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AVANÇOS BIOTECNOLÓGICOS: USO DE PEPTÍDEOS DE AUTO MONTAGEM E DISPOSITIVOS MICROFLUÍDICOS "TOOTH-ON-A-CHIP"APLICADOS À ODONTOLOGIA. ESTUDO *IN VITRO* E REVISÃO DE ESCOPO.

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BIOTECHNOLOGICAL ADVANCES: USE OF SELF-ASSEMBLING PEPTIDES AND TOOTH-ON-A-CHIP MICROFLUIDIC DEVICES APPLIED TO DENTISTRY. IN VITRO STUDY AND SCOPING REVIEW

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Orientador: Prof. Dra. Regina Maria Puppin Rontani
Coorientador: Prof. Dr. Lourenço Correr Sobrinho

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RESUMO

Avanços biotecnológicos como a produção de peptídeo de auto montagem, P_{11}-4, tem sido amplamente estudado em diferentes aplicações que vão desde materiais surfactantes até a regeneração de tecidos. Este peptídeo tem como vantagem a capacidade de se auto montar quando submetido a queda do pH, sendo dose dependente. Na odontologia, já foi comprovado seu uso para remineralização do esmalte dentário e em estudos mais recentes, o uso na dentina como um biomodificador capaz de aumentar a espessura das fibrilas colágenas e diminuir a degradação do colágeno. Além disso, o mesmo provou não ser citotóxico às células semelhante a odontoblastos MDPC23 e estimular a migração celular. Nesse sentido, a primeira parte desta tese investigou o efeito do P_{11}-4 em baixas concentrações (<1 mg/ml) sobre células tronco da papila apical (SCAP). Para tal, neste estudo, foram realizados testes de citotoxicidade pelo uso do reagente MTT nos tempos experimentais de 24, 48 e 72 hs. Para a análise de deposição e quantificação dos nódulos minerais, foram utilizados o corante de alizarina e cloreto de cetilpiridínio, respectivamente, para o tempo experimental de 30 dias. E por fim, para a análise da expressão gênica, os osteo marcadores RUNX2, ALP e OCN foram analisados em termocicladora com leitor de fluorescência pelo método da amplificação em tempo real (qPCR) no tempo experimental 3 e 7 dias. Na segunda parte do presente trabalho foi realizada uma revisão de escopo sobre dispositivos microfluídicos e seu uso na área odontológica com materiais dentários. Essa tecnologia se apresenta como uma boa alternativa às limitações impostas pelo uso de testes in vitro em cultura 2D. O objetivo deste artigo foi identificar os possíveis *gaps* dessa nova metodologia em ascensão na área odontológica, que em sua maior parte, vem sendo utilizada para testes de citotoxicidade de materiais dentários. De maneira geral, na primeira parte do trabalho de tese, o peptídeo P_{11}-4 em baixas concentrações (10 μg/ml, 100 μg/ml and 1 mg/ml) não demonstrou toxicidade às células e induziu maior deposição mineral na concentração de 100 μg/ml. Os resultados de expressão gênica demonstraram que o uso na concentração de 10 μg/ml foi suficiente para causar ligeiro aumento dos níveis expressos de RUNX2 e OCN em 3 dias, apesar de diminuir a expressão do gene ALP no tempo de 3 e 7 dias. Já os resultados que se referem à revisão de escopo, podemos concluir que as limitações encontradas ao uso dos chips em odontologia são: ausência de padronização na fabricação dos dispositivos, aquisição e validação dos resultados, que também influenciam com a restrita funcionalidade dos mesmos. O alto custo e a demanda de profissionais qualificados demonstram ser os principais fatores na baixa publicação de artigos utilizando-se desta tecnologia.

Palavras-chave: peptídeos; células-tronco, microfluídica

ABSTRACT

Biotechnological advances such as the production of a self-assembling peptide, P_{11}-4, have been widely studied in different applications ranging from surfactant materials to tissue regeneration. This peptide has the advantage of being able to self-assemble when submitted to a drop in pH, being dose dependent. In dentistry, its use has been proven to remineralize dental enamel, and in more recent studies, its use in dentin as a bio modifier able to increase the thickness of collagen fibrils and decrease collagen degradation. Moreover, it has proven to be non-cytotoxic to MDPC23 odontoblast-like cells and stimulate cell migration. In this sense, the first part of this dissertation investigated the effect of P_{11}-4 at low concentrations (<1 mg/ml) on stem cells of the apical papilla (SCAP). For this purpose, in this study, cytotoxicity tests were performed by the use of the MTT reagent at the experimental times of 24, 48 and 72 hrs. For the deposition analysis and quantification of mineral nodules, the alizarin dye and cetylpyridinium chloride were used, respectively, for the experimental time of 30 days. Finally, for the analysis of gene expression, the osteo markers RUNX2, ALP and OCN were analyzed in a thermocycler with fluorescence reader by the method of real-time amplification (qPCR) at the experimental times of 3 and 7 days. In the second part of this work, a scoping review was conducted on microfluidic devices and their use in dentistry with dental materials. This technology presents itself as a good alternative to the limitations imposed by the use of in vitro tests in 2D culture. The objective of this article was to identify the possible gaps of this new methodology rising in dentistry, which has mostly been used for cytotoxicity testing of dental materials. Overall, in the first part of the dissertation work, the P_{11}-4 peptide at low concentrations (10 µg/ml, 100 µg/ml and 1 mg/ml) showed no toxicity to cells and induced the highest mineral deposition at a concentration of 100 µg/ml. The gene expression results showed that the use of the 10 µg/ml concentration was sufficient to cause a slight increase in the expressed levels of RUNX2 and OCN in 3 days, in spite of decreasing the ALP expression in 3 and 7 days. Regarding the results that concern the scope review, we can conclude that the limitations found to the use of chips in dentistry are: lack of standards in the manufacturing, acquisition and validation of results that also restrict the chip functionality. The high cost and the demand for specialized professionals are the main factors behind the low number of articles published using this technology.

Keywords: peptides; stem cells, microfluidics

SUMÁRIO

1 INTRODUÇÃO

Diversas estratégias biomiméticas, como os análogos sintéticos de proteínas não colagênicas, têm sido desenvolvidas para alcançar a remineralização do colágeno, inspiradas no comportamento da matriz proteica nos processos de biomineralização. Tais análogos sintéticos, na forma de peptídeos, foram projetados espelhando-se as características anfifílicas das proteínas não colagênicas (NCP´s), por exemplo DMP1 e fosfoproteína da dentina (DPP). Algumas destas macromoléculas freqüentemente contêm ácido aspártico, ácido glutâmico, serina fosforilada e treonina, sítios esses que se acredita estarem envolvidos na deposição mineral devido à alta afinidade pelo Ca^2 + e pelo colágeno.

Com a finalidade de atuar na biomineralização, este deve ser um peptídeo relativamente simples, com reconhecimento molecular, capaz de se auto-montar em estruturas funcionais complexas. (Whitesides *et al* 2002). Além disso, deve apresentar uma sequência peptídica relativamente curta similar em relação às proteínas naturais, podendo permitir um crescimento melhor e mais ordenado de cristais minerais (Chien *et al* 2017).

Nesse sentido, o P_{11}-4 (Ace-QQRFEWEFEQQ-NH2), pertencente ao grupo dos peptídeos que apresentam a conformação *ß-sheet*, foi projetado para se auto montar e formar fibrilas tridimensionais em resposta aos estímulos do ambiente, principalmente pH. (Aggeli *et al* 1997; Aggeli *et al* 2003). Este processo de auto montagem é impulsionado pela ligação intramolecular de Hidrogênio que surge do esqueleto peptídico, juntamente com interações adicionais entre cadeias laterais específicas (Aggeli *et al* 2001; Kayser *et al* 2004). Esses peptídeos oferecem uma nova geração de biopolímeros bem definidos, com uma gama de aplicações potenciais (Firth *et al* 2006). O P_{11}-4 mostrou-se muito promissor como um *scaffold* injetável em aplicações de engenharia de tecidos duros, incluindo seu uso como um lubrificante injetável para osteoartrite e já está em uso clínico como tratamento regenerativo para cárie dentária, em esmalte, baseado na capacidade de nuclear minerais de hidroxiapatita *de novo* (Bell *et al* 2006; Kirkham *et al*. 2007; Kind *et al* 2017; Firth *et al* 2006; Brunton *et al* 2013).

Dentre outras aplicações na odontologia, o P_{11}-4 comprovou ser capaz de aumentar a resistência de união de sistemas adesivos à dentina afetada por cárie (Barbosa-Martins *et al* 2018) e inibir a atividade proteolítica da dentina, além de aumentar a espessura da fibrila

colágena. (de Souza *et al* 2019) e produzir o aumento da mineralização na interface resina/dentina, diminuição da degradação enzimática e da nanoinfiltração (Moreira et al 2021). Ademais, estudos recentes, demonstraram que o seu uso em células semelhantes a odontoblastos (MDPC 23), foi capaz de induzir a formação de nódulos minerais e estimular a migração celular, agindo de forma muito semelhante à DMP1 (Araújo et al 2022), e agregado a *scaffold* de polidoxanona foi capaz de induzir a formação óssea *in vivo* (De Souza Araújo et al 2022). A maior vantagem física distinta do P_{11}-4 sobre outros *scaffolds*, reside no fato de que quando em estado líquido, o P_{11}-4 retorna ao seu estado original de gel, sem separação de fases (Carrick *et al* 2007), oferecendo um *scaffold* biomimético capaz de se encapsular (Saha *et al* 2019). Nesse sentido, uma combinação sinérgica de um biomaterial apropriado com uma fonte celular adequada resultaria na regeneração de tecidos com uma composição bioquímica e mecânica semelhante à do tecido original (Horii *et al* 2007, De Sousa Araújo et al 2022).

É fato que após o desenvolvimento das macromoléculas é necessário avançar no conhecimento de fatores de crescimento e moléculas sinalizadoras implicadas na regeneração de diferentes estruturas dentárias, bem como das células envolvidas nesse processo. Devido às suas propriedades intrínsecas (auto-renovação e imunomodulação), as células-tronco são a principal fonte de uso no que diz respeito à regeneração de tecidos. (Bianco et al 2000; Kaigler *et al* 2013). Melhores resultados em regeneração dental são encontrados com células tronco mesenquimais de origem dental (DSC´S) comparadas aos outros tipos de células tronco mesenquimais, uma vez que as DSC´S são as células progenitoras genuínas dos tecidos dentais, podendo dar origem a linhagens como odontoblastos, cementoblastos e osteoblastos. Além disso, derivam diretamente de células da crista neural tornando-as insubstituíveis por células tronco derivadas da medula óssea ou tecido adiposo por exemplo (Morsczeck *et al* 2017), muito embora as células tronco de origem óssea sejam consideradas o padrão ouro das células tronco adultas (Zhang W e Yelick 2012). Comparando-se as células tronco de origem óssea com as DSC´s confirmou-se diversas similaridades como alta taxa de proliferação e imunomodulação (Gronthos *et al* 2000, Seo *et al* 2004, Zhang *et al* 2009).

Até o momento, vários tipos de células-tronco adultas foram isoladas dos dentes, incluindo células-tronco da polpa dental (DPSCs) (Gronthos *et al* 2000), células-tronco de dentes decíduos humanos esfoliados (SHEDs) (Miura *et al* 2003), células-tronco do ligamento periodontal (PDLSCs) (Seo *et al* 2004), células-tronco progenitoras do folículo (DFPCs) (Morsczeck *et al* 2005), e células-tronco da papila apical (SCAPs) (Sonoyama *et al*

2006). Também chamada de *pad like tissue* (Sonoyama *et al* 2008), a presença de células tronco da papila apical foram identificadas em virtude dos dentes tratados endodonticamente continuarem a formação radicular. Esse tecido papilar é basicamente a parte apical da papila dental anteriormente derivada do mesênquima dental (Nanci *et al* 2012), sendo que parte apical da papila dental, permanece após a formação de pelo menos dois terços da raiz. Histologicamente, a papila apical pode ser vista precisamente à apical ao diafragma epitelial, como uma zona rica em células que o separa do diafragma epitelial da polpa. Desde que se tornaram objeto de estudo primeiramente por Sonoyama *et al* 2006, estas expressaram marcadores positivos para STRO-1, o qual refere-se ao mesênquima, principal marcador utilizado para detectar células tronco mesenquimais (MSCS). Em suma, a expressão de STRO-1 positivo para SCAP´s foi uma evidência que células tronco podem estar presentes nos tecidos apicais dos dentes (Huang *et al* 2008). Assim como as DPSC´s e SHED´s, as SCAP´s são capazes de se diferenciar em células tipo odontoblastos e a produzir o complexo dentina-polpa *in vivo*. Além do que, as SCAP's exibiram maiores taxas de proliferação *in vitro* comparadas as DPSC´s e SHED´s. (Zhang e Yelick 2012), superior plasticidade e potência comparada as DPSC´S (Yuan *et al* 2014), alta capacidade remineralizadora (Bakopoulou *et al* 2011) e a possibilidade de obtenção de uma linhagem odontoblástica (Cordeiro *et al* 2008). Uma das vantagens, senão a maior das SCAP´s, é a acessibilidade a uma circulação colateral apical, como resultado, conseguem sobreviver a tratamentos endodônticos e até mesmo necrose pulpar (Huang *et al* 2008; Diógenes e Hargreaves, 2017). Justifica-se, portanto, como os dentes imaturos com polpa necrótica são capazes de realizar o desenvolvimento/apexogénese radicular, até mesmo com sinais e sintomas persistentes indicativos de periodontite apical, elucidando ainda mais a natureza resistente à infecção das SCAP's (Chrepa *et al*., 2017, Lin *et al*, 2018). Por fim, sabendo-se do potencial das SCAP's em ensaios que envolvem mineralização e o potencial de indução da deposição de minerais pelo P_{11}-4 em células, a primeira parte do trabalho avaliou o uso de diferentes concentrações do peptídeo na estimulação da produção de minerais pelas SCAPs.

No entanto, atualmente muito se discute sobre as limitações dos ensaios in vitro que ocorrem em garrafas ou em placas de cultura de monocamadas (cultura 2D),sobretudo porque distanciam-se da realidade dos modelos *in vivo*. Por conseguinte, a cinética do crescimento e adesão celular são diretamente influenciados pela sua dimensão (2D). Nota-se nesse tipo de cultura a ausência de polaridade celular, e as interações celulares, bem como a presença da matriz extracelular, deturpadas. Além disso, oxigênio e gradientes químicos não são

distribuídos equitativamente, afetando diretamente o fenótipo e a função celular (Sayde et al 2021).

Frente ao exposto, os dispositivos microfluídicos através do uso da biotecnologia visam compensar as limitações do cultivo em monocamadas. Tal proeza ocorre, pois, através de sistemas de *organ on chip* é possível mimetizar as condições fornecidas pelo ambiente *in vivo* pelo controle das forças físicas como tensão de cisalhamento e compressão mecânica e bioquímicas que ocorrem no ambiente celular (Lacombe et al 2022). Esses ensaios realizados em micro escala proporcionam uma oportunidade única de atingir níveis elevados do controle do microambiente celular, garantindo assim, o suporte de vida dos tecidos além de observar diretamente o comportamento de células e tecidos (Leung et al 2022). Dentre as muitas vantagens da utilização desta tecnologia, podem ser citadas a sustentabilidade, uma vez que, a manipulação de reagentes acontece em microescala havendo economia de reagentes, e o seu uso como substituto aos experimentos em animais, que além de não ser capaz de reproduzir a complexidade do sistema fisiológico humano (Akhtar et al 2015), refletindo portanto na baixa taxa de sucesso de novos fármacos quando em fase de testes em humanos.(Lacombe et al 2022). Somado a isto, recentemente o uso de animais em pesquisa vem sendo alvo constante de discussões e enfrenta questões éticas severas (Probst et al 2018). Diante disso, pode-se observar o massivo investimento e interesse das indústrias farmacêuticas no desenvolvimento de *chips* para inserção de novos fármacos.

Estruturalmente o *chip* consiste de microcanais de espessura similar a um fio de cabelo, capaz de guiar e manipular volumes líquidos mínimos (picolitros até mililitros) dentro de seus microcanais (Manz et al 1990; Harrison et al 1992; Whitesides et al 2006). Embora estes sistemas de análise sejam muito mais simples que órgãos e tecidos complexos, eles se tornaram uma alternativa interessante ao cultivo de células 2D, pois, permite recriar a fisiologia humana e patologias dentro do chip.

Desse modo, o desenvolvimento do *chip* está diretamente atrelado com o desenvolvimento da engenharia, biomarcadores, biomateriais e uso de células (Koyilot et al 2022). Nesse sentido, destacam-se o uso de células pluripotentes induzidas (IPS), células tronco adultas e embrionárias como as mais exploradas nesses dispositivos. Todavia, células do tipo IPS são as mais utilizadas nesse setor, uma vez que, podem ser obtidas por pacientes com fenótipos pré determinados por patologias (Koyilot et al 2022). Outros autores, relatam ainda o potencial superior das células IPS na sua habilidade de diferenciação e propriedades biomiméticas

comparadas à outras fontes celulares (Becker et al 2017). No entanto, quando voltamos para a odontologia destacamos o uso de células tronco de origem dental.

Apesar do desenvolvimento dos dispositivos microfluídicos terem ganhado destaque nos anos 1990 (Sin et al 2008), na odontologia o primeiro *chip* surgiu em 2016, com a finalidade de estudos de biofilme dental. Coincidentemente, neste mesmo ano, o Fórum Econômico Mundial reconheceu a tecnologia do *organ on chip* como uma das dez principais tecnologias emergentes de 2016. Desde então podemos observar que esta plataforma tem sido cada vez mais utilizada por pesquisadores na área odontológica e sua inserção para testes de materiais dentários está em foco crescente. Contudo, ainda é possível observar algumas limitações referente aos *chips* ao qual até o momento não foram explorados, por exemplo como na escolha dos materiais utilizados para fabricação, aquisição e validação dos resultados bem como o uso destinado a ensaios de biologia molecular dentro do *chip*.

Em suma, este trabalho de tese foi organizado em dois capítulos, abordando os efeitos de um peptídeo de automontagem em diferentes concentrações (10 μg/ml, 100 μg/ml e 1 mg/ml) sobre a citotoxicidade, análise da deposição e quantificação de minerais por SCAP's e analisar por meio de uma revisão de escopo as limitações apresentadas por dispositivos microfluídicos na odontologia. Em virtude do assunto ser relativamente novo no campo dos materiais dentários, a revisão de escopo aqui apresentada visa a identificação dos *gaps* referentes a metodologia de fabricação dos mesmos e identificação dos materiais dentários já testados *on chip*. As hipóteses testadas foram: 1. O peptídeo de auto-montagem P_{11}-4 é citocompatível e capaz de induzir a mineralização por células tronco da papila apical. 2. O uso de dispositivos microfluídicos para odontologia apresenta limitações referentes à prototipagem dos chips, ao material de escolha para a fabricação, e sobre a aquisição e validação dos resultados.

2 ARTIGOS

2.1 Artigo: The Self Assembling peptide P_{11}-4 influences viability and osteogenic differentiation of stem cells of the apical papilla (SCAP).

Authors

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Abstract

Objective: to analyze the effect of P_{11}-4 on cell viability and osteogenic capacity (mineral deposition and RUNX2, ALP, OCN genes expression) of SCAPs.

Methods SCAPs were seeded in contact with P_{11}-4 (10 µg/ml, 100 µg/ml and 1 mg/ml) solution. Cell viability was verified using MTT (n = 7). Mineral deposition was tested using Alizarin Red (*n* = 4). Real-Time PCR with SYBR green was used to quantify the gene expression levels of conventional osteogenic markers (RUNX2, ALP, OCN) on day 3 and day 7. Data were analyzed by Kruskall Wallis followed by multiple comparisons and T-test for gene expression with α=0.05.

Results All concentrations tested (10 µg/ml, 100 µg/ml and 1 mg/ml) were not cytotoxic at time 24 and 48h. After 72h a slight decrease in cell viability for the lower concentration of 10 µg/ml was observed. 100 µg/ml P_{11}-4 presented the highest mineral deposition. However, qPCR tracing of P_{11}-4 (10 µg/ml), has shown upregulation of RUNX2 and OCN at 3d and downregulation of ALP at 3 and 7d.

Conclusion: P_{11}-4 did not affect cell viability, induces mineral deposition of SCAP cells and upregulates the expression of RUNX2 and OCN genes at 3 days, while downregulates ALP expression at 3 and 7 days.

Keywords: Biomineralization, stem cells from the apical papilla, self-assembling peptide, osteogenic differentiation, tooth regeneration.

Clinical significance: Based on the results obtained in this study it can be stated that self assembling peptide P_{11}-4 is a potential candidate to induce mineralization on dental stem cells for regenerative purposes and also for a clinical use as a capping agent without compromising the cells health.

1- Introduction

Nowadays, a lot has been discussed around the biomineralization/mineralization/remineralization process in dental tissues due to the increase in the development of novel bioinspired materials in order to restore the mineral loss of injured dental structures. Such materials can partially reproduce the mineral formation due to the ability to mimic biomolecules functionality or even stabilize mineral phases involved in the mineralization process (1).

Certain peptides are designed to self-assemble into hierarchical structures, and have high affinity for inorganic molecules. Furthermore, they can directly control the morphology, size and composition of mineralized materials (2). Since their discovery in 1989 (3), self assembling peptides has been used in several applications ranging from surfactant materials to wound healing in regenerative medicine (3,4).

The self assembling P_{11}-4 peptide (Ace-QQRFEWEFEQQ-NH2), bioinspired in the amelogenin telopeptide at the C-terminal, involves 11 amino acids capable of forming antiparallel β-sheet structures under specific triggers (5). In dentistry, the application of this peptide has been investigated in the process of enamel remineralization, in a non classical way, also named as biomimetic remineralization (6). We can mention its use on the enamel remineralization, when applied to the treatment of initial caries (7), and on dentin when its use has promoted the remineralization of the dentin-like caries affected on the dentin/resin interface (8). Previous research also has shown the dentin conditioned with P_{11}-4 as a potent inhibitor of the proteolytic activity of collagen fibrils and able to enhance the collagen thickness of dentin (9). To which concerns the association of this peptide with cells, recent evidence has proved its success on periodontal regeneration (10), capability to induce cell migration and mineral deposition of, odontoblast-like cells (MDPC-23) similarly to DMP1 (11), and increase the in vivo osteo regeneration after 8 weeks (12).

The use of mesenchymal stem cells (MSCs) for regenerative purposes are extensively used and most of the time in association with cell responsive materials. That way, stem cells from the apical papilla (SCAP) have been a promising source for tissue engineering since their discovery by Sonoyama et al in 2006 (13). Also named by them as “pad-like tissue”, those cells are easily extracted from the immature root of third molars (Fig1A-B). In addition, recent findings suggest superior plasticity and potency compared to those of Dental Pulp Stem

Cells (DPSCs)(14), higher mineralization capacity (15) and also able to obtain an odontoblastic lineage from them (16).

To induce mineral deposition by cells, the peptide must be able to induce cell migration and differentiation as well. During this process, mesenchymal stem cells pass from a nonspecific state to a morphologically and functionally specific state, culminating in the production of extracellular matrix and formation of mineral nodules. In dental practice, this process that leads to the formation of mineral nodules induced by the peptide may be useful for future therapy to replace calcium hydroxide in tooth repair as a pulp capping agent, also envisioning tooth regeneration (11).

Hence, considering the gap of studies relating the use of the P_{11}-4 as a mineralization inducer of SCAP cells, the aim of this present in vitro study was to verify the cell viability and osteogenic ability based on mineral deposition of SCAPs cells, using quantitative real-time polymerase chain reaction (qPCR) of 3 osteo genes RUNX2, ALPL,OCN and alizarin red staining during 30 days. We hypothesize that P_{11}-4 at low concentrations, under 100ug/ml, induces the osteogenic differentiation of SCAP cells, thus the mineral deposition, without a cytotoxic effect.

2.Materials and Methods

2.1 P_{11}-4 solutions preparation

The self-assembling peptide P_{11}-4 solution (Credentis AG, Windisch, SWI) was prepared by dissolving the lyophilized powder in 50µl of sterile distilled water at 25 °C, as recommended by the manufacturer, reaching a 10mg/ml P_{11}-4 solution, then the fresh solution was dissolved in the cell culture media (Supplemented alpha-MEM) for each experiment according to the work concentration desired.

2.2- Cell culture

A characterized SCAP cell line (RP89), between the passage 3-4 were cultivated in cell culture flasks 75 cm^2 along with α-MEM culture medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% FBS (GIBCO™, Life Technologies) and 1% of penicillin/streptomycin (P/S) antibiotic (GIBCO™, Life Technologies) previously filtered with a 0.22 μm filter. The flasks were incubated at 37 °C and 5% CO_2. When cells reached 80% of confluence 0.05% Trypsin were used to detach cells. After the process of cell counting, the cells were plated according to the experimental cell density required. (Fig 1)

2.3 Cell viability

The methyl thiazolyl tetrazolium (MTT) (Sigma–Aldrich) assay was used for assessment of cell viability according to the manufacturer's instructions. For this purpose, the cells were cultured in a 96-well plate at a density of 10^4 cells/well and treated according to the respective concentration of P_{11}-4: 10 μg/ml,100 μg/ml and 1 mg/ml (n=7) and incubated (37°C and CO_2 5%) at 24, 48 and 72 h. After each period of incubation, the medium was aspirated, and the cells were washed with sterile phosphate buffer solution (PBS). Then, MTT solution (0.3mg/ml) was added to each well and incubated (37°C and 5% CO_2) for 4 h. After this period, the overlaying medium was removed, and replaced with 150 μl of isopropyl alcohol. The optical density (OD) values for each well were measured on a microplate reader (Biochrom Asys UMV340, Biochrom Ltd. Cambridge, UK) at 570 nm wavelength.

2.4Assessment of osteogenic differentiation

The cells were cultured at a density of $5x10^4$ cells/well in 24-well plate and after exposure to P_{11}-4, osteogenic–odontogenic medium including regular medium containing 10 mM beta-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 10 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) and 50 μg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells. A group with only odontogenic/osteogenic medium and a separate group containing only alpha-MEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive

and negative controls, respectively. The test groups were P_{11}-4 in the concentration of 10 µg/ml, 100 µg/ml and 1 mg/ml (*n*=4)The culture medium was changed every 72 h. After 30 days, Alizarin Red S staining protocol was followed to measure mineralization [29,30]. Briefly, after the culture period, the cells were washed with PBS and fixed with 4% paraformaldehyde for 1 h at room temperature. The monolayer was washed twice with PBS, and then 1 ml of 40 mM Alizarin Red S solution (pH=4.2) was added to each well, left at room temperature for 20 min under gentle shaking. After, the solution was aspirated, and the wells were washed with distilled water 4 times to remove the unincorporated dye. Then, the wells were photographed with a camera (Nikon 3200, Nikon, Tokyo, Japan) to accomplish the qualitative assessment. To quantify the staining, 500 µl of Cetylpyridinium chloride (CPC) solution (37°C), previously prepared with sodium phosphate buffer, were dispensed to each well, and the plate was covered with aluminum paper and left for 30 min to 1h at room temperature with shaking. The absorbance was read in triplicate using 100 µl of each well in a 96- well plate at 562 nm on a microplate reader.

2.5 Assessment of the expression of osteo genes

After treatment of the cells with P_{11}-4 (10 µg/ml), RNA extraction was performed by a silica column method (PureLink® RNA Mini Kit, Life Technologies®, Carlsbad, CA, EUA) mini kit life technology after 3 days and 7 days and the amount of extracted total RNA was quantified by NanoDrop at 260 and 280 nm wavelengths. RNA was, then, treated with DNAse (DNase I, Invitrogen®, Waltham, MA, USA) using the concentration/purity (A260 / 280) and analyzed by spectrophotometer.The cDNA was then synthesized by SuperScriptTM IV First-Strand Synthesis System cDNA synthesis kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The expression of RUNX Family Transcription Factor 2 (RUNX2), alkaline phosphatase (ALPL), and osteocalcin (OCN) genes was quantified by using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as the housekeeping gene (Table 1). The cDNA was quantified by real-time polymerase chain reaction (StepOne™ RealTime PCR System-Applied Biosystems, Foster City, CA, EUA) using specific primers. The gene expression of specific markers in different experimental groups was normalized against the control group and the relative gene expression used the comparative Cq method (2-ΔΔCq) (Applied Biosystems, 2008).

Table 1-Reverse transcription-quantitative polymerase chain reaction primers, in which (F) means Forward, and (R) Reverse. (MT) melting temperature.

Gene	Primer sequence	MT
GAPDH	(F) GGTGGTCTCCTCTGACTTCAACA (R) GTTGCTGTAGCCAAATTCGTTGT	83
RUNX2	(F) TCTTAGAACAAATTCTGCCCTTT (R) TGCTTTGGTCTTGAAATCACA	73.9-74.1
OCN	(F) AGCAAAGGTGCAGCCTTTGT (R) GCGCCTGGGTCTCTTCACT	83.9-84.1
ALPL	(F) GACCTCCTCGGAAGACACTC (R) TGAAGGGCTTCTTGTCTGTG	83

2.6 Statistical analysis

All data obtained from all the experiments were submitted to the normality test using Shapiro Wilk or Kolmorov-Smirnov according to the respective sample size to evaluate data distribution. Then, data were submitted to Kruskall Wallis test for cell viability and mineral quantification followed by multiple comparisons. Unpaired T-test with Welch's correction were used for relative gene expression comparisons. Data were analyzed in GraphPad Prism 9.4.1 software (La Jolla USA) and $P < 0.05$ was considered to indicate a statistically significant difference between the groups.

3. Results

3.1 MTT assay

The cell viability percentage of the groups during the experimental time of 24, 48 and 72 h are shown in Fig 2. At 24 h no statistically differences between the groups were observed ($p>0.05$). On the other hand at 48 h, the concentration of 10 µg/ml P_{11}-4 was significant lower than the control group ($p=0.0446$) and the concentration of 100 µg/ml P_{11}-4 ($p=0.0071$), but still with a cell viability close to 60%. 100 µg/ml P_{11}-4 showed significantly higher percentage values of cell viability compared to 1 mg/ml P_{11}-4 ($p=0.0006$), but no

statistically difference with the control group. The 1 mg/ml P_{11}-4 concentration exhibited significantly lower cell viability compared to the control group (p=0.0058) although still represents a percentage higher than 60%. At 72h all groups showed decreasing cell viability, including the control group. However, the only one that was significantly lower than the control group was 10 μg/ml P_{11}-4 group. It is notable that 10 μg/ml group, the lowest concentration used, showed for all time elapsed the lower cell viability. It is important to note that even the low cell viability was noted, the percentage of cell viability was higher than 50% after 72 h for all groups.

3.2 Mineral deposition

After 30 days, the 100 μg/ml P_{11}-4 presented statistical differences compared to the control group, although no statistically significant differences were found compared to the lower concentration of 10 μg/ml (Fig 3). For this reason, as a preliminary result the lower concentration of P_{11}-4 was also tested for osteo markers involved during the osteogenic differentiation.

3.3 Relative Gene Expression of Osteo-Markers

The gene expression of osteo markers RUNX, OCN, ALPL was evaluated after 3 and 7 days of SCAP treatment with 10 μg/ml P_{11}-4 (Fig 4). At the experimental period of 3d there was an up-regulation of RUNX2 and OCN, although a down-regulation of alkaline phosphatase (ALP) was observed at the same time. Conversely, after 7 days of treatment, no significant difference was found between the treated and control groups for RUNX2 and OCN markers. However the treated group presented again, down regulation of ALP.

4. Discussion

In this study we investigated the influence of P_{11}-4 on the cell viability, osteogenic differentiation through the conventional colorimetric assay, Alizarin red and the molecular regulatory response by tracing the expression levels of osteo genes, also involved in odontogenic differentiation.

P_{11}-4 has already proved their safe use in DPSCs (17), Human Periodontal Ligament fibroblasts (hPLF) (10) and odontoblasts-like cells (MPDC23) (11). Indeed, at present the development of novel molecules with certain osteogenic potential claims for the use of undifferentiated cells, being a promising strategy especially in an attempt to dentin remineralization for deep caries lesions.

For this study we used SCAP cells, and as neural crest derived mesenchymal stem cell (MSCs) (18) have high proliferative activity and capability of differentiation into neuronal cells, chondroblasts, adipocytes and osteoblasts (18-21), being a very good source for dental reparative and regenerative processes (22). The results obtained are in accordance with previous findings where the cell viability had already been tested on dental stem cells (10,17). In addition, P_{11}-4 regardless of the concentration tested, did not affect the proliferation of SCAP cells as evidenced by the methylthiazolyldiphenyl-tetra-zolium bromide salt (MTT) analysis.

After 24h cells being treated with a medium supplemented with P_{11}-4 , no significant difference was found between the groups (Fig 2A). After 48h can be seen that the intermediary concentration of P_{11}-4 maintained the same cell viability as the control group. Although after 48h differences were found between the groups (Fig 2B), the percentage of cell viability was maintained at levels higher than 60%. Nevertheless, a slight decrease for the concentration 10 µg/ml after 72h has been shown (Fig 2C). In fact, the results prove the concentration influence of P_{11}-4 acting directly on cells.

In order to test the ability of P_{11}-4 as an inducer of osteogenic differentiation in SCAP cells, we tested if the increase of the concentration could induce higher quantity of calcium deposits, and our data, at first, refute the statement, due to the intermediary concentration of 100 µg/ml has shown better results compared to the control(Fig 3A). However, surprisingly the microscopy's images suggest that the concentration can dictate the pattern of mineral morphology. The lower concentration (10 µg/ml) presented big dark spots (Fig 3E) while the intermediary (100 µg/ml) produced several mineral nodules of spherical shape (3F). In Addition, the concentration of 1 mg/ml has shown the presence of calcium deposits with irregular shape (3D). Hence, we can assume that the major quantity of spheroidal mineral deposits is associated with the nature of the peptide directly influencing the ions affinity and nucleation sites (23). As previously reported by Thomson et al (24) the mechanism whereby the peptide modulates the mineral deposition, resides in its chemical structure composed by

four central glutamic acid residues negatively charged. Thanks to this negatively charged surface of P_{11}-4, the binding of the divalent calcium ions causes the high mineral deposition.

Regarding the expression of osteo markers, it is time dependent, and correlates the different stages of odontogenic differentiation. We present here early mineralization genes directly involved in the mesenchymal cell differentiation besides, the polemic gene OCN, which in the past was often cited by many as a later osteo differentiation gene (25) and a relevant evidence in hard tissue regeneration (26). Since 2009, Nakamura and co-workers (27) have mentioned OCN role as an early osteoblastic differentiation marker in MSCs cultured under osteoinductive conditions.

RUNX2 is considered the master gene for odonto/osteoblast differentiation because it controls the differentiation of mesenchymal cells to pre-osteoblasts and is requested for the expression of non collagenous proteins such as Osteocalcin (OCN) (28,29). ALP, in turn, is responsible for the continuous development of the odonto/osteoblast cells (30). In contrast, OCN, is a calcium binding protein that regulates the mineralization ability of cells and the formation of mineral nodules(27,30). Our data show the capability of even the lowest concentration of P_{11}-4 (10 µg/ml) upregulates the early mineralization markers such as RUNX2 and OCN at 3d (Fig 4A), representing an increase of 37% and 27%, respectively. On the other hand, we observe downregulation of the ALP gene at 3d and 7d (Fig 4B). Those findings confirmed those stated in the literature, as the early mineralization markers are more expressed at 3d (31). However, at 7d the results indicate that the lower concentration of P_{11}-4 isn't effective to promote upregulation of early mineralization genes. However, a downregulation of the ALP marker was also observed for both experimental times. Since the ALP marker is the main responsible for the development of odontoblast/osteoblast during differentiation pathways, it is necessary to test gene expression in periods longer than 7 days in order to confirm the negative effect, as well as to use the cells from different donors. Besides, in order to confirm the best molecular regulatory response by the peptide, higher doses > 10 µg/ml must be precisely verified.

In short, our results suggest the use of this peptide as a potential candidate for dentin remineralization and mineralization facing stem cells.. Also, P_{11}-4, as a smart biomaterial, is able to respond to pH changes, temperature and ionic strength being today one of the most promising strategies to promote tissue regeneration (10).

Our findings can guide the next generation of biomaterials that incorporate the self assembling peptide P_{11}-4 as potential drug candidate to induce mineralization on dental stem

cells for regenerative purposes and also for a clinical use as a capping agent. Further investigation must be conducted to explore the molecular mechanisms of P_{11}-4 in SCAP's later mineralization genes.

5. Conclusion

Overall, the results presented here confirm the tested hypothese. P_{11}-4 has shown to be non cytotoxic for SCAP cells for all the concentrations tested and induced mineral deposition after 30 days. In addition, P_{11}-4 exhibited upregulation of RUNX2 and OCN genes at 3 days and downregulation of ALP at the same period. At 7d, no significant difference was found in the expression values of RUNX2 and OCN in the treated and control groups, but downregulation of ALP was evidenced by the treated group.

Credit Authorship contribution statement

Jessica Rodrigues Camassari: Conceptualization, Methodology, Formal analysis,Data curation Validation, Investigation, Writing – original draft, Writing – review & editing. **Iago Torres Cortês de Sousa:** Methodology, and Formal Analysis of gene expression. **Karina Kogo Müller:** Methodology, and Formal Analysis of gene expression, Supervision. **Regina Maria Puppin Rontani:** Conceptualization, Methodology, Formal analysis, Validation, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration.

Conflicts of Interest

The authors declare no conflict of interest.

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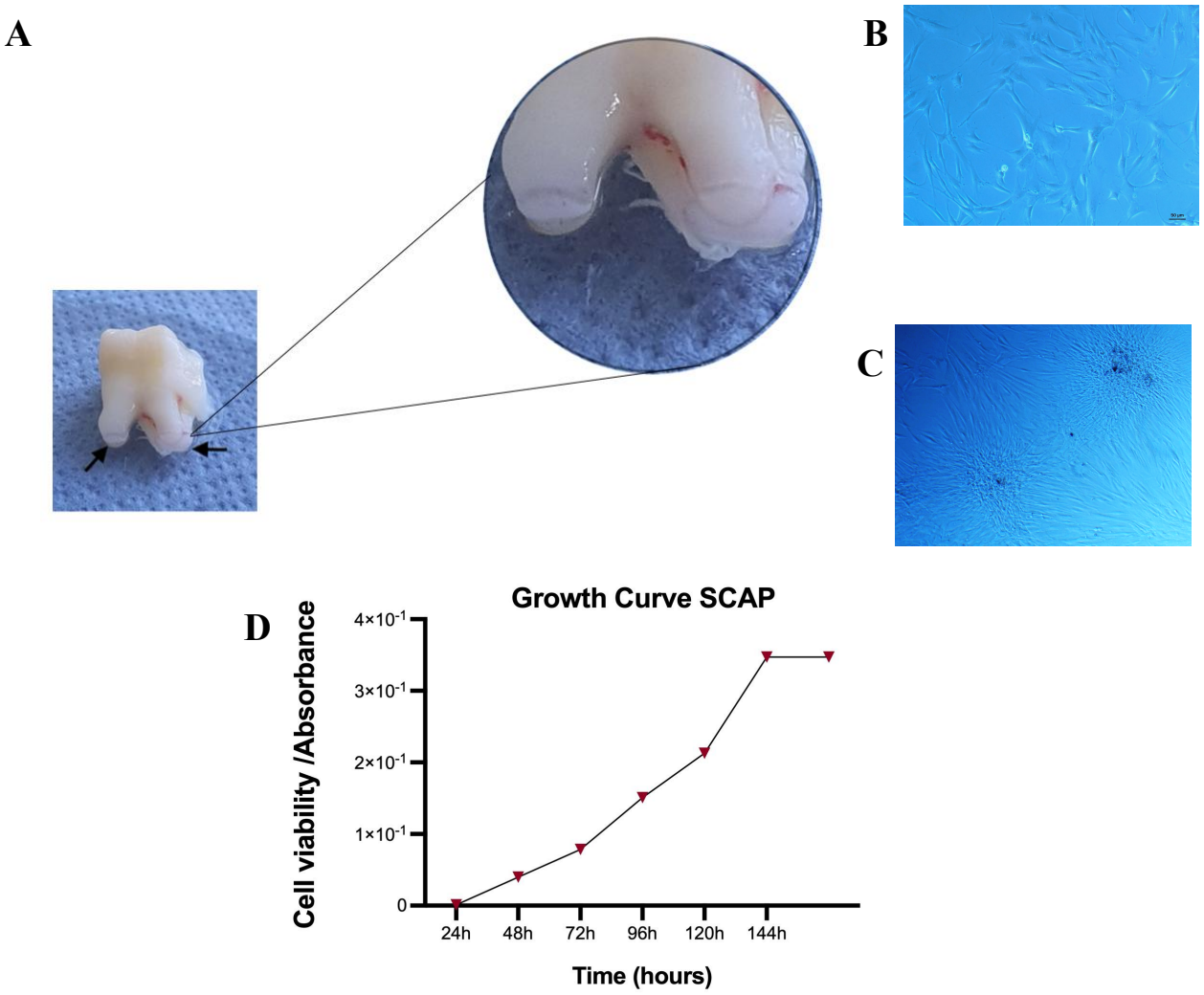

Fig. 1 – (A) Merely Illustrative photo of Apical Papilla localization in Impacted third molar at the stage of root development. (d) SCAP cultures after 3 days of cell culture with alpha MEM, FBS 10% and 1% of P/S. (B) Cells are morphologically elongated similar to fibroblast-like or stellate in shape, with numerous cytoplasmic processes (Scale Bars 50 mm). (C) Cells in adherent monolayers under the osteogenic medium culture(13 days), the cells tend to form aggregates of high density in colony-like clusters with small black spots.(D) Typical growth curves of SCAP cultures plated in a density of 5 x 10^3 cells/well at passage 3 and 6 days. The proliferation of SCAP cells cultures is related not only to their higher growth rate, as shown by the inclination of the growth curves, but also to their smaller size, which allows for more cell growth before cultures reach confluence (plateau phase).

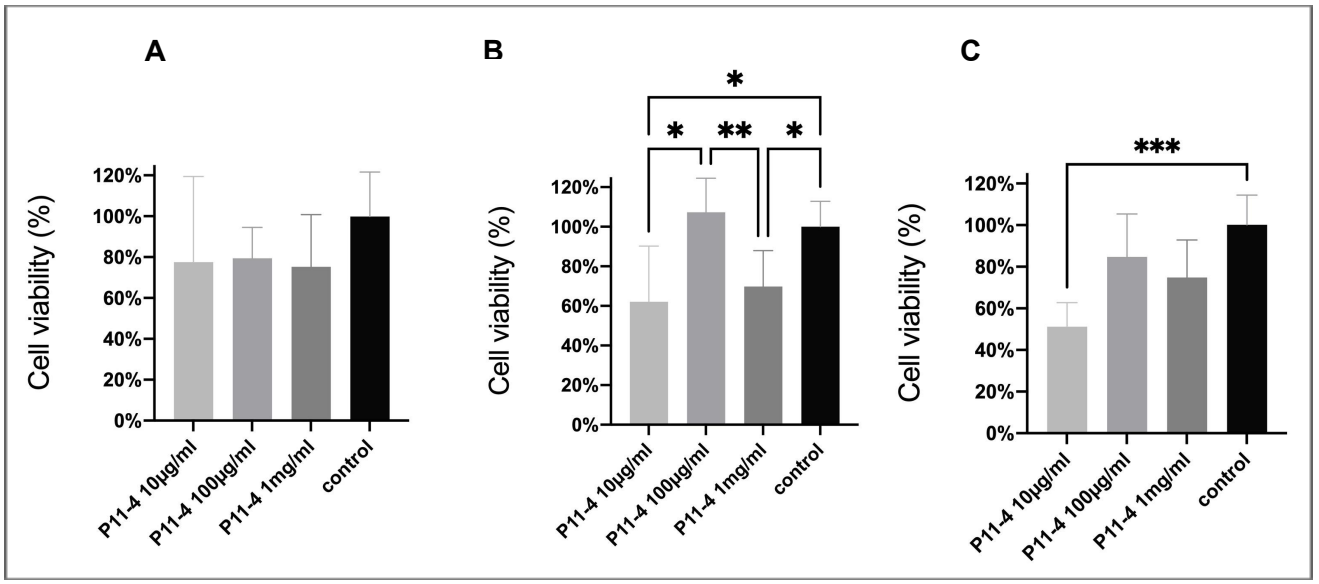

Fig. 2- Mean and standard deviation of SCAP cell viability (%) after 24 h (A), 48 h (B) and 72 h (C) . Asterisks: Kruskall Wallis (24 h), followed by multiple comparisons test (48 h) and (72 h),represent statistical difference, (*$p < 0.05$) (**$p < 0.0001$) (***$p < 0.0001$) between groups according to each time point. P_{11}-4 has shown to be non cytotoxic for the SCAP cells due to the concentrations being higher or equal to 50% of cell viability.

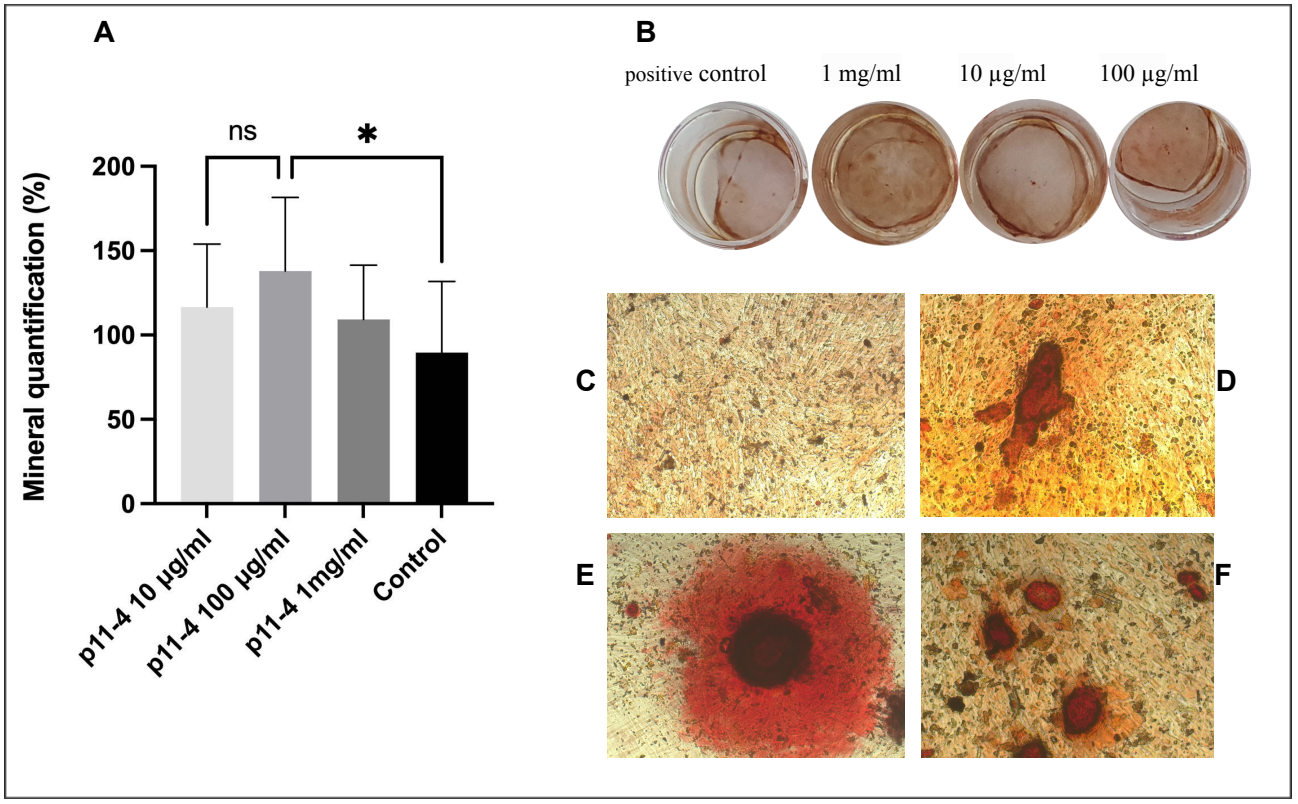

Fig. 3- (A)Quantification of Alizarin Red staining as proportion of Alizarin-red-positive surface (in %). Values were reported as mean±SE fold change in optical density (at 570nm) Asterisks:Kruskall Wallis followed by multiple comparisons by Dunns, *$p < 0.05$. Fig (B) plate picture after Alizarin Red Staining, it's not possible to clearly see differences between groups without adequate magnification. Microscopic views of Alizarin Red S staining of human stem cell apical papilla (hSCAP), cultured for 30 days. Positive control (just osteogenic medium) (C) and osteogenic medium + P_{11}-4 1 mg/ml (D) osteogenic medium + P_{11}-4 10 µg/ml (E), osteogenic medium + P_{11}-4 100 µg/ml (F). Calcium deposits (red color) are evident in SCAP cells but with different patterns in mineralization nodules according to the self assembling peptide P_{11}-4 concentration. (E) shows the presence of a unique big dark spot, (D) presence of mineral nodules of irregular shape and (F) several round mineral nodules.

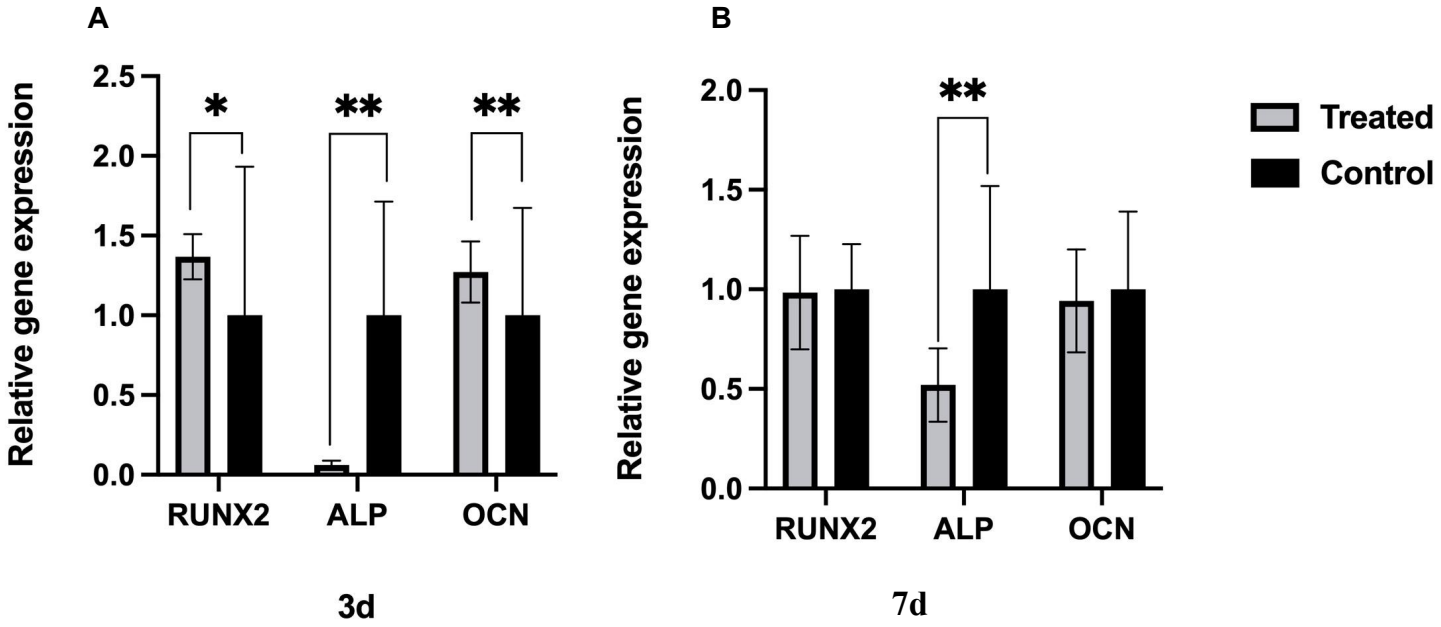

Fig. 4-Gene expression for the osteo markers RUNX2, ALPL, OCN in SCAP cells normalized to the housekeeping gene GAPDH.The gene expression was assessed at days 3 and 7. Levels of gene expression are provided as mean±SE, *$p<0.05$, **$p < 0.0001$,(Unpaired T-test with Welch's correction). Upregulation of RUNX2 and OCN expression levels were significantly higher for 3d and downregulation of ALP for both 3d and 7d. However, there was no significant difference in the values expression of RUNX2 and OCN at 7d in the treated and control groups.

2.2 Artigo: An overview on dental biomaterials used in novel dental-chips for soft and hard tissue regeneration

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Abstract

Purpose of review: Microfluidics devices have gained much attention, due to the similarity of in vivo conditions and animal rights concerns. In dental research, they are also named as dental chips or tooth-on-a-chip. By contrast, it is not well explored the material influence on dental chip fabrication. Thus, we present here a review of the current dental materials tested on chips, besides the pros and cons of the disposable materials for chip production.

Recent findings: Recent studies suggest the use of microfluidics for dental materials such as HEMA, Acid phosphoric, Adper Scotch bond, SE BOND, Silver Diamine Fluoride, silicate cements, EDTA-chitosan, and new regenerative drugs. PDMS and PMMA are the main materials for chip manufacturing and the bottom up approach elected for cell injection.

Summary: The use of microfluidics for dental materials testing is expanding. However, a gap can be observed between the standardization of prototype fabrication and results validation.

Keywords: dental chip, dental materials, tissue regeneration, microfluidic devices, tooth-on-chip, dental stem cells.

Introduction

The tooth is a complex organ described by mineralized tissue surrounding the pulp, which in turn, is fully innervated and vascularized being responsible for the tooth vitality [1]. Dentin and pulp form a unified tissue named as pulp-dentin complex where the peripheral odontoblasts through their odontoblast process maintain intimate contact with the pulp [2]. Proper to this configuration a severe injury condition on pulp dentin complex can lead to pulp tissue death [3] and if remain untreated, tooth loss.The most common disease that affects teeth is dental caries, a biofilm induced disease that destroys the mineralized tissues from tooth, forming a cavitation [4]. The most clinical practice is to clean the cavity and restore with a biomaterial that from time to time needs to be replaceable and consequently put the patient at risk of pulp exposure in deep cavitations [5]

Nowadays, the majority of the dental research has been driven in biomaterials for regeneration in a way to preserve and keep the tooth in the cavity. Characterization of cell-material interaction by using laboratory cell culture techniques is the first biological analysis necessary after biomaterial development. Biomaterial structures must allow viable cells to adhere to its surface, spread throughout its porous network, and proliferate. The conventional system to evaluate biocompatibility and cytotoxicity of dental materials has been so far employed right to the simplicity and the several well established protocols in the literature for the dental tissues [6]. It includes cell cultured on plates [7-9], the in vitro pulp chamber [10] , dentin barrier tests [11], ex vivo models (12), entire human tooth culture [13] and decellularized mucosal tissue [14]. One disadvantage of those models is the limited controllability of the morphological and metabolic events through time when cells are in contact with the biomaterial. Consequently, the obtained data in relation to the tooth ability to respond to different biomaterials may not be so reliable.

Microfluidic is the science that manipulates flows in micro-sized channels(10-100μm) and is directly related to drug discovery [15]. Dental chips are microfluidics devices, which derive from Organ-on-a-chip (OOC), and integrate tissue engineering when combining biomaterials and cell analysis inside the platforms mimicking human organs [16]. In general those devices are manufactured with a compartment for cell culture and several microchannels responsible for the management of substances and measurable signals. Allow the creation of a whole cellular microenvironment as well as their responses in front of desirable triggers [17]. This novel technology (OOC) has emerged due to the necessity to

replace animal studies, once most of the drug tests fail when applied to clinical models since their efficacy can not be proven in humans, such as the case of neuroprotective drugs [15] . It also has been driven by the ethical limitations of animal experiments [18] and the industry commitment to 3Rs of animal usage in research [19]. This system is more sophisticated compared to conventional monolayer cell culture and allows reproducibility of tissue levels, hard enough, to be achieved with conventional 2D or 3D cell culture models [20,21]. Comparing to animal models and in vitro models those devices are more clinically relevant because of their ability to reconstruct cell layer geometry on a biological scale and ability to monitorate cellular interactions with microenvironmental parameters such as fluid shear stress, mechanical load and biochemical concentration gradients that are often controlled, providing an environment more similar to in vivo conditions(6).

All over the years microfluidic has presented to the tissue engineering field a more sustainable and assertive way of research due to the manipulation and consumption of small fluids inside chips, which allows reagents saving. In addition, after an efficient chip design, the fabrication is fast and allows high sensitivity combined with cost-effectiveness and no animals evolved [22-24] .

The first OOC is dated in the late of 1990s used in studies of human physiopathology. [25] However just years after that, this device gained more attention with the first lung on chip [26] that proved to recreate an epithelial/endothelial barrier in an elastic PDMS membrane mimicking respiratory movement. Between the first OOC and the first dental chip we can observe a huge space around 16 years. In fact, dental research started slowly in basic research with chip models for biofilm studies [27], and from now on we can observe the use of this platform for testing conventional and responsive dental materials up to new regenerative ones.

To the best of our knowledge, this is the first review to summarize all the studies that used the technology of microfluidic devices with the purpose of dental research in the last 5 years. Moreover, although most of the papers elucidates the use of this platform for testing dental materials, there is not enough information about the material characteristics for their fabrication. It's imperative to understand the advantages and disadvantages of each material to develop the most suitable material for dental materials testing.

In this review, we intend to provide a summary of the main findings on dental chips, together with the materials already tested inside and their biological response. For that, we classify the studies on chips by classes : 1- First class: basic research, 2- class: dental

materials applied inside chip and 3 - class Tissue Regeneration. In Addition, in the last part we present an overview of the present limitations and challenges of this novel technology in order to guide the next generations of works in this field.

Literature Search

For this review, data were collected online using Pubmed, Scopus and Embase databases from the past 5 years. Key articles with dental chip relation were selected and analyzed for this review. All dental chip papers were located using the following keywords ("tooth on chip" OR "organ on chip dentistry" OR "dental chip" OR "microfluidic co culture device" in the title or abstract were retrieved. Just in vitro studies were included. Figure 1 shows the articles distribution during the past 5 years searched.

First Class of microfluidic devices- Basic Research

In short, the first papers were related to basic research not being applied to clinical state. In 2016 with Raymond Lam et al [27] who developed a microfluidic device named as the "artificial teeth device" with the purpose of characterizing the biofilm growth under different conditions such as sucrose concentrations and dissolved oxygen. The great achievement in the obtained results of this paper guided the next generation of microfluidic devices to analyze biofilm interaction. Indeed, there is a consensus about the success in the use of microfluidic systems for oral microbial biofilm purposes, mainly because it is possible to reproduce different microenvironments through the control of shear stress [28,29] temperature, nutrient [30] compounds [31] and oxygen levels [32,33].

In 2019, Niu et al produced one chip for odontoblasts cells, the chip was made by soft lithography and PDMS (Table 1) the aim of this study was to allow the growth of odontoblasts process in vitro and for that they verified if the geometric constraint of different microchannels size (2, 4, 6, and 8 μm) of the chip can mimic the dentin tubules and so the

odontoblasts process growth induced on chip. The results showed that the size of 2 μm is the ideal to make the odontoblasts form the odontoblasts process, and the morphology of those was similar to the same cells in vivo and expressed the protein marker AQP4 . According to the authors, once the odontoblasts process is directly related to the dental hypersensitivity, this chip could be enhanced in the future and be used for dental diseases modeling and dentin repair. A more detailed account of other papers related is fully mentioned on Table 1.

Second Class- Chips applied to dental materials

The first dental chip for dental materials testing was reported in 2018, by Rahmini et al [34] The chip enabled to study cell viability of oral mucosa after exposure to 25mM of HEMA and streptococcus mutans, the results showed lower cell viability when exposed to HEMA and lower transepithelial electrical resistance after contact with the bacteria. For the chip design it (Table 1) is possible to observe eight-three channel devices and each culture chamber consists of a main channel and two parallel side channels. The selected chip material was PDMS.

In 2020, França et al [35] developed the first experimental chip, which enabled the reproduction of the dentin interface with biomaterials. The chip consists of four chambers separated by one dentin slice. The aim of the group was to analyze SCAP cells' response to conventional dental materials treatment with HEMA, phosphoric acid and Adper Scotch bond used in clinical dentistry, cells were tested inside chip for cell cytotoxicity, cell morphology and metabolic activity, according to the correspondent treatment. For the purpose of results validation they used as a control group the conventional monolayer culture (off chip). The data collected has shown cells after treatment inside the chip with differences in quantities and morphology compared to the cells treated off chip. After the treatment with HEMA (10mM) cell results on chip showed a similar behavior to clinical findings, contrary, off chip cells were more sensitive to the material. Cells in lower numbers and with polygonal morphology were found off chip. Considering the metabolic activity, the chip exhibited higher cell values. Despite the limitations of this study, this was the first device to simulate dental

treatments with real follow up and to show that even the cells being on monolayer on chip, they were more reliable to clinic conditions.

Ongoing in the same topic Ly et al [6] published in 2021, an oral mucosa chip to evaluate the cell response of different HEMA concentrations 1.56-25mM. The biggest goal was to reproduce multilayered configuration, in other words, the culture of two different cell types. The chip consisted of three microchannels fabricated by PDMS (Table 1). Keratinocytes and fibroblasts embedded collagen hydrogel were used for the HEMA treatment inside the chip, Also they used the cell culture on plate as a control. The results proved that the mucosa chip developed was useful in determining a higher sensitivity cell responses to HEMA in a dose dependent manner compared to conventional plates.

Vurat MT et al [36] diversified when used bioprinting technology for creating a microtissue model similar to ligament alveolar bone PDL-AB biointerface. Periodontal ligament layer was created by bioprinting hPDL suspended in gel-MA and alveolar bone layer by hOB suspended in Gel-MA/HAp-MNP. The microfluidic platform consists of two separate layers with inlet, outlet and a reservoir. The chip was produced in PDMS and by Soft lithography (Table 1). All chip components were autoclaved before the microfluidic culture phase. The microtissue constructs were cultivated under flow inside the microfluidic platform in the experimental time of 10 days. Interestingly, besides the positive expression of STRO+ for the hPDL layer and Osteocalcin for the osteoblasts cells from the hybrid layer construct, They verified interconnexion between the cells from the microtissue suggesting the capability of the bioinked tissue to promote the cell migration and one possibility to explain that, is proper to the gel degradation under the microfluidic culture device. Cell interactions with Tetracycline inside chip have demonstrated no significant cytotoxic effect, this finding is very important in guiding the clinical treatment of periodontal diseases.

In 2021, Nara and co-workers [1] innovated by testing inside chip the cell viability and proliferation of DPSCs after exposure to commercial silicate cements: Theracal, Pro root and Biodentine commonly used for the protection of pulp dentin. Those materials belong to the family of mineral trioxide aggregate (MTA) and became very popular due to the capacity to induce tertiary dentin formation and the mineral barrier. Although they share similarities, each cement will have their own particularities related to setting time, handling and release of Ca. The chip has changed from the previous one [35] used by the authors, and some differences related to angulation and channel size can be observed. The authors also correlate the pH variation with the TGFβ liberations. The biggest goal here was to test the antimicrobial

properties of PRO ROOT with a biofilm model inside chip. The results proved that their chip model was able to analyze the antibiofilm activity of PRO ROOT, which was also the bioactive material to induce the highest liberation of TGFβ during 7 days,this growth factor is well known for being related to the signaling events in the cell migration and tertiary dentin formation.

Exploring another dental material on chip, Hu S et al [37], was the first to publish the cytotoxicity effect of 38% of SDF on hDPSC and gingival equivalents. Also they verified the dentin thickness effect on the SDF penetration. For that, the authors mention the use of a tooth on chip with a dentin barrier. The most striking characteristic from this device compared to others dental chips already described in the literature, it's the manufactured material of choice, the thermally bonding four microstructured poly(methyl methacry- late) (PMMA) (Table 1). Also, the chambers and microchannels within the PMMA sheet were fabricated by computer numerically controlled (CNC) micromilling. The significant data obtained has proven the cytotoxicity effect of SDF to hDPSCs at lower concentrations (0.001%), besides to penetrate dentin with thickness lower than 1.0mm resulting in death of pulp cells. Mucosal corrosion test with gingival cells was confirmed by the disruption of the epithelial integrity histological images.

Recently, Zhout and coworkers [38] used the microfluidic chip to simulate the pulp dentin complex and develop an interaction between the material, dentin slice and cell. In the attempt to improve dentin bonding through the use of new molecules able to demineralize just the extra fibrillar components of collagen, they compared the bonding effect of EDTA-chitosan, phosphoric acid and SE-BOND. Microfluidics chips were manufactured by PDMS with a pulp chamber of 4mm diameter and 1mm groove (Table 1). Dentin slice with 0.5mm of thickness was used and inserted into the dental pulp cell chamber. Interestingly, specifically this paper is the first paper to correlate mechanical testes with the cell images on chip in the same paper. Overall, these results confirm that EDTA-chitosan can be a better alternative to the conventional phosphoric acid. Furthermore, the mechanical tests performed showed better results for the bonding strength with EDTA-chitosan when compared to phosphoric acid and SE-Bond. With regard to cytotoxic results, EDTA-chitosan has proved to be less cytotoxic compared to the other materials, which was later complemented by the chip images where hDPSCs did not change their morphology being less irritant to the cells than phosphoric acid, that, in turn presented a rounded shape to the cells.

Third Class - Tissue Regeneration

A Brazilian group was the pioneer in matching experimental scaffolds for tissue regeneration with 3D chips [39]. In this specific case they developed scaffolds within the association of β-glycerophosphate (βGP) and calcium-hydroxide with chitosan (CH) to formulate a porous bioactive scaffold suitable as a cell-homing platform for dentin regeneration. The complexity is such, mainly because the scaffold characterization is necessary previously the cell interaction inside the chip. However, a good strategy to save time made by the authors was the use of a pulp on chip (Table 1) industrially produced by KYATEC, known for developing 3D cell culture dispositives. This dispositive was able to simulate intra-pulpal pressure by the connection with a column reservoir. The results concern the higher porus size effects with the higher proliferation cell rates, which has already been described in the literature as the surface topography effect in guiding the adhesion, spread, proliferation and cell differentiation [40-43]. Taken together, those results suggest new possibilities for dentin tissue regeneration by the use of experimental low cost scaffolds, but totally efficient in what is proposed.

Fabrication

During the material selection some aspects must be taken into consideration, based on the model functionality and materials properties. Some of the most essential properties are: toxicity, which means that the material should not be toxic for the cells in long term cell culture. Transparency, for good optical analysis. Gas permeability, to supply oxygen for the cells. Asides from aspects related to the costs and manufacturing procedure that must be accessible and previously considered [44]. The most common materials used for biomedical chip fabrication are silicon, glass, polymers such as PDMS and thermoplastics as Polyestyrene (PS), poly(methyl methacrylate) (PMMA), polycarbonate (PC) or cyclic olefin copolymer (COC) [45], and recently hydrogels [46]. Frequently, those devices are found in a hybrid shape made of two or more materials [47]. Historically organs on chips made by silicon gained popularity due to its fabrication technique, although their opacity restricts the use in the conventional inverted microscopes [45,48]. Glass by contrast appeared as an inert, robust and transparent material, although glass is considered a cheap source its manufacturing process is expensive and complex, moreover frequently requires cleanrooms [45-49].

Hydrogels are natural materials and gained popularity due to the similarity to the extracellular matrix (ECM) but their low stiffness impairs the manufacturing process and long term cell cultures [46]. Despite the material contrasts, differences regarding the industry and laboratory purposes are frequently found. Costs of production, easy handling and reliability are some of the key aspects taken into account by the industries. However, for laboratory research, the material usually focuses on prototyping simplicity and their performance [50]. That way, PDMS, PMMA, glass/silicon are commonly used in laboratory production [51]. Contrary to the expectations, dental research has not deeply explored the chip materials available, according to the literature just PDMS and PMMA has been the choice material and for this reason a general brief of both polymers (PDMS and PMMA) characteristics for microfluidics devices will be detailed above. All the other materials available for the biomedical field, as well as, the common polymers and their pros and cons are presented on Table 2.

Poly-(dimethyl-siloxane)—PDMS

PDMS is the most common material used for OOC fabrication [48]. The low cost and easy manufacturing by soft lithographic methods made this material the first choice by the researchers [52]. PDMS chips can be manufactured by preparing using simple mixing, casting and heating steps to replicate microchannels and structures of molds [53-54] .This silicon based elastomer (PDMS) has important properties such as, optical transparency, high gas permeability, robustness, biocompatibility, and low toxicity being vastly used for biomedical applications [55]. Some studies also mention the PDMS elasticity favoring the mechanical stimulation to cells [56] Even though it has several advantages, one inherent limitation is related to the hydrophobic nature that increases the adsorption and absorption of hydrophobic molecules [57] that can negatively affect the results of toxicity and pharmacokinetics/pharmacodynamics tests [58]. Besides, its incompatibility with organic solvents can provide significant changes in the microchannel's structure [59], nonetheless, a classical approach to modify surface properties of PDMS has already been described as the use of plasma treatment, UV treatment and coating [60], metal coatings such as titanium oxide and gold [61], sol gel coating [62], and creation of Si-O-Si bonds via sinalization techniques [63].

Poly(methyl methacrylate) (PMMA)

PMMA is classified as a thermoplastic material [64-65], and as such, it can be remodeled when heated close to their glass transition temperature, and also keep their shape when cooled [66]. PMMA is considered with better solvent compatibility characteristics than PDMS [51], and without molecule absorption [47]. Also is optically transparent [45], presents good mechanical properties [47], good rigidity [67] and allows prototyping at a small scale of production [45-65]. All those qualities combined make PMMA a very good material choice for organ on chip systems [45, 46-47]. However, a very important issue is that, unlike PDMS, it can not be treated with ozone or plasma oxygen because it becomes an incompatible polymer [47].

Sterilization methods

Before proceeding to cell culture inside microfluidics devices the selection of the correct way of sterilization is crucial in order to avoid microbial contamination, similarly to the conventional 2D cell culture. Particularly, a device failure for contamination is a huge financial loss. The majority of plastics, including PMMA, can not be autoclaved and not even use ethanol due to the risk of being partially dissolved. PDMS also can adsorb ethanol when exposed to periods longer than overnight. UV sterilization is not indicated for opaque materials, as silicon because UV light can not penetrate properly. A good alternative to the exposed methods described above is the use of gamma irradiation and ethylene oxide sterilization [67].

Cell culture and Injection on chips

Another important theoretical aspect is the cell culture and injection method on chip. In general the most common cell sources for microfluidics devices are: primary cells, adult or pluripotent stem cells and also immortalized cell lines from animal or tumor derived [68].

For dental chips odontoblasts, keratinocytes, fibroblasts, DPSC, SCAP, PDLSC (periodontal ligament) and osteoblasts were already used (Table 1). For each individual platform an injection mode is required. They can be classified as bottom-up approaches where inject single cells suspensions, building block assembly when you injects preformed organoids and spheroids or explant integration used for the injection of micro fragments from biopsies [68]. Although we have all those methods of injection, just the bottom up approach is often used in dental chips.

Limitations and Challenges

So far, in dental chips no one was able to reproduce the entire organ physiology on chip, as also not seen in the other fields [69]. The construction of a chip with the whole tooth organ physiology will be an important issue for future research. Regarding the development of dental chips by the academy, it depends directly on the multidisciplinarity between a big team composed of engineers, biologists and academics and even though those labs are already producing dental chips, it is still limited to small quantities such as one dispositive per day [70]. Moreover once the device design varies from one project to another, there's no golden standard in data analysis and quality control. Soon, to support their use in biological research it is necessary to delve into standardization protocols as well as the availables ones for 2D cell culture.

Concerning the chip material, even though inert coat application in PDMS allows his use [71],this material is far away from being the perfect material proper to its limitations [72] as already explained previously. The search for the ideal material boosts the development of new and improved ones and consequently drives the use of four dimensional printing, known as intelligent materials able to change their shape and reactivity according to the environment being a promising approach for future chips [73].

Regarding data acquisition and validation, the larger amount produced by those dispositives requires collaboration and use of artificial intelligence and machine learning for a deep comprehension of results and experimental design [70].

The literature has shown great advances with the use of microfluidics in dentistry, however there is a big gap in which concerns the use of those platforms in molecular biology

The key challenge for the dental research community is to develop a chip model able to analyze gene expression, since the conventional RNA extraction model is restricted to a bigger quantity of cells and a larger amount of reagents. So new molecular biological systems on chip may give a different and sustainable direction of the novel research on the subject.

Conclusions

Here we have discussed all the dental chips available for dental research. Due to its intrinsic complexity, the number of papers using this methodology still is few. This microfluidic branch offers a world of possibilities ranging from the material selected, device design, cell injection and even data analysis. Step by step, dental research advances within the challenges claimed by microfluidics. However this technology requires high financial investment, developing time and highly qualified professionals confining the present use domain in a small selected group of researchers. For the future, the use of microfluidic will allow the personalized medicine to integrate cells from the patient, as IPS cells, therefore tracking the disease onset, progress and treatment.

Declarations

Conflict of Interest: The authors declare no competing interests.

Human and Animal Rights and Informed Consent: This article does not enclose studies performed in human or animal subjects performed by any of the present authors.

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• Of importance

•• Of major importance

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of the organ on chip devices taking into account concepts of design fabrication, materials properties and the main applications of this dispositive.

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Table 1- Last Dental publications (> 5 years) with microfluidics devices and the main characteristics such as material type, cells and chip design. Remover a figura e citar a figura no artigo de referencia.

References	Year	Generation	Chip material	cells used
Oral mucosa-on-a-chip to assess layer-specific responses to bacteria and dental materials.Rahimi C, Rahimi B, Padova D, Rooholghodos SA, Bienek DR, Luo X, Kaufman G, Raub CB.	2018	2nd generation	PDMS	Keratinocyte and fibroblasts
Microfluidic Chip for Odontoblasts in VitroNiu L, Zhang H, Liu Y, Wang Y, Li A, Liu R, Zou R, Yang Q..	2019	1st generation	PDMS	Odontoblasts
Human dental pulp stem cells exhibit enhanced properties in comparison to human bone marrow stem cells on neurites outgrowth.Pagella P, Miran S, Neto E, Martin I, Lamghari M, Mitsiadis TA	2020	1st generation	PDMS	DPSC, BMSC
The tooth on-a-chip: a microphysiologic model system mimicking the biologic interface of the tooth with biomaterials.França CM, Tahayeri A, Rodrigues NS, Ferdosian S, Puppin Rontani RM, Sereda G, Ferracane JL, Bertassoni LE.	2020	2nd generation	PDMS	SCAP
Analysis of Tooth Innervation in Microfluidic Coculture Devices.Pagella P, Mitsiadis TA.	2020	1st generation	PDMS	Trigeminal Ganglia (TG) and tooth germs
An Oral-mucosa-on-a-chip sensitively evaluates cell responses to dental monomers.Ly KL, Rooholghodos SA, Rahimi C, Rahimi B, Bienek DR, Kaufman G, Raub CB, Luo X.	2021	2nd generation	PDMS	Keratinocytes and fibroblasts
Biomaterial and Biofilm Interactions with the Pulp-Dentin Complex-on-a-Chip.Rodrigues NS, França CM, Tahayeri A, Ren Z, Saboia VPA, Smith AJ, Ferracane JL, Koo H, Bertassoni LE.	2021	2nd generation	PDMS	hDPSCs
Chitosan in association with osteogenic factors as a cell-homing platform for dentin regeneration: Analysis in a pulp-in-a-chip model. Bordini EAF, Cassiano FB, Bronze-Uhle ES, Alamo L, Hebling J, de Souza Costa CA, Soares DG.	2022	3rd generation	Acrylic (PMMA)	hDPSCs
Characterization of silver diamine fluoride cytotoxicity using microfluidic tooth-on-a-chip and gingival equivalents.Hu S, Muniraj G, Mishra A, Hong K, Lum JL, Hong CHL, Rosa V, Sriram G	2022	2nd generation	PMMA	hDPSCs
Development of a multicellular 3D-bioprinted microtissue model of human periodontal ligament-alveolar bone biointerface: Towards a pre-clinical model of periodontal diseases and personalized periodontal tissue engineering.Vurat MT, Şeker Ş, Lalegül-Ülker Ö, Parmaksiz M, Elçin AE, Elçin YM	2022	2nd generation	PDMS	hPDLFs and osteoblasts

References	Year	Generation	Chip material	cells used
EDTA-chitosan is a feasible conditioning agent for dentin bonding.Zhou, Y., Zhao, Y. & Han	2022	2nd generation	PDMS	hDPSCs

Fig1 A- Distribution of the articles using dental chips during the past 5 years.The current trend is the increase of the number of publications using these devices and clinical materials testing. **B**-Timeline shows the emergence of dental materials using microfluidics devices starting in the year of 2020. Dental materials already tested within chips include: HEMA, acid phosphoric, Adper Scothbond, ProRoot, Biodentine, Theracal, Silver Diamine Fluoride, EDTA-chitosan.

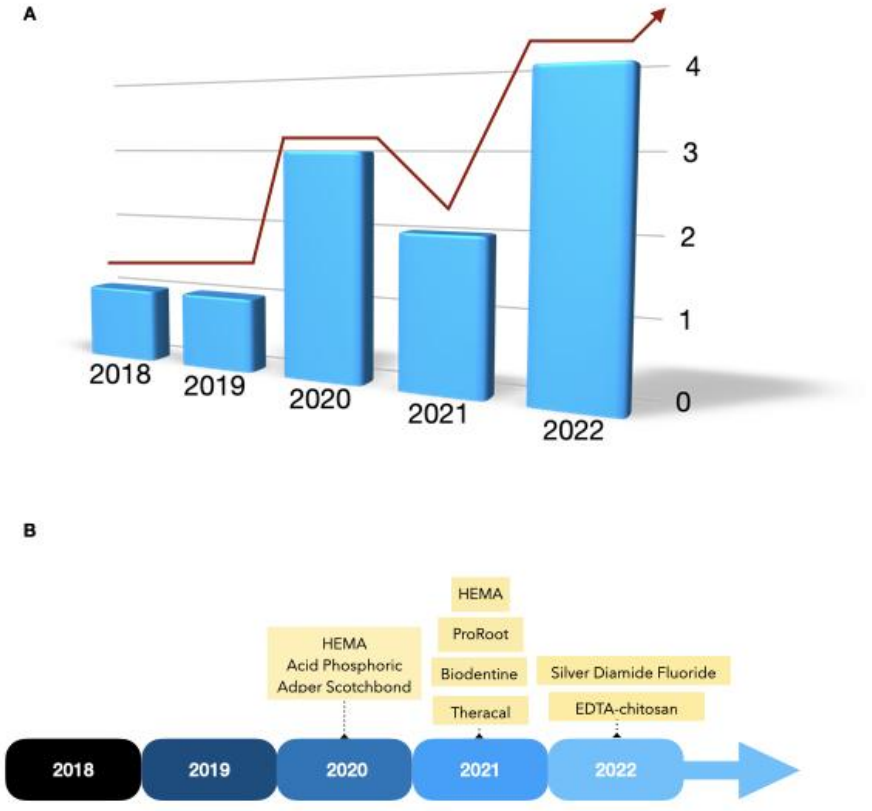

Table 2- Comparison of several available materials for the fabrication of microfluidic platforms in the biomedical field. Adapted from the literature references (41-62,67-69).

Material	Class/ examples	Advantage	Disadvantage
Glass	Inorganic soda-lime glass, borosilicate glass, and fused quartz	transparency, resistance to mechanical stress, hydrophilicity and biocompatibility	low gas permeability*, high cost,delay in fabrication process
Silicon	Inorganic crystal silicon (SCS), polycrystalline silicon, silicon dioxide,	good mechanical properties, low cost	opacity
PDMS	Organic:elastomers	biocompatibility, low cost, easy surface modification, gas permeability, low toxicity, optical transparency	adsorption and absorption of hydrophobic molecules,incompatibility to organic solvents.
Thermoplastics	Organic: PMMA, PS, polycarbonate (PC) or cyclic olefin copolymer (COC).	Transparency, low cost	rigidity, hard to reproduce complex structures.
Hydrogels	Organic: collagen, gelatin, silk, and synthetic (e.g., PVA (polyvinyl alcohol), PEG (polyethylene glycol))	biocompatibility,adjustable degrability, high permeability	lower mechanical properties

3. DISCUSSÃO

A Cárie dental é a doença infecciosa mais prevalente na cavidade oral e é considerada como um dos fatores mais relevantes relacionados à perda do elemento dental (Frencken et al 2017). Embora a natureza fisiológica da dentina apresenta mecanismos de auto reparação, como no caso de pequenas lesões, se não existe um controle rigoroso da lesão de cárie,um quadro irreversível pode ser instaurado (De Souza Araújo et al 2022). Nesse sentido, o tratamento padrão para tratamento de lesões de cárie profundas envolve o capeamento pulpar e ou procedimento restaurador após remoção do tecido necrótico (Schwennicke et al 2016). Os dados revelam que anualmente aproximadamente 500 milhões de procedimentos restauradores são realizados no consultório (Heintze et al 2012). No entanto, embora a maior parte dos materiais restabelecem forma e função, sem controlar ou erradicar a doença cárie, e ainda falham por serem incapazes de promover a regeneração tecidual (de Souza Araújo et al 2022).

Histologicamente, os tecidos dentários mineralizados são compostos por uma fase orgânica composta de proteínas, minerais e água (Bertassoni et al 2017; De Melo Pereira et al 2018). Assim como osso, a dentina apresenta 90% de colágeno tipo I e 10% de proteínas acídicas não colagênicas, ou também chamadas de NCP´s (Bertassoni et al 2017). Cientificamente é comprovado que o próprio colágeno e as proteínas NCP´s são os maiores responsáveis pelo direcionamento e formação dos cristais de hidroxiapatita (Bonucci et al 2012). Pode-se dizer, que o colágeno tipo I atua como um arcabouço capaz de estruturar a precipitação desses cristais, e as NCP´s responsáveis pela sua precipitação, orientação e seu crescimento (He et al 2004).

Dessa forma, materiais bio inspirados ascendem como uma alternativa aos procedimentos restauradores convencionais embora ainda seja um grande desafio reproduzir as condições intrínsecas da mineralização que necessariamente envolve a interação do colágeno com as NCP´s e por fim formar estruturas biomineralizadas (Aytac et al 2021).

Os peptídeos de auto montagem (SAP), também classificados como materiais inteligentes são capazes de interagir com o microambiente e formar estruturas tridimensionais que atraem minerais durante o processo de mineralização (Kyle et al 2010; Maude et al 2012; Aggeli et

al 1997; Aggeli et al 2003). Esses peptídeos podem ser desenhados sinteticamente em um estado sólido com cadeias de aminoácidos específicas que, por sua vez, apresentam similaridades e ou são inspiradas nas proteínas nativas do tecido de interesse (Kyle et al 2010; Rivas et al 2019). Além disso, os peptídeos de auto montagem também demonstraram potencial de formar fibrilas nanoestruturadas (Kyle et al 2010), e induzir a mineralização (Kirkham et al 2007; Kind et al 2017).

O P_{11}-4, pertencente aos SAPs, é definido como um oligopeptídeo organizado na seguinte sequência CH3CO-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH2(Aggeli et al 2003). Outrossim, P_{11}-4 é anfifílico e capaz de alterar seu estado de fluido newtoniano para gel em função do pH, com sítios negativos (Aggeli et al 2003) e formar fitas do tipo Beta quando exposto a um estímulo de ordem fisiológica (Barco et al 2018). Em relação à sua função na mineralização, esses peptídeos têm sido caracterizados como modelos ideais para a deposição de hidroxiapatita em virtude de se assemelhar-se a proteínas ricas em fosfoserina envolvidas na mineralização (Rivas et al 2019; Eren et al 2018).

Nesse contexto, após o desenvolvimento do peptídeo de auto montagem P_{11}-4, previamente à utilização clínica deste peptídeo de maneira a modificar a superfície da dentina, testes convencionais *in vitro* ao qual envolve citotoxicidade celular frente a um tratamento com determinado material são exigidos. Em suma, no que concerne ao P_{11}-4, alguns testes celulares já foram realizados e demonstraram que o peptídeo de auto montagem não é um material citotóxico dentre as células múltiplas células previamente testadas (Koch et al 2022; Araújo et al 2022; Saha et al 2019). Testes celulares envolvendo o uso de células tronco dentais surgem como uma alternativa interessantíssima à regeneração dental frente a sua fácil obtenção, uma vez que, podem ser obtidas de dentes extraídos rotineiramente na clínica por razões ortodônticas ou no caso da dentição decídua pelo processo natural de esfoliação (Honda et al 2022). Uma outra vantagem explícita do uso de células tronco dentais reside no fato de que as questões éticas e morais são ínfimas quando comparadas a células tronco embrionárias, por exemplo. Ademais, do ponto de vista biológico, essas células são capazes de se diferenciar em linhagens dentais como odontoblastos, cementoblastos e osteoblastos.

A diferenciação do tipo osteogênica é um ensaio relativamente simples que comumente combina cultura de células mesenquimais com novos materiais com a finalidade de provar a sua eficácia em induzir a produção de nódulos minerais pelas células em questão. Para tal, o

uso de técnicas de expressão gênica associada a metodologias complementares como as que envolvem o uso do corante de Alizarina são frequentemente utilizados para se confirmar ou contestar a diferenciação osteogênica. Em caso afirmativo, o aumento dos níveis de expressão de gênica de marcadores osteogênicos são vinculados a quantificação e deposição de nódulos minerais.

Neste trabalho avaliamos a efetividade da utilização do P_{11}-4 em baixas concentrações na diferenciação osteogênica de células SCAP. Nenhuma toxicidade celular considerável foi relacionada aos resultados, o que corrobora os resultados dos estudos anteriores. Embora o uso de baixas concentrações tenha se mostrado capaz de produzir nódulos minerais como os ensaios de deposição e quantificação dos nódulos minerais por corante de alizarina, algumas diferenças foram encontradas no padrão morfológico destes minerais sendo dependente da concentração utilizada. No que diz respeito a expressão gênica, ainda o uso de baixas concentrações sugere ser um fator limitante a modular altas expressões dos marcadores osteogênicos. Por exemplo, os resultados obtidos neste trabalho sugerem que para o RUNX2,

no tempo experimental de 3 dias um ligeiro aumento na expressão deste marcador pode ser identificado. Contrariamente, para o gene ALP no tempo de 3 dias observa-se uma redução dos valores expressos, sugerindo que baixas concentrações podem causar um efeito negativo para certos genes de mineralização, como o mencionado em questão.

Além disso, é importante destacar que os experimentos do trabalho mencionado acima utilizaram-se de células de apenas um único doador, o que pode influenciar nos resultados até aqui obtidos. Com base na literatura e nas contribuições que esse trabalho de Tese traz, com modelos *in vitro*, os próximos passos envolvem estudos celulares com concentrações mais altas de P_{11}-4 e com células de diferentes doadores para eliminar possíveis divergências nos resultados.

No segundo trabalho discorremos, em uma revisão de literatura, sobre uma alternativa eficaz a ensaios de cultura celular convencional do tipo 2D: dispositivos microfluídicos de cultura celular. O motivo reside na falha que os modelos de cultura 2D padrões para desenvolvimento de novos fármacos não apresentam sucesso quando em fase de testes pré clínicos (Lacombe et al 2022). A perda financeira de grandes indústrias farmacêuticas alavancaram a busca e o desenvolvimento a uma tecnologia que fosse eficaz na testagem de novos fármacos (Ma et al 2022). De fato, ensaios de cultura 2D não conseguem mimetizar as

condições biológicas do tecido de interesse e o uso de experimentação animal se contrapõe às questões éticas (Probst et al 2018). Na odontologia, muitos pesquisadores têm se utilizado de dispositivos microfluídicos também chamados de *tooth on chip*, para ensaios de citotoxicidade de materiais dentários clínicos e experimentais (Rahimi et al 2018; França et al 2020; Ly et al 2021; Rodrigues et al 2021, Bordini et al 2022; Hu et al 22; Zhou et al 2022). Ainda que, essa plataforma de teste seja rotineiramente utilizada, esta apresenta limitações que até o presente momento não foram expostas confirmando a hipótese inicial. Primeiramente observamos que não existe padronização na confecção desses dispositivos e isto se deve ao fato de que existem diversos materiais disponíveis para confecção do mesmo. No entanto, observamos que a indicação do material varia de acordo com o *design* e finalidade do experimento. Por exemplo, na odontologia os artigos apenas se utilizaram do PDMS Poly-(dimethyl-siloxane) e PMMA Poly(methyl methacrylate) **(**PMMA) para confecção dos dispositivos. Embora produzir chips em PDMS seja relativamente simples, este apresenta como principal desvantagem sua natureza hidrofóbica, o que consequentemente pode levar a absorção e adsorção de moléculas, sendo indesejável para a testagem de drogas (Toepke et al 2006). No entanto, muitos se utilizam da modificação da superfície do PDMS com plasma, tornando-o um polímero compatível para determinados ensaios (Zhou et al 2010). O segundo material em ascensão para confecção de chips para pesquisa odontológica é um polímero termoplástico, o PMMA. Comparado ao PDMS, o PMMA apresenta maior compatibilidade de solventes e sem absorção de moléculas. Além disso é opticamente transparente (Nielsen et al 2020), apresenta boas propriedades mecânicas (Nahak et al 2022), boa rigidez (Leung et al 2022) e permite a prototipagem em pequena escala de produção (Ren et al 2013). No entanto, ao contrário do PDMS, este não permite modificação de superfície com ozônio ou plasma de oxigênio porque se torna um polímero incompatível (Nahak et al 2022).

Ademais, o uso de dispositivos microfluídicos na odontologia apresenta restrições referentes à validação e obtenção dos resultados obtidos. Em virtude da complexidade do protótipo de cada *chip* ser dependente da pergunta de cada problema, não podemos padronizá-lo como um todo. Além disso, podemos afirmar que na pesquisa odontológica, o uso desses dispositivos ainda é extremamente restrito a testes de citotoxicidade celular e imagens microscópicas. Ensaios envolvendo biologia molecular, como extração de RNA dentro do chip*,* são de suma importância para o avanço das pesquisas. Contudo, ainda é um desafio pois a cultura celular dentro do chip é restrita a quantidade celulares reduzidas

(aproximadamente 10 mil células). Portanto podemos mencionar que uma possível explicação para todas essas limitações está diretamente relacionada à complexidade que envolve o desenvolvimento do *tooth on chip*, necessitando de uma equipe multidisciplinar composta de biólogos/ dentistas, engenheiros e físicos altamente capacitados.

Simultaneamente ao identificarmos limitações também podemos observar que a utilização destes dispositivos se iniciou com a pesquisa na área básica de biofilme dental e interações celulares,e posteriormente, iniciou-se os trabalhos envolvendo odontoblastos *in vitro* (Lam et al 2016;Rahimi et al 2018), propriedades celulares de DPSCs comparadas a medula óssea (Pagella et al 2020) e inervação dental (Pagella et al 2020). Todos esses artigos podemos classificar como *chips* de 1ª geração. Na 2ª geração aparece o uso destes dispositivos para testes de citotoxicidade celular e morfologia frente a materiais dentários clínicos e experimentais. Recentemente uma 3ª geração do uso do *chip* o inclui para a testagem de materiais com fins regeneradores. Nossos resultados mostram que entre os materiais dentários já testados dentro do *chip* estão: *HEMA*, *Acid phosphoric*, *Adper Scotch bond*, *SE BOND*, *Silver Diamine Fluoride*, cimentos de silicato, *EDTA-chitosan*, e materiais experimentais com propriedades regeneradoras.

Outrossim, sabendo-se da importância da esterilização em ensaios de cultura celular, esta revisão também evidencia os métodos de esterilização do tipo radiação Gama e esterilização UV como os mais indicados para ambos os materiais (PDMS e PMMA). O PMMA devido sua natureza plástica não permite sua esterilização em autoclave. Já para o PDMS a esterilização em etanol 70% é contra indicada pois pode absorver moléculas. (Leung et al 2022)

No que concerne aos métodos de injeção celular no chip, encontramos a seguinte classificação: *Bottom-up*, no qual as células são inseridas em suspensões, inserção em blocos, como no caso da injeção de organóides ou esferóides pré-formados ou explantes quando micro fragmentos de biópsias são inseridos dentro do chip (Rogal et al 2022). Contudo, apesar das opções, na odontologia apenas artigos que se utilizam da técnica *bottom-up* de injeção celular foram encontrados.

Portanto, podemos concluir que embora esses dispositivos microfluídicos tenham sido favoráveis ao proporcionar dados mais condizentes com a realidade humana, ainda é preciso padronizar a fabricação, desenvolver chips mais funcionais e precisos. Por outro lado,

empresas especializadas no assunto têm obtido sucesso em desenvolver e comercializar chips para cultura 3D, a exemplo podemos citar empresas como *Emulate* (EUA) e *TissueUse* (Alemanha). Futuramente, o uso dos chips permitirá a personalização dos tratamentos ao permitir o uso de células do próprio paciente (Danku et al 2022).

4. CONCLUSÃO

1- O P_{11}-4 em nas concentrações de 10 μg/ml, 100 μg/ml and 1 mg/ml não foi citotóxico às células tronco dentais da papila apical (SCAP), demonstrou capacidade de induzir nódulos de mineralização na concentração 100 μg/ml e aumentar ligeiramente os níveis de RUNX2 mesmo quando utilizado a menor concentração de P_{11}-4 (10 μg/ml) no tempo experimental de 3 dias.

2- De acordo com os resultados obtidos a partir da revisão, confirmamos as limitações impostas pelo uso do chip em odontologia, são elas: ausência de padronização na prototipagem, aquisição e validação de resultados e restrição na funcionalidade, sendo restrita apenas a ensaios de citotoxicidade celular e caracterização morfológica. Outros fatores limitantes secundários: alto custo e demanda de profissionais extremamente qualificados, o que também demonstra ser o fator responsável pelo baixo número de artigos encontrados na odontologia utilizando-se dessa metodologia.

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ANEXO 1 - Comprovante de submissão artigo 1

This is an automated message.

Journal: Journal of Dentistry
Title: The Self Assembling peptide P11-4 influences viability and osteogenic differentiation of stem cells of the apical papilla (SCAP).
Corresponding Author: Dr. Regina Maria Puppin Rontani
Co-Authors: Jessica Rodrigues Camassari, DDS, MSc, PHD student; Iago Cortes; Karina Müller
Manuscript Number: JJOD-D-23-00168

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