-specific biding sites with 5% bovine serum albumin (BSA; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min. The hAPCs were incubated with the respective primary antibody (1:50, 5% BSA; Santa Cruz Biotecnology) at 4ºC overnight. After this step, they were washed and incubated with FITC-conjugated secondary antibody (1:100, 1% BSA; Santa Cruz Biotecnology) for 1 h, followed by nuclear counter-staining with Hoescht (1:5000; Invitrogen, Eugene, Oregon, USA) at room temperature, for 15 min. Images were obtained in fluorescence microscope (Leica DM 5500B, Nussloch GmbH, Nussloch, Germany) (23).

**Active Cell Migration Assay:** For this assay, hAPCs ($3x10^4$) were seeded on the upper surface of FluoroBlok cell culture inserts (8 μm pores; Corning) and incubated at 37°C and 5% $CO_2$ for 2 h. Then, the lower surface of the inserts was placed in close contact with the hydrogel applied in the bottom of 24-well plate (n=4). After 24 hours, the cells on both upper and lower surfaces of the inserts, as well as on the hydrogels, were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and incubated with a specific fluorescent probe in 2% BSA (1:20; Actin Red 555, Life Technologies, Grand Island, NY, USA) for cytoskeleton detection. Then, incubation with Hoescht (1:5000; Invitrogen) was performed for nuclear counter-marking. Fluorescence microscope images (Leica DM 5500B) of the FluoroBlok membrane (upper and lower surfaces) and hydrogels were obtained for descriptive analysis of the active cell migration assay. For quantitative analysis, 4 images were obtained in different fields on the upper surface of each insert in order to count the number of cells (ImageJ software) (23). The arithmetic average of the values obtained was determined for each sample, with this data being considered an experimental unit (n=4/group).

**Alamar Blue Assay:** After time intervals of 1, 3 and 7 days, cells on hydrogel (n=8) were incubated with α-MEM supplemented with 10% Alamar Blue solution (Thermo Fisher Scientific, Waltham, MA, USA) at 37ºC

and 5% $CO_2$ for 4 h. Then, an aliquot of this solution was transferred to 96-well plate and the fluorescence was read at 570 nm excitation and 610 nm emission (Synergy H1; BioTek, Winooski, VT, USA). The positive control in each time-point was considered as 100%.

**Live/Dead Assay:** After time intervals of 1, 3 and 7 days, cells on hydrogel (n=4) were incubated α-MEM supplemented with 4 mmol/L ethyl homodimer-1 and 2 mmol/L calcein AM (Live/Dead Viability/Cytotoxicity Kit; Invitrogen, Carlsbad, CA, USA) at 37ºC and 5% $CO_2$ for 30 min. The hydrogel containing the cells was analyzed under a fluorescence microscope (Leica DM 5500B).

**F-actin Assay**: After time intervals of 1, 3 and 7 days, cells on hydrogel were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) and incubated with specific fluorescent probe to actin filaments staining (1:20; ActinRed$^{TM}$ 555 ReadyProbesTM reagent; Life Technologies) in 2% BSA (Santa Cruz Biotechnology) for 30 min. Then, incubation with Hoescht (1:5000; Invitrogen) in phosphate-buffered saline was performed to stain cell nucleus for 15 min. The hydrogel containing the cells was analyzed under a fluorescence microscope (Leica DM 5500B).

**Real-time Polymerase Chain Reaction (PCR) Assay:** After a time interval of 7 days, the total RNA (n=4) of the cells was extracted using the RNAqueous-Micro Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Next, cDNA synthesis was performed from 500 ng of RNA from each sample using the reverse transcription technique, following the instructions of the supplier of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using a set of pre-designed primers and probes (Gene expression assays, Applied Biosystems) to detect target genes (ITGA5, ITGAV, COL1A1, COL3A1) using the TaqMan system (TaqMan Universal PCR Master Mix, Applied Biosystems) with StepOne Plus equipment (Applied Biosystems) and using the cycling conditions optimized by the manufacturer. The cycle threshold (CT) values for each sample were calculated using the thermal cycler software and analyzed using the $2^{\Delta\Delta CT}$ method. The results were normalized for the expression of the constitutive gene (GAPDH) and expressed as a fold change in relation to the positive control (Collagen Group).

## 4 CONCLUSÕES

De acordo com as metodologias empregadas no presente estudo laboratorial, os quais resultaram na preparação dos artigos científicos 1*, 2* e 3* que foram submetidos para publicação em periódicos qualificados pela CAPES e de forte impacto para a área do conhecimento, foi possível concluir que:

1. As concentrações de 5 e 10 µg/mL de fibronectina pode atuar como um potente agente bioativo sobre hAPCs, estimulando a adesão, proliferação e espalhamento celular, bem como a síntese de colágeno e expressão de genes relacionados à regeneração pulpar;

2. Scaffolds tubulares, formados por nanofibras alinhadas a base de 10% de poli-caprolactona e revestidas com uma dose quimiotática e bioativa de fibronectina (10 µg/mL) para as hAPCs, apresentou-se como um promissor biomaterial com potencial para ser aplicado na regeneração pulpar de dentes com rizogênese incompleta;

3. A incorporação de fibronectina (10 µg/mL) num hidrogel à base de colágeno/gelatina na proporção de 8:2, resultou no desenvolvimento de um biomaterial com ação quimiotática e bioativa sobre hAPCs, o qual tem potencial para ser aplicado no preenchimento do canal radicular de dentes com rizogênese incompleta e estimular a regeneração do tecido pulpar.

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* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf

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# ANEXOS

**ANEXO A** - COMITÊ DE ÉTICA

Aprovação do Projeto pelo Comitê de Ética em Pesquisa da Faculdade de Odontologia de Araraquara – FOAr/UNESP.

UNESP - FACULDADE DE ODONTOLOGIA - CAMPUS ARARAQUARA

**PARECER CONSUBSTANCIADO DO CEP**

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** Síntese e avaliação de biomateriais associados a proteínas da matriz extracelular para a regeneração do tecido pulpar

**Pesquisador:** Carlos Alberto de Souza Costa

**Área Temática:**

**Versão:** 5

**CAAE:** 80806617.3.0000.5416

**Instituição Proponente:** Faculdade de Odontologia de Araraquara - UNESP

**Patrocinador Principal:** Financiamento Próprio

**DADOS DA NOTIFICAÇÃO**

**Tipo de Notificação:** Envio de Relatório Parcial

**Detalhe:**

**Justificativa:** Relatório Parcial para acompanhamento do projeto de pesquisa em andamento.

**Data do Envio:** 27/10/2020

**Situação da Notificação:** Parecer Consubstanciado Emitido

**DADOS DO PARECER**

**Número do Parecer:** 4.402.834

**Conclusões ou Pendências e Lista de Inadequações:**

Sem pendências.

**Considerações Finais a critério do CEP:**

Relatório Parcial APROVADO em reunião de 13 de novembro de 2020.

**Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

| Tipo Documento | Arquivo | Postagem | Autor | Situação |
|---|---|---|---|---|
| Envio de Relatório Parcial | Relatorio_Parcial_2.pdf | 27/10/2020 18:48:39 | Carlos Alberto de Souza Costa | Postado |

**Situação do Parecer:**

Aprovado

**Necessita Apreciação da CONEP:**

Não

ARARAQUARA, 17 de Novembro de 2020

**Assinado por:**
**Andréa Gonçalves**
**(Coordenador(a))**

**ANEXO B -** PUBLICAÇÃO 1

Comprovante da situação da Publicação 1* no Journal of Applied Oral Science.

Journal of Applied Oral Science

JAOS
JOURNAL OF APPLIED ORAL SCIENCE

**Bioactivity effects of extracellular matrix proteins on apical papilla cells**

| | |
|---|---|
| Journal: | *Journal of Applied Oral Science* |
| Manuscript ID | JAOS-2021-0038.R1 |
| Manuscript Type: | Original Article |
| Please use keywords available at <a href= 'http://decs.bvs.br/' target='new'> http://decs.bvs.br/</a>.: | Guided Tissue Regeneration, Dental pulp, Fibronectin, Laminin, Collagen |
| | |

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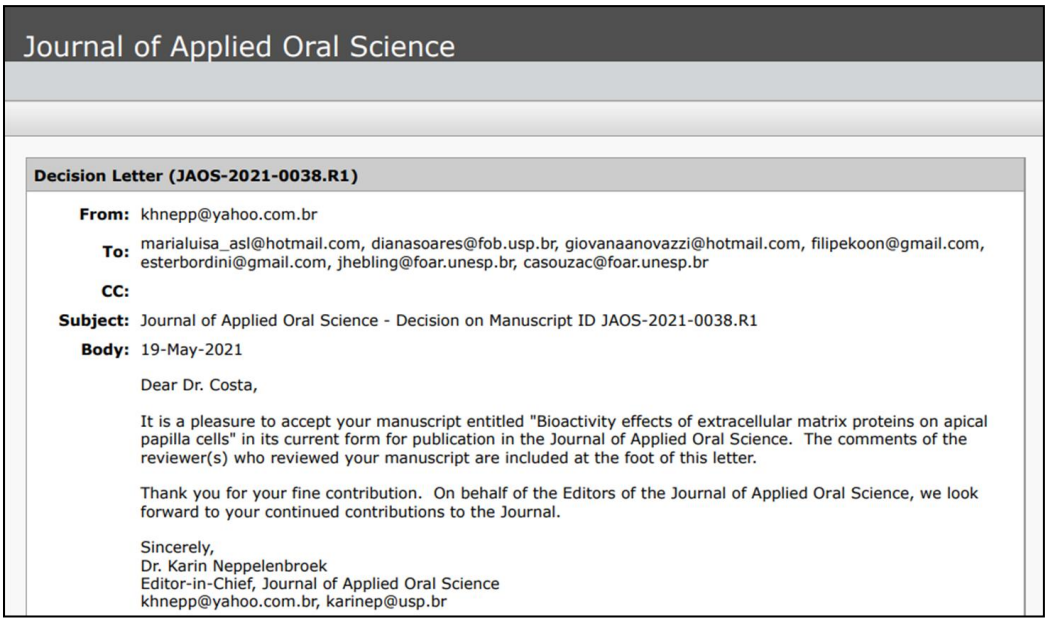

Journal of Applied Oral Science

**Decision Letter (JAOS-2021-0038.R1)**

**From:** khnepp@yahoo.com.br

**To:** marialuisa_asl@hotmail.com, dianasoares@fob.usp.br, giovanaanovazzi@hotmail.com, filipekoon@gmail.com, esterbordini@gmail.com, jhebling@foar.unesp.br, casouzac@foar.unesp.br

**CC:**

**Subject:** Journal of Applied Oral Science - Decision on Manuscript ID JAOS-2021-0038.R1

**Body:** 19-May-2021

Dear Dr. Costa,

It is a pleasure to accept your manuscript entitled "Bioactivity effects of extracellular matrix proteins on apical papilla cells" in its current form for publication in the Journal of Applied Oral Science. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Journal of Applied Oral Science, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Karin Neppelenbroek
Editor-in-Chief, Journal of Applied Oral Science
khnepp@yahoo.com.br, karinep@usp.br

**ANEXO C** - PUBLICAÇÃO 2

Comprovante da situação da Publicação 2* no Journal of Biomedical Materials Research: Part B - Applied Biomaterials.

Received: 16 July 2020 | Revised: 28 September 2020 | Accepted: 8 December 2020
DOI: 10.1002/jbm.b.34785

ORIGINAL RESEARCH REPORT

# Development of fibronectin-loaded nanofiber scaffolds for guided pulp tissue regeneration

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**Funding information**
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Numbers: 302047/2019-0, 408721/2018-9; Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant/Award Numbers: 2017/14210-8, 2017/22739-9

**Abstract**

Fibronectin (FN)-loaded nanofiber scaffolds were developed and assessed concerning their bioactive potential on human apical papilla cells (hAPCs). First, random (NR) and aligned (NA) nanofiber scaffolds of polycaprolactone (PCL) were obtained by electrospinning technique and their biological properties were evaluated. The best formulations of NR and NA were loaded with 0, 5, or 10 μg/ml of FN and their bioactivity was assessed. Finally, FN-loaded NR and NA tubular scaffolds were prepared and their chemotactic potential was analyzed using an in vitro model to mimic the pulp regeneration of teeth with incomplete root formation. All scaffolds tested were cytocompatible. However, NR and NA based on 10% PCL promoted the highest hAPCs proliferation, adhesion and spreading. Polygonal and elongated cells were observed on NR and NA, respectively. The higher the concentration of FN added to the scaffolds, greater cell migration, viability, proliferation, adhesion and spreading, as well as collagen synthesis and gene expression (ITGA5, ITGAV, COL1A1, COL3A1). In addition, tubular scaffolds with NA loaded with FN (10 μg/ml) showed the highest chemotactic potential on hAPCs. It was concluded that FN-loaded NA scaffolds may be an interesting biomaterial to promote hAPCs-mediated pulp regeneration of endodontically compromised teeth with incomplete root formation.

**KEYWORDS**
apical papilla cells, fibronectin, nanofibers, pulp regeneration, tissue engineering

## 1 | INTRODUCTION

The integrity of dental enamel and dentin is important for maintaining both the function and esthetic appearance of teeth, and once this is damaged, the dentin-pulp complex is exposed to several injuries. These may be caused by microbial, traumatic, and iatrogenic stimuli as well as the toxic effects caused by dental materials and their components.[1] When teeth with incomplete root formation are subjected to intense pulp damage capable of causing irreversible pulpitis or even necrosis, specific endodontic therapy is necessary to promote physiological apexification. In this clinical situation, all pulp tissue should be mechanically/chemically removed and the root canal filled with an intracanal medication that stimulates biological sealing of the root apex through mineralized tissue deposition.[2] This procedure interrupts the physiological apexification, which may compromise the stability of teeth and root wall resistance to masticatory efforts. Thus, different tissue regeneration strategies have appeared as promising alternatives to ensure the biological continuity of apical root formation.[3,4]

Cell-free therapy, which consist of using biomaterials associated with potent signaling agents for guided tissue regeneration, has been widely evaluated in the last decades. When introduced into the damaged tissue area, one may expect that such biomaterials can induce migration of resident cells as well as stimulate the adhesion, proliferation, and differentiation of cells capable of synthesizing a new local tissue.[5] This modality of therapy appears to be very promising for

  

**ANEXO D** - PUBLICAÇÃO 3

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BASIC RESEARCH – BIOLOGY

# Fibronectin-loaded Collagen/ Gelatin Hydrogel Is a Potent Signaling Biomaterial for Dental Pulp Regeneration

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## ABSTRACT

**Introduction:** Guided tissue regeneration has been considered a promising biological strategy to replace conventional endodontic therapies of teeth with incomplete root formation. Therefore, in the present study, a collagen/gelatin hydrogel either containing dosages of fibronectin (FN), or not, was developed and assessed concerning their bioactive and chemotactic potential on human apical papilla cells (hAPCs). **Methods:** Hydrogels were prepared by varying the ratio of collagen and gelatin (Col/Gel; v/v), and used to establish the following groups: Collagen (positive control); Col/Gel 4:6; Col/Gel 6:4; Col/Gel 8:2. The viability, adhesion, and spreading of cells seeded on the hydrogels were evaluated. Different concentrations of FN (0, 5, or 10 μg/mL) were incorporated into the best formulation of the collagen/gelatin hydrogel selected. Then, the hAPCs seeded on the biomaterials were assessed concerning the cell migration, viability, adhesion and spreading, and gene expression of ITGA5, ITGAV, COL1A1, and COL3A1. **Results:** The Col/Gel 8:2 group exhibited better cell viability, adhesion and spreading in comparison with Control. Higher values of hAPC migration, viability, adhesion, spreading and gene expression of pulp regeneration markers were found, the higher the concentration was of FN incorporated into the collagen/gelatin hydrogel. **Conclusion:** Collagen/gelatin hydrogel with 10 μg/mL of FN had potent bioactive and chemotactic effects on cultured hAPCs. *(J Endod 2021;■:1–6.)*

## SIGNIFICANCE

This new biomaterial, applied inside the root canal of teeth with incomplete root formation, would be expected to stimulate the migration of human apical papilla cells (hAPCs) and to act as a temporary scaffold for new local pulp-like connective tissue formation.

## KEY WORDS

Hydrogel; collagen; gelatin; Fibronectin; pulp regeneration

Various protocols for guided regeneration of damaged tissue have been extensively assessed and successfully applied in diverse fields of health sciences, especially in medicine and dentistry. Overall, this innovative strategy associates biomaterials with potent signaling agents capable of stimulating the migration of cells to the implantation site[1,2]. Furthermore, by means of specific biological mechanisms, an adequate biomaterial for guided tissue regeneration should be able to improve cell adhesion, migration, proliferation, and differentiation, as well as stimulate the local synthesis and deposition of a new functional tissue[3,4].

Considering all scientific data available regarding cell induction therapies, one may suggest that this type of biological strategy could also be used for regenerating the pulp tissue of young teeth with incomplete root formation. For this purpose, a bioactive material should be inserted into the root canal to stimulate the inward migration and proliferation of apical papilla cells, to replace the lost connective tissue with a new pulp-like tissue[1,5]. Duarte et al[6] demonstrated that 60 days after inducing pulp necrosis, a specific biomaterial used to fill the root canals induced the formation of vital pulp-like tissue in the apical third of roots. Several *in vivo* studies have also shown pulp-like tissue formation inside root canals that were filled with scaffolds containing cells, and then implanted into subcutaneous connective tissue of rats[7–10].

Researchers have reported that hydrogels are interesting alternatives to develop biomaterials for pulp regeneration because of their easy application technique and adequate adaptation in sites with irregular anatomy[11,12]. In addition, hydrogels are very similar to extracellular matrix of connective tissues because they have high humidity and rheological properties that provide them with plasticity[13–15]. Within tissue engineering strategies, collagen and gelatin have been considered as interesting natural sources to mimic the connective tissue[1,2,5]. Because biocompatible hydrogels inserted into damaged tissues are

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