UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

Flávia Gomes Matos

Avaliação de superfícies de titânio-15 molibdênio submetidas a funcionalização com estrôncio: análises in vitro de propriedades físico-químicas e biológicas

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Dissertação apresentada à Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara para a obtenção do título de Mestre em Odontologia, na Área de Periodontia

Orientador: Joni Augusto Cirelli

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DADOS CURRICULARES

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“Não fui Eu que lhe ordenei? Seja forte e corajoso! Não se apavore, nem desanime, pois o Senhor, o seu Deus, estará com você por onde você andar. ” *

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RESUMO

Tratamentos de superfície, desenvolvimento de novas ligas e funcionalização das superfícies dos implantes com elementos bioativos têm sido objetivos de estudos recentes na área da Odontologia visando contribuir para o processo de osseointegração. Nesse trabalho, dois estudos investigaram a influência na resposta celular in vitro e de propriedades físico-químicas de ligas de Ti-15Mo submetidas a (1) funcionalização com estrôncio pelo método hidrotérmico previamente submetidas ou não ao tratamento químico por ácido (H_3PO_4) e base (NaOH) e a (2) dois métodos de aplicação de estrôncio método hidrotermal e método *magnetron co-sputtering.* Testes de composição química e topografia demonstraram que ambas as técnicas foram efetivas na aplicação de estrôncio. Em (1) o método hidrotermal conciliado a tratamento prévio com ácido e base apresentou resultados de maior quantificação de estrôncio com liberação mais lenta além de melhores resultados nas análises de molhabilidade, energia de superfície e resistência a corrosão. Os ensaios biológicos verificaram que em (1) células pré-osteoblásticas MC3T3-E1 apresentaram características morfológicas de maior espraiamento e proliferação em superfícies com adição de estrôncio. Em (2) células humanas do ligamento periodontal os resultados demonstraram maior proliferação e espraiamento em superfícies tratadas pelo método *magnetron co-sputtering.* Os estudos demonstraram que a adição de estrôncio contribuiu para os eventos celulares e, conciliadas com tratamento químico prévio à adição de estrôncio, promoveram melhorias nas propriedades físico-químicas de superfícies de Ti-15Mo. Além disso, os métodos de aplicação e forma química do estrôncio revelaram influenciar nas respostas celulares iniciais. Análises sobre a influência dos tratamentos in vivo são necessárias.

Palavras – chave: Titânio. Implantes dentários. Estrôncio.

Matos FG. Evaluation of titanium-15 molybdenum surfaces submitted to strontium functionalization: in vitro analysis of physical-chemical and biological properties. [dissertação de mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2020.

ABSTRACT

Surface treatments, development of new alloys and functionalization of implant surfaces with bioactive elements have been the objectives of recent studies in the field of Dentistry aimed at contributing to the process of osseointegration. In this dissertation, two studies investigated the influence on the in vitro cellular response and physical-chemical properties of Ti-15Mo alloys submitted to (1) strontium functionalization by the hydrothermal method previously submitted or not to chemical treatment with acid (H_3PO_4) and alkali (NaOH) and (2) two methods of strontium application: hydrothermal method and magnetron co-sputtering method. Tests of chemical composition and topography demonstrated that both techniques were effective in the application of strontium. In (1) the hydrothermal method reconciled the previous treatment with acid and alkali presented results of greater strontium quantification with slower release and better results in the analysis of wettability, surface energy and corrosion resistance. The biological tests verified that in (1) preosteoblastic cells MC3T3-E1 presented morphological characteristics of greater spreading and proliferation on surfaces with strontium addition. In (2) human periodontal ligament cells the results showed greater proliferation and spreading on surfaces treated by the magnetron co-sputtering method. The studies demonstrated that the addition of strontium contributed to cellular events and, combined with chemical treatment prior to the addition of strontium, promoted improvements in the physical-chemical properties of Ti-15Mo surfaces. In addition, the methods of application and chemical form of strontium were shown to influence initial cell responses. Analyses on the influence of in vivo treatments are necessary.

Keywords: Titanium. Dental implants. Strontium.

SUMÁRIO

1 INTRODUÇÃO

O sucesso da osseointegração dos implantes dentários depende de fatores relacionados com o estado geral de saúde do paciente, quanto ao uso de medicamentos controlados e a presença de doenças sistêmicas[1]. Da mesma forma, esse sucesso é dependente das características associadas ao material implantado, tais como tratamentos de superfície e ligas de titânio[2].

O uso de ligas metálicas de titânio tem demonstrado propriedades mecânicas superiores em relação ao uso de titânio comercialmente puro[3]. Amplamente utilizada em implantes para substituição de elementos dentários perdidos e em próteses ortopédicas, a liga Ti-6Al-4V apresenta alta resistência à corrosão e propriedades mecânicas adequadas[4,5].

A exposição de implantes de Ti-6Al-4V em meio fisiológico, no entanto, gera preocupação quanto à possibilidade de citotoxicidade causada pela dissolução de óxidos seguido pela liberação de íons alumínio[6] e vanádio[7] para os tecidos peri-implantares. Além disso, a liga Ti-6Al-4V apresenta alto módulo de elasticidade quando comparada ao titânio comercialmente puro e com outras ligas[8]. Assim, o desenvolvimento de ligas de titânio compostas por elementos não tóxicos como o tântalo (Ta), nióbio (Nb), zircônio (Zr) e molibdênio (Mo), tem como objetivo substituir o uso de ligas de Ti-6Al-4V excluindo a possibilidade de contaminação por aqueles íons metálicos e apresentando módulo de elasticidade mais compatível ao módulo de elasticidade ósseo[8-11].

Estudos prévios demonstraram que ligas de titânio-15molibdênio (Ti-15Mo) apresentam maior estabilidade eletroquímica, baixo módulo de elasticidade, e resistência à corrosão em meio fisiológico simulado, em comparação à liga de Ti-6Al-4V[12]. Além disso, a concentração de molibdênio em ligas de titânio influencia a estrutura cristalina do metal[9,13]. A estrutura cristalina do titânio pode ser classificada em alfa e beta e são importantes nas propriedades mecânicas das ligas. A fase α está relacionada à estrutura estável em temperatura ambiente. No entanto, quando submetidas a altas temperaturas transforma-se em fase β[14].

Oliveira et al.[9] e Ho et al.[15] demonstraram que a partir de uma concentração de 10% de molibdênio (Ti-10Mo) a presença da fase alfa de titânio já não se verifica, sendo observada apenas a fase beta. O alfa-titânio é muito susceptível à distorção e

sobreaquecimento, fato que direcionou as pesquisas recentes ao desenvolvimento de titânio β.

Assim, além de estabilizar a fase beta, a liga de titânio com uma concentração de 15% de molibdênio (Ti-15Mo) se destaca entre as outras ligas de Ti-Mo devido a sua excelente resistência à corrosão e sua boa combinação de propriedades mecânicas e resistência ao desgaste[16].

As características da superfície do implante também são fatores que afetam as propriedades dos materiais sendo determinantes para a incorporação do osso no implante[17]. Assim, molhabilidade, rugosidade, topografia e sua composição são propriedades físico-químicas da superfície que exercem influência sobre os eventos celulares durante o processo de cicatrização[18].

A molhabilidade da superfície é importante no processo de adesão celular[18,19]. Uma essencial grandeza na avaliação da molhabilidade é o ângulo de contato. O ângulo de contato é definido como o ângulo formado entre a superfície sólida e a tangente do líquido no ponto de contato dos dois. Esse ângulo é dependente da tensão superficial do líquido e da energia de superfície do material. Alguns autores relatam que ângulos maiores que 65° caracterizam superfícies hidrofóbicas[20], enquanto outros indicam hidrofobicidade acima de 90°[21]. De forma geral, os estudos sugerem que quanto menor o ângulo de contato, mais hidrofílica é a superfície tendo maior molhabilidade e maior energia de superfície favorecendo a adesão celular[21].

Além da molhabilidade, a rugosidade da superfície influencia na adesão, mas também no espraiamento celular e adsorção de proteínas[22]. A rugosidade promove uma topografia de maior área de superfície que podem alterar a orientação, o crescimento, metabolismo e diferenciação das células osteoblásticas[19].

A composição química da superfície pode estimular a regeneração óssea, induzindo repostas específicas da osseointegração em células e tecidos[23]. A composição química também influencia na molhabilidade das superfícies e depende dos tratamentos realizados nas superfícies[24].

Assim, tratamentos tais como irradiação por raio laser[25], anodização[2], ataque ácido e tratamento alcalino[26] têm sido realizados nas superfícies dos implantes afim de otimizar as características desses materiais para a osseointegração.

O tratamento ácido nas superfícies de titânio é realizado principalmente com ácido clorídrico, sulfúrico, fluorídrico ou a mistura entre eles[2], e afeta as características

do material como rugosidade, molhabilidade/energia livre de superfície demonstrando promover maior osteogênese durante a remodelação óssea[27].

No tratamento alcalino com NaOH, ocorre precipitação de hidroxiapatita através da formação de uma camada de titanato de sódio, tornando os materiais bioinertes em bioativos. Há formação de grupos funcionais bioativos (OH^-) devido à troca iônica Na^+ da superfície do titanato de sódio com os íons H_3O^+ na solução. Os grupos Ti-OH formados combinam-se com os íons Ca^{2+} do fluido e formam titanato de cálcio amorfo que se converte em apatita cristalina[28].

Pesquisas tem reportado os efeitos do tratamento ácido/alcalino na expressão gênica osteoblástica e observado que esse tratamento induz um comportamento osteocondutivo mais pronunciado, cujas superfícies mostraram a formação de características superficiais micro e nanométricas[26,29,30].

Embora os implantes dentários tenham geralmente altas taxas de sucesso, certas condições sistêmicas do paciente podem levar a condenação dos implantes. Em pacientes idosos, além de acelerar a perda óssea, o acúmulo de adipócitos da medula óssea pode interferir na estabilização do implante[31]. Além disso, os pacientes diabéticos e osteoporóticos, por exemplo, apresentam dificuldades no processo de reparação óssea[32-35].

O desenvolvimento de superfícies de implantes com elementos bioativos, como o estrôncio (Sr), tem sido discutido como uma alternativa para contribuir na superação do desafio da osseointegração em ossos de má qualidade. Além disso, a otimização do processo inicial de osseointegração permitiria a utilização de protocolos de carga mais precoces.

O estrôncio é caracterizado como um metal alcalino terroso e, devido à sua semelhança com o cálcio, é possível incorporar o Sr^{2+} na fase mineral do osso[36]. Com estudos desde a década de 1950 por McCaslin e Janes[37] no seu uso como tratamento para pacientes com osteoporose grave sob a forma de ranelato de estrôncio, o estrôncio apresenta-se como um elemento fundamental na remodelação óssea, estimulando a formação e inibindo a reabsorção óssea. Sua administração oral, entretanto, pode apresentar efeitos adversos de diarréia, cefaléia, náuseas[38] tromboembolismo venoso[39] e hipersensibilidade[40].

Na Odontologia, a funcionalização da superfície de titânio com estrôncio tem sido uma alternativa de incorporação local em revestimentos sobre implantes, a fim de minimizar os efeitos sistêmicos adversos. Além disso, alguns estudos que

implementaram o estrôncio em suas superfícies observaram maior estímulo à diferenciação das células mesenquimais para a linhagem osteogênica[41], aumento da formação óssea e redução da atividade osteoclástica e reabsorção óssea[42].

O estrôncio tem sido incorporado na superfície de titânio por métodos, tais como anodização[43], hidrotérmico[44] e *magnetron co-sputtering*[45]. O método hidrotermal é uma alternativa simples e de baixo custo e provou ser viável na incorporação de elementos bioativos, tais como estrôncio e cálcio, em superfícies de titânio[46-48].

Estudos in vitro e in vivo em animais saudáveis[49,50] observaram uma influência positiva na diferenciação celular e uma melhor integração óssea nos implantes sob tratamento hidrotérmico com estrôncio. Lin et al.[44] analisaram superfícies de titânio com tratamento hidrotermal com estrôncio em coelhos osteoporóticos e encontrou efeitos positivos no processo de osseointegração precoce.

Zhou C et al, 2019[51] verificou, in vitro, uma ação antiadipogênica das superfícies de titânio com adição de estrôncio, pelo processo hidrotermal, suprimindo a produção de gotículas lipídicas e a expressão de adipocinas. Os resultados in vivo foram de acordo com os resultados in vitro, demonstrando que as superfícies de estrôncio melhoraram significativamente a osseointegração e inibiram a formação de tecido adiposo na tíbia de ratos idosos.

O método de *magnetron co-sputtering* é a técnica de deposição física a vapor (*Physical Vapour Deposition - PVD*) mais amplamente utilizada. A deposição física a vapor compreende um grupo de técnicas de revestimento a vácuo que são utilizadas para depositar uma película fina de substâncias que melhoram as propriedades e o desempenho dos materiais apresentando vasta aplicação industrial, dentre elas, em materiais da esfera da saúde. O processo de *sputtering* refere-se à remoção de material atomizado de um sólido por bombardeamento energético de suas camadas superficiais por íons ou partículas neutras[52].

Diversos autores têm utilizado o método de *magnetron co-sputtering* para aplicação de estrôncio em superfícies de titânio[53-55]. Andersen et al.[56] prepararam 4 grupos de superfícies funcionalizadas com estrôncio pelo processo de *magnetron co-sputtering* com revestimentos de diferentes espessuras. A análise da liberação controlada de estrôncio e a caracterização físico-química das superfícies revelaram uma superfície nanotopográfica colunar, considerada essencial para a liberação controlada de Sr, já que esta estrutura produz uma área superficial altamente efetiva. Além disso, foi observado um aumento na liberação de Sr com o aumento da

espessura do revestimento o que também aponta para uma relação entre morfologia e liberação de Sr, pois mostra que a liberação não está ocorrendo apenas nos primeiros nanômetros da superfície do revestimento.

Testes biológicos in vitro revelaram que a aplicação de estrôncio em implantes pelo método *magnetron co-sputtering* induz a proliferação celular e viabilidade sem mostrar citotoxicidade das superfícies testadas[56]. Em testes in vivo, indicaram um aumento significativo da formação óssea periimplantar, sendo que os níveis séricos sanguíneos avaliados por espectrometria de absorção atômica (*Atomic absorption spectrometry - Aas*) não indicaram efeitos sistêmicos do estrôncio[54].

Considerando o exposto, visando estimular a osseointegração em pacientes com ossos de má qualidade, novos estudos que conciliem materiais de excelentes propriedades mecânicas e biocompatíveis, como implantes de ligas de Ti-15Mo, com tratamentos de superfícies promissores a osseointegração, como a adição de estrôncio, são necessários e relevantes para a Implantodontia.

2 PROPOSIÇÃO

Considerando a importância do processo de osseointegração e a influência das propriedades dos materiais na resposta biológica, o objetivo desse trabalho foi avaliar superfícies da liga Ti-15Mo nos aspectos físico-químicos e respostas celulares após diferentes tratamentos de superfície, descritos a seguir:

a) Funcionalização das superfícies com estrôncio pelo método hidrotermal previamente submetidos ou não ao ataque ácido com H_3PO_4 e alcalino com NaOH (Publicação 1)

b) Funcionalização das superfícies com duas técnicas de aplicação de estrôncio: *magnetron co-sputtering* e hidrotermal (Publicação 2).

3 PUBLICAÇÕES

O desenvolvimento da dissertação resultou em dois trabalhos: 1) Strontium-loaded titanium-15molybdenum surface enhances MC3T3-E1 osteoblastic activity in vitro e 2) Effect of two different methods of surface functionalization of Ti-15Mo alloy with strontium in the behavior of human periodontal ligament cells.

3.1 Publicação 1*

Strontium-loaded titanium-15molybdenum surface enhances MC3T3-E1 osteoblastic activity in vitro

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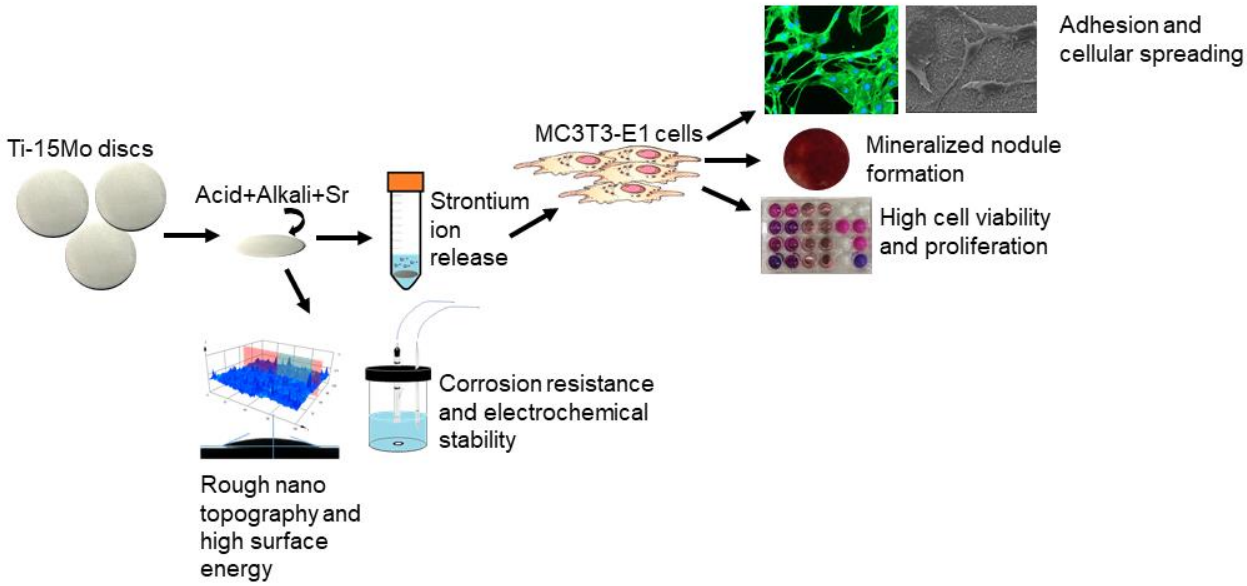
Abstract

The biochemical interactions of the osseointegration process between the titanium surface and the bone cells are influenced by the physicochemical properties of the surface as well as by the composition of the titanium alloy. The purpose of this study was to evaluate physical, chemical and biological characteristics of titanium-15 molybdenum discs submitted to chemical treatment with phosphoric acid (H_3PO_4), followed by alkaline (NaOH) and hydrothermal treatment with strontium compared to discs with smooth surfaces and with chemical treatment without strontium functionalization. The alloy used in this study demonstrated to stabilize the crystalline structure in beta phase, as showed the results of the X-ray diffraction analysis. Tests of chemical composition by X-ray dispersive energy spectroscopy showed that the hydrothermal method is feasible for the addition of strontium on titanium surfaces. Furthermore, confocal laser microscopy analysis indicated that the surfaces with strontium showed greater roughness and when combined acid/alkaline treatment with strontium, they obtained an improvement in surface energy and resistance to corrosion. This combination was also beneficial for greater strontium retention on surfaces according to inductively coupled plasma optical emission spectrometry tests. Cellular responses (MC3T3-E1) indicated that all surfaces were viable for cell culture, allowing adhesion and proliferation, but in groups with strontium, greater spreading and formation of mineralization nodule were observed. The findings in this study indicated that the addition of strontium on the surfaces of ti-15Mo by the hydrothermal method combined with previous acid/alkaline treatment contributed to the improvement of the physicochemical properties of the materials, besides optimizing the cellular events.

Keywords: Titanium-molybdenum alloy, Strontium, Surface properties, Osteoblasts, Cell proliferation

Graphical abstract



Statement of Significance

The seek for optimization of the osseointegration process aims at reducing the healing period especially in patients who present poor bone quality, and contributing to the installation of early protocols.

The osseointegration process is influenced by the characteristics of the implanted material such as titanium alloys and surface treatments. Thus, the biofunctionalization of implant surfaces with strontium aims to use the molecular properties of this element to favor bone formation and prevent bone loss. In addition, the development of materials that present not only excellent mechanical properties, but also biocompatibility, such as Ti-15Mo alloy implants, is important to contribute to the short and long term success of implants.

1. Introduction

The functionalization of titanium-based materials has become largely accepted in view of the current trend towards the reduction of total treatment time spent in the oral rehabilitation with titanium implant-supported prosthesis [1], and the seek for enhanced bone-to-implant anchorage of femoral stem after total hip arthroplasty [2].

Beyond patient's favorable general health condition, the success of implants' osseointegration relies on mechanical stability and biochemical interactions between the bone cells (mesenchymal stem, osteoblasts, osteoclasts, and osteocytes) and titanium surface [3, 4]. Otherwise, bone ingrowth will not occur and the fibrous tissue will take place in the early phase of osseointegration [5]. In this respect, the development of feasible methods of loading titanium implants with bioactive molecules with osteogenic activity is of utmost importance [6].

The osteogenic activity of titanium-based materials loaded with inorganic molecules (calcium, phosphate, strontium, magnesium) have been investigated by several studies [7-9]. These molecules are incorporated into the titanium surfaces by methods such as physical vapor deposition [10], laser irradiation of femtoseconds [8], hydrothermal method [11] and has demonstrated antibacterial, angiogenic and osteogenic properties [12]. Calcium and phosphate ions applied on surfaces have improved osteoconductivity and aid bone apatite formation [13]. Studies show that strontium influences the mechanisms of bone remodeling, stimulating its formation and inhibiting bone resorption [14, 15]. This chemical element has already being used as treatment for patients with severe osteoporosis [16]. Furthermore, its application on implant surfaces contributed to greater cellular adhesion and to the expression of indicators of osteoblast differentiation and mineralization, such as ALP, osteopontin OPN, osteocalcin OCN [14, 17] enhancing early osseointegration.

The biochemical interactions of osteoblastic cells with the implant surface are essential for bone repair and are influenced by the physicochemical characteristics of the surface. Characteristics such as wettability have shown better results in strontium treated implants [18]. Similarly, surface modifications subjected to acid and alkaline treatment showed improved hydrophilicity, surface free energy and roughness [19], promoting greater bone-implant contact during bone remodeling [20].

Studies have shown that surface energy and hydrophilicity have a positive impact on cell adhesion and spreading by homogeneously inducing proliferation and mineralization at the surface [21]. The increase of the contact area by roughness promoted by surface treatments also improves cell-substrate interaction due to fibronectin adsorption and serum proteins favoring contact osteogenesis [22]. These implant properties are highly dependent not only on surface modifications, but also on titanium and its metallic composition. Commercially pure Ti showed significantly less roughness compared to alloy titanium [23]. Ti-6Al-4V alloy showed better wettability

performance compared to other titanium alloys (Ti-6Al-2Zr-1Mo-1V, Ti-15Mo-3Al-2.7Nb-0.2Si) [24].

However, the dissolution of ions in physiological environment generates concern about the biocompatibility of these alloys [25]. Studies have shown that, although Ti-6Al-4V alloy is widely used due to its mechanical advantages [26], but the release of Al and V ions can be potentially toxic to cells, inducing the release of inflammatory mediators in tissues around the implant [27], compromising distance osteogenesis. In addition, studies reported the possible occurrence of neurological disorders by the presence of aluminum ions [28]. In this context, the development of titanium alloys using molybdenum (Ti-Mo) has been presented as a viable alternative with similar mechanical and corrosion resistance, but with greater biocompatibility than other alloys [29, 30].

Studies showed that the concentration of molybdenum in titanium alloys influences the crystalline structure of the metal [31, 32]. Titanium alloy with a concentration of 15% molybdenum (Ti-15Mo) presents itself as a beta phase stabilizer and stands out among other Ti-Mo alloys due to its excellent corrosion resistance and good combination of mechanical properties and wear resistance [29].

Therefore, considering the positive results of acid-alkali titanium surfaces and the potential role of bioactive molecules in both distance and contact osteogenesis, this study sought to investigate the impact of strontium-charged Ti-15Mo by the hydrothermal method regarding physical-chemical aspects and in vitro behavior of mouse pre-osteoblastic cells (MC3T3-E1).

2. Materials and methods

2.1. Specimens and surface physicochemical properties

2.1.1. Sample preparation

In this study, discs (area: 12.2 mm; thickness: 2 mm) were obtained from a Ti-15Mo bar (ATI Alvac, Monroe, NC, USA) and used to characterize surface properties and for cell culture experiments. Four groups were obtained: **US** (untreated sample), **Ac+Alk** (acid+alkali), **Ac+Alk+Sr** (acid+alkali+strontium) and **US+Sr** (strontium). The

discs were mechanically polished with 600 to 1500 grit SiC and then ultrasonically washed with acetone, alcohol and deionized water.

The experimental surfaces were treated with phosphoric acid (H_3PO_4) concentrated at 80°C for half an hour and alkaline treatment with NaOH at 60°C for 24h, as previously described [33]. After that, a strontium solution was prepared by diluting $Sr(OH)_2.8H_2O$ in Milli-Q water to give the final concentration of 60 mM. The Ti-15Mo samples were placed in 100 mL of the previously prepared strontium solution, and the hydrothermal treatment were performed in autoclave, with constant temperature and pressure (121°C and 1.0 to 1.5 Kgf/cm^2) for 1 hour. All the samples were sterilized by UVA/C irradiation (Veco VLFS-12) for 1 hour each side [34].

2.1.2. Surface characterization

Surface morphologies and semi-quantitative chemical composition were observed by scanning electron microscopy (SEM) (JEOL JSM-6610LV, JAPAN) and energy dispersive X-ray spectroscopy – EDS (THERMO SCIENTIFIC 6743A-1UES-SN, USA) and 2 discs from each group were used. Measurements of roughness, magnification images and reconstructions of the surfaces studied in 3D format on the surfaces of the two samples of each group were performed with the OLYMPUS - LEXT OLS 4000 laser confocal microscope (Federal University of São Carlos - UFSCar)

For the X-ray diffraction (XRD) measurements 3 samples from each group were used and the experiment was performed as described [33] with a range of 5–90° in 2 theta (x). The contact angle / surface energy (Ramset-Hart, USA), were performed in three samples of each group using water and diiodomethane to analyze its hydrophilicity. The average contact angle with both liquids was determined by DROPimage CA software, Rame-Hart, USA.

2.1.3. Corrosion resistance and electrochemical stability

These analyses were performed in a 0.9% NaCl solution (m/m) at room temperature, using an electrochemical cell three electrodes. One work electrode composed of the material under study, one against platinum electrode and one silver reference electrode silver chloride (Ag/AgCl KCl 3mol/L) [35].

The open circuit potential (OCP) was measured and registered for 1,5 h during the stabilization of the samples in the electrolyte. The electrochemical behavior was analyzed through the potentiodynamic response of the alloys and individual components. Potentiodynamic polarization scans were carried out at a scan rate of 1 mV/s in the range from –0.3 to +3.0 V.

Two samples were used from each group and after the corrosion tests were analyzed by SEM to evaluate the modifications caused on the surfaces.

2.1.4. Strontium ion release

Three samples from each group were submerged in 3 mL of phosphate buffered saline (PBS) solution (37°C, pH 7.4). The PBS solution containing Sr released was analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo Fisher Scientific iCAP 7000, USA) at intervals of 1, 3, 5, 7 and 14 days as previously described [36]. For total strontium surface quantification, the samples of Ac+Alk+Sr and US+Sr groups were submitted, in triplicate, to an extraction process using 10mL of nitric acid 30% and the values were determined by ICP-OES (Thermo Fisher Scientific iCAP 7000, USA) [37].

2.2 Osteoblast activity

To evaluate the cell behavior, a mouse calvaria-derived preosteoblast cell line (MC3T3-E1), were plated in α-MEM, supplemented with 10% fetal bovine serum, antibiotics (Penicillin 100 U/mL and Streptomycin 100 µg/mL), and osteogenic inducers (2 mmol/L of ascorbic acid and 10 mM/L β-glycerophosphate), in a humidified atmosphere of 5% CO_2 at 37°C [23]. All the tests used cell passage between 6 to 7.

2.2.1 Cellular morphology

Cells were seeded at a density of $2x10^4$ cells in the surface of discs allocated into 24-well plates. The cell morphology and spreading were evaluated by scanning electron microscopy analysis (SEM) (JEOL JSM-6610LV, JAPAN) after 1 and 3 days of incubation. Two samples from each group were used for each period analyzed. Cells were washed in PBS, fixed with glutaraldehyde 2% in pure α-MEM for 1 hour, washed

in PBS again and dehydrated in increasing concentrations of ethanol (until 100%). After drying in a vacuum desiccator, samples received gold deposition (Denton Vaccum Desk V, 120 seconds, current 20 mAmps) [9].

2.2.2. Cellular adhesion and spreading

In order to assess the stage of adhesion and spreading at 1, 3 and 7 days of cells culture, the samples were evaluated by direct fluorescence with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) and 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes), for nuclear staining. Cells were seeded at a density of $2x10^4$ cells in discs and allocated into 24-well plates and then they were fixed with 4% formalin solution for 10 min. The cells were washed in PBS and permeabilized with T-PBS (1% Triton X-100 in PBS; Sigma-Aldrich) for 5 min. The T-PBS were removed and the cells were incubated with Alexa Fluor 488 and DAPI for 20 min and 5 min, respectively. The cells were visualized in inverted fluorescence microscopy (Evos fl, AMG Micro). The analyses were carried out in duplicate in each period.

2.2.3. Cellular viability and proliferation by Alamar Blue (AB)

Cell viability and proliferation was evaluated by a colorimetric assay (Alamar Blue- Molecular Probes, USA). $5x10^4$ cells were seeded in the discs and evaluated after 1, 3, 7, 10, 14 and 21 days. At each time point, Alamar Blue was added to 10% of the total volume of each well and incubated for 4 hours. After this, the absorbance of the supernatant was measured by spectrophotometer at a wavelength of 540 nm and 600 nm. The number of viable cells was associated with the level of reduction and expressed as a percentage reduction of Alamar Blue according to the manufacturer's formula. This experiment was performed in duplicate and repeated at three different times.

2.2.4. Mineralized nodule formation

To evaluate the amount of mineral deposited by the cultivated cells, $5x10^4$ cells were sown on discs and the calcium was quantified by the Alizarin red (Sigma-Aldrich) on day 21.

The discs were washed with PBS and fixed with 70% alcohol for 1 hour at 4°C and then stained with 1 mL (40 mmol/L, pH 4.2) of Alizarin red solution (Sigma-Aldrich) per disc at room temperature for 5 min. The excess solution was removed with distilled water. For quantification of the mineralized nodules, 1 ml of 10% cetylpyridinium chloride (Sigma-Aldrich) solution per disc at room temperature was applied. Then, 150 µL of solution from each sample were transferred to a 96-well plate and read in a spectrophotometer (Spectramax M, Molecular Devices) at wavelengths of 550nm and analyzed with the equipment software (SoftMx Pro, Molecular Devices). For this experiment, two discs from each group were used and the experiment was repeated three times. A control without cells was also necessary to visualize the false results due to the possibility of interaction between Sr and Alizarin red.

2.3. Statistical analysis

GraphPad Prism 6.0 for Windows (GraphPad Software Inc., La Jolla, USA) was used for statistical evaluation. Statistical analyses were performed by means of parametric or non-parametric tests for independent data and more than two samples (ANOVA or Kruskal-Wallis test, respectively, in case of normal sample distribution and homogeneity of variances), followed by multiple comparison tests, when applicable. The minimum statistical significance was established in $P \leq 0.05$.

3. Results

3.1. Surface morphology

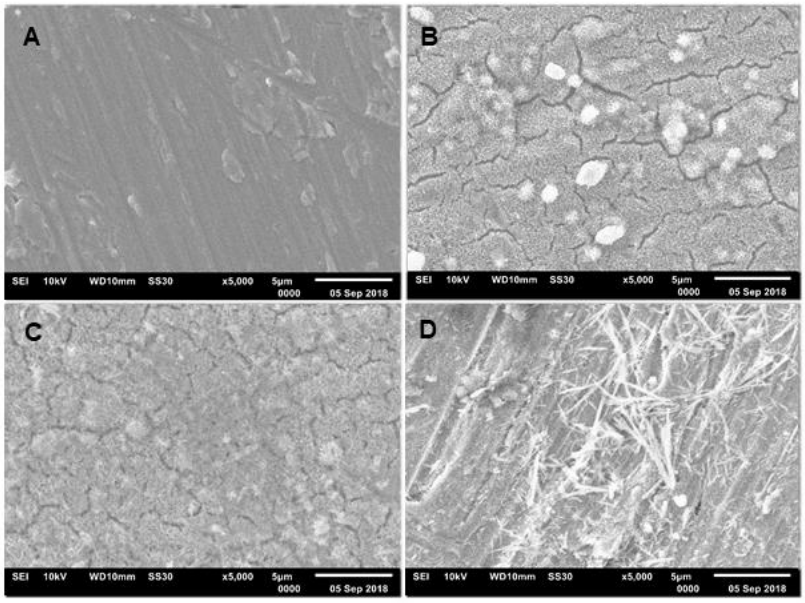


Fig. 1. Scanning electron microscope images of Ti-15Mo samples with different treatments. (A) US, (B) Ac+Alk, (C) Ac+Alk+Sr and (D) US+Sr surfaces at a magnification of x5000.

Fig. 1 shows the different surface morphologies of the investigated samples by SEM. The US and US+Sr samples showed oriented lines caused by grit paper. The group with the acid etching follow by alkaline treatment presented a nanotopographic formation and this aspect continued after the strontium deposition (Ac+Alk+Sr).

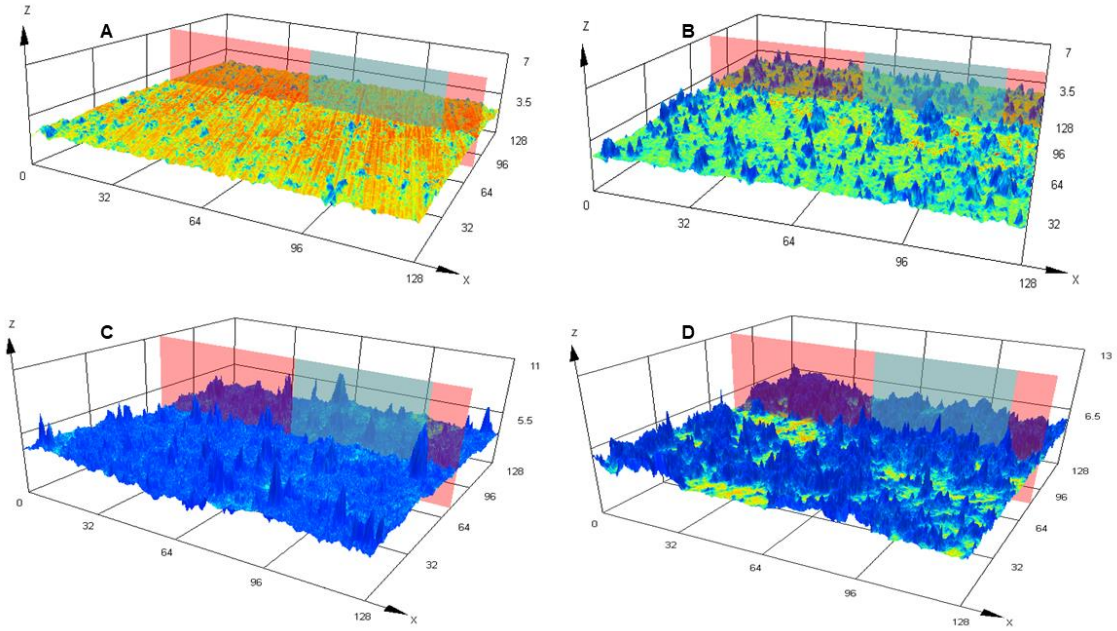


Fig. 2. 3D images constructed by laser scanning confocal microscopy show the surface roughness of the samples (A) US, (B) Ac+Alk (C) Ac+Alk+Sr and (D) US+Sr.

3D images of the different groups and measures of roughness were obtained from laser confocal microscopy (Fig. 2). The mean roughness values were Ra(µm) 0,06; Ra(µm) 0,10; Ra(µm) 0,25; Ra(µm) 0,47 for the US, Ac+Alk, Ac+Alk+Sr and US+Sr groups, respectively. It is possible to observe that the groups with strontium addition presented the highest values of roughness when compared to the other groups, however all groups are classified as nanotopographic.

3.2. Surface chemical analysis

The groups Ac+Alk+Sr and US+Sr showed a homogeneous distribution of the structures with crystallized aspect on its surface (Fig. 1). The X-ray Dispersive Energy Spectroscopy assay demonstrated that these structures are strontium (Fig. 3).

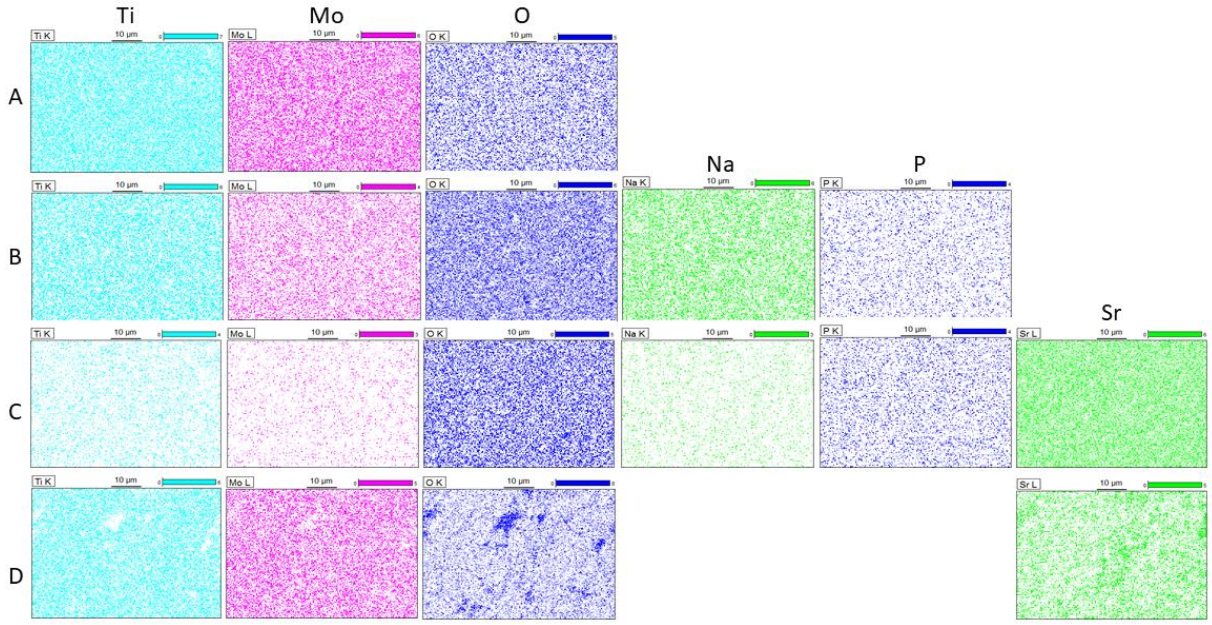


Fig. 3. Elemental mapping analysis by energy dispersive X-ray spectroscopy (EDS) of the samples (A) US, (B) Ac+Alk (C) Ac+Alk+Sr and (D) US+Sr. Elements detected: titanium (Ti), molybdenum (Mo), oxygen (O), sodium (Na), phosphorus (P) and strontium.

The distribution map of the elements by energy dispersive X-ray spectroscopy (EDS) demonstrated the presence of titanium, molybdenum and oxygen in all samples. The elements corresponding to the alkali/acid treatment (sodium, phosphorus) and strontium were identified.

Fig. 4 shows the XRD peaks intensities of the investigated samples. All surfaces presented with beta titanium peaks and the presence of alpha phase titanium was not observed. The surfaces US+Sr and Ac+Alk+Sr suggest the formation of peaks of strontium oxide hydrated, represented by the peaks corresponding to the blue bars.

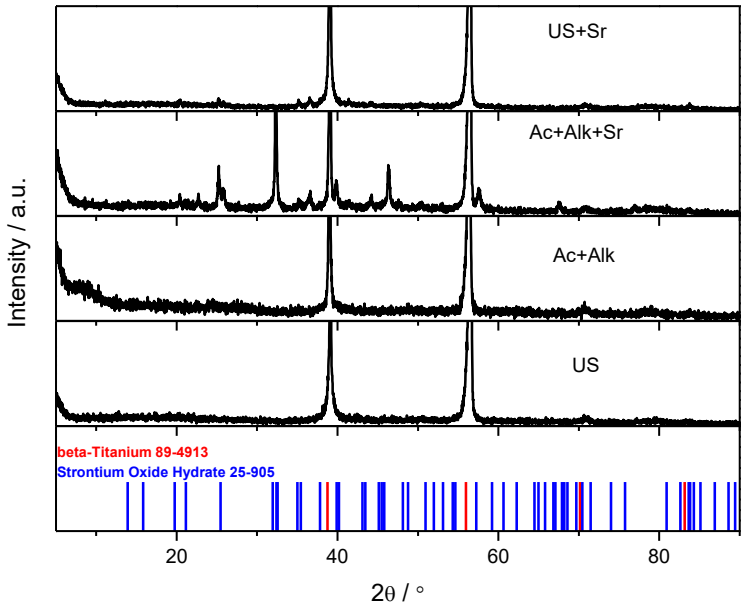


Fig. 4. X-Ray Diffraction (XRD) of samples US, Ac+Alk, Ac+Alk+Sr and US+Sr. Corresponding peaks the red bars refer to the titanium beta phase. The blue bars refer to the peaks of possible formation of strontium oxide hydrated.

3.3. Wettability and surface free energy

Contact angle results and surface energy are shown in Table 1. The samples submitted only to acid/base treatments were the most hydrophilic among the groups. Groups treated with acid/alkali (Ac+Alk and Ac+Alk+Sr) presented the best values of surface energy without significant difference between them.

Table 1. Results of contact angle with water and diiodomethane and surface energy of the different samples. γ_S^p, γ_S^d and γ_S indicate the values of the polar, dispersive and total surface energy components (mN/m) respectively.

			Surface energy (mN/m)					
	Contact angle (±SD)		**Geometric mean**			**Harmonic mean**		
Groups	**Water**	**Diiodomethane**	γ_S	γ_S^d	γ_S^p	γ_S	γ_S^d	γ_S^p
US	72.13	40.88	45.77	39.16	6.60	51.74	39.76	11.99
Ac+Alk	18.06	33.30	74.90	42.80	32.10	78.87	43.09	35.78
Ac+Alk+Sr	33.85	19.66	70.98	47.88	23.10	76.54	47.92	28.62
US+Sr	64.51	47.80	47.02	35.49	11.53	52.84	36.47	16.36

3.4. Corrosion resistance and physicochemical stability

The results of the electrochemical corrosion tests are shown in Fig. 5. The open-circuit potential curves and potentiodynamic polarization were performed in 0.9% NaCl solution to verify the corrosion resistance of the surfaces. After the corrosion tests, the samples were analyzed by scanning electron microscopy (Fig. 6) and elemental analysis by EDS (Fig. 7).

The open circuit potential measurements (Fig. 5A) show that the treated samples stabilize the potential in the first hours of immersion while there is oscillation for the polished sample. Fig. 5B shows that the group submitted to acid/alkaline treatment followed by hydrothermal treatment with strontium (Ac+Alk+Sr) showed greater resistance to corrosion due to lower anodic current density, standing out over the other groups.

It is possible to observe in Fig. 6 that the surfaces with treatment suffer less impact when submitted to corrosion tests. The EDS test showed that the strontium remained on the surface even after corrosion tests (Fig. 7).

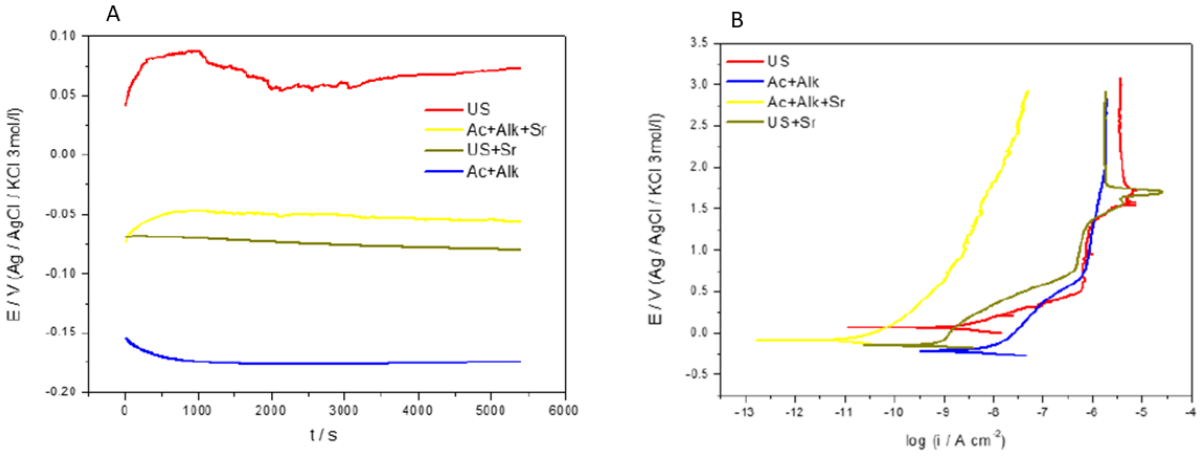


Fig. 5. Electrochemical corrosion tests. (A) Open circuit potential measurements (B) Potentiodynamic polarization curves.

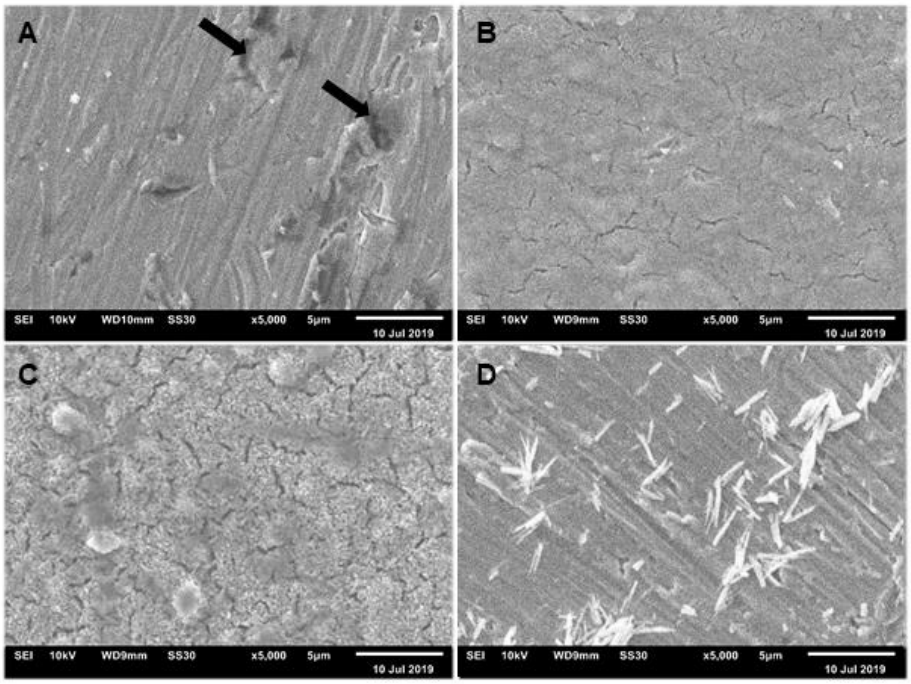


Fig. 6. Scanning Electronic Microscopy (SEM) after corrosion tests. (A) US, (B) Ac+Alk (C) Ac+Alk+Sr and (D) US+Sr x5000 magnification. Localized pit corrosion is indicated in group A.

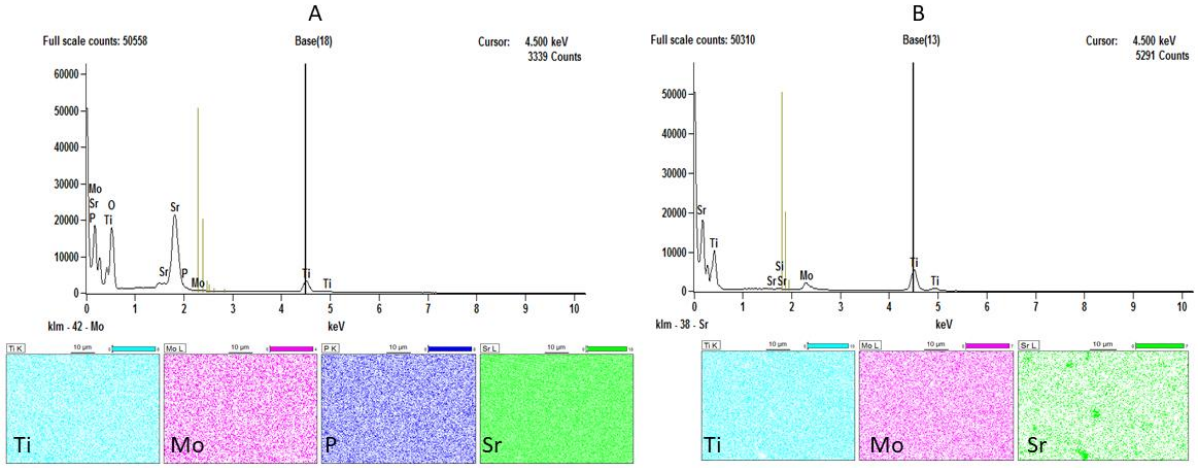


Fig. 7. Elementary analysis by X-ray dispersive energy spectroscopy (EDS) of samples after corrosion tests. (A) Ac+Alk+Sr and (B) US+Sr x5000 magnification

3.5. Quantification of Sr^{+} release

The results of controlled release of strontium and total strontium concentration assays are presented in Fig. 8. In Fig. 8A, the highest strontium release occurs in the initial periods (1 to 3 days) and in 14 days practically all strontium concentration was

released from the sample, optimizing the cellular adhesion and spreading already in the first days of analysis (Fig. 9 and Fig. 10).

The group with chemical treatment prior to the addition of strontium (Ac+Alk+Sr) retained more strontium on its surface with statistically significant differences to the group without chemical treatment (US+Sr). Approximately 50 mg/L and 35 mg/L of total strontium concentration were detected in the samples, respectively (Fig. 8B).

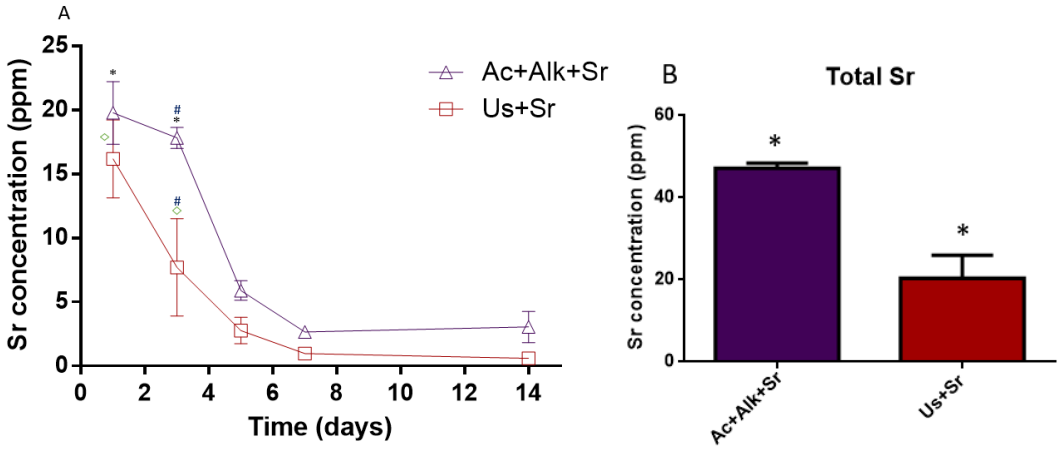


Fig. 8. (A) Non-cumulative strontium release (◊ Statistical differences with all the other periods p<0,05; # Statistical differences between groups in the same period p<0,05; * Statistical differences with the periods 5, 7 and 14 days in the same group p<0,05); (B) Total strontium of groups Ac+Alk+Sr and US+Sr (* Statistical differences between groups p<0,01).

3.6. Cells morphology, spreading and proliferation

Cell adhesion was observed in all groups through analysis of SEM and fluorescence (Fig. 9 and Fig. 10, respectively). In the groups with strontium addition, the cells presented elongated polygonal morphology and long cell extensions. In 7 days, all groups presented complete proliferation on the disc.

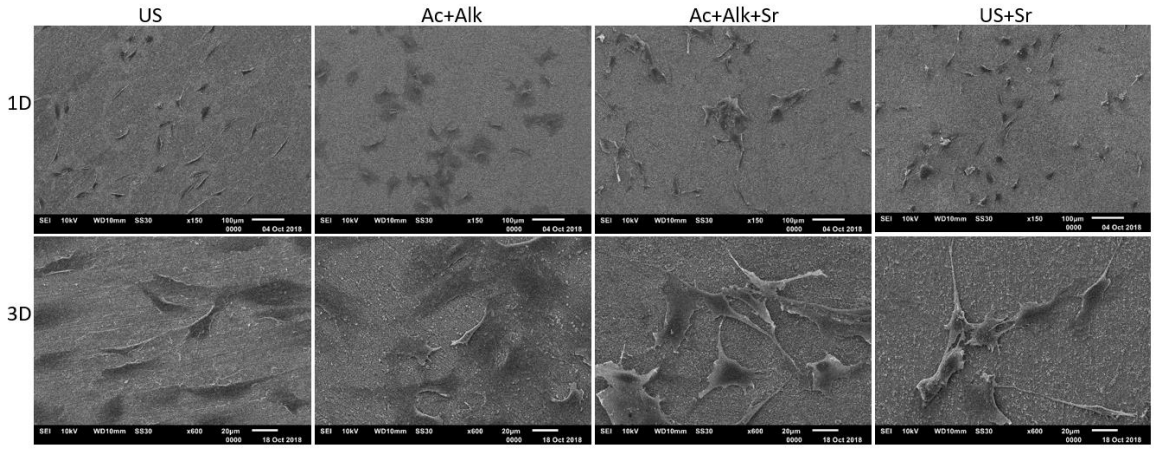


Fig. 9. Scanning electron microscopy (SEM) of discs with MC3T3-E1 cells in 1 and 3 days of culture. In day 1, magnification of x150, scale 100 µm. In day 3, magnification x600, scale 20 µm.

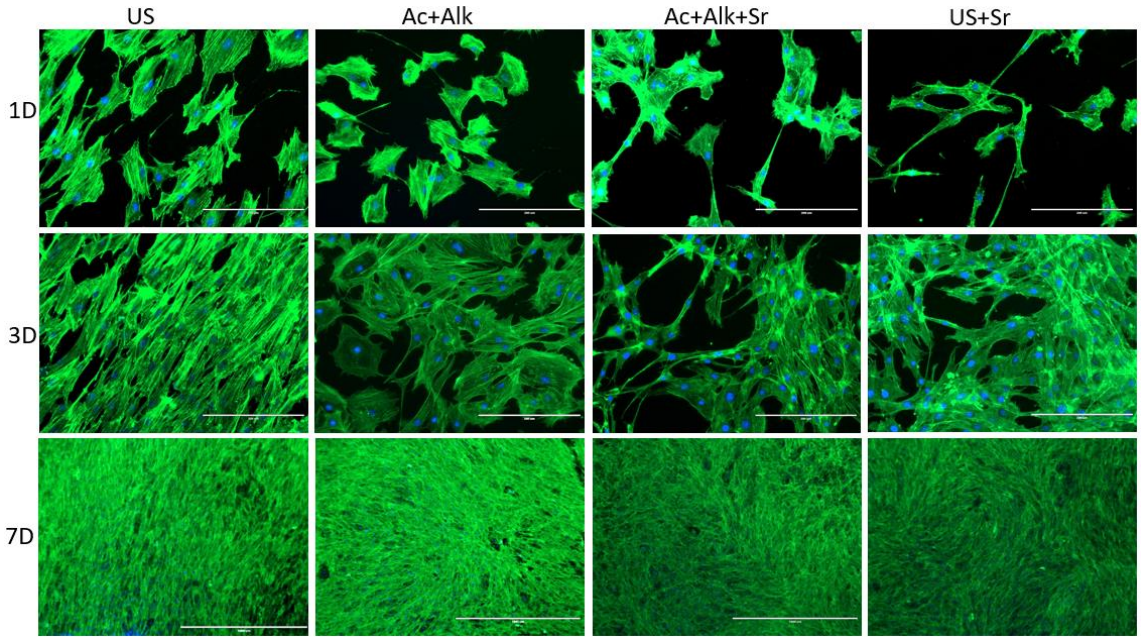


Fig. 10. Discs with fluorescence stained cells. MC3T3-E1cells marked with DAPI (blue) and Alexa Fluor 488-conjugated phalloidin (green). The images show cellular adhesion after the incubation period of 1, 3 and 7 days of the different groups. In 1 and 3 days, magnification x20, scale 200 µm. In 7 days, magnification x4, scale 1000 µm.

Fig. 11 indicates cell proliferation and viability through the Alamar Blue test The percentage of reduction means the cellular metabolization of the reagent (AB) by an oxide-reduction chemical reaction. This reaction reflects a color change from blue to red medium and is detectable by a spectrophotometer at wavelengths of 540 and 600nm.

It is possible to observe that the group with acid/alkaline (Ac+Alk) treatment presented significant difference from the other groups in most of the periods. The best results were verified in the strontium treatment groups and in the control group.

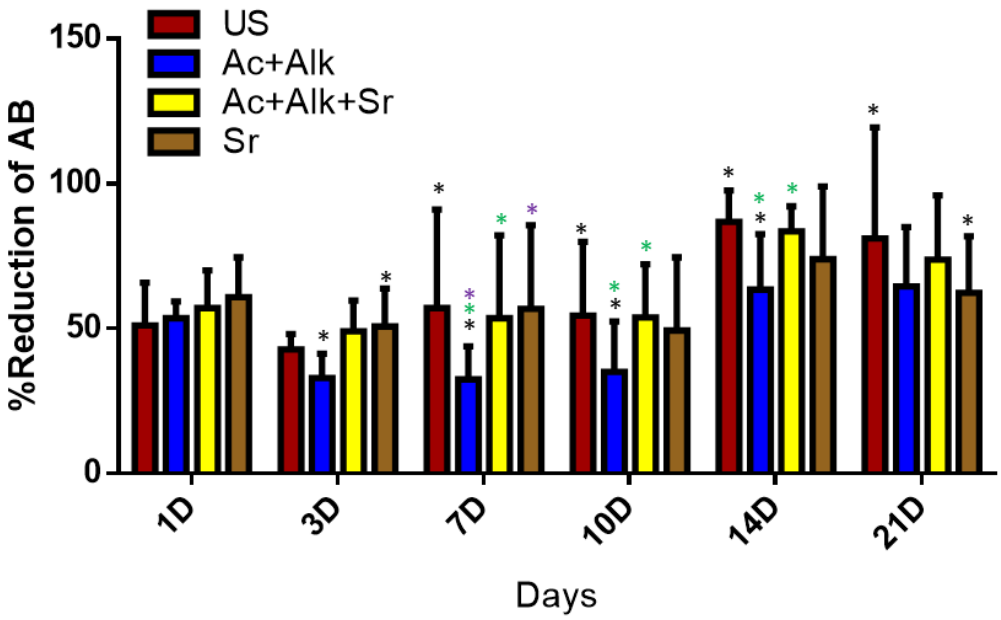


Fig. 11. Results of the cellular viability test (Alamar Blue) in the periods of 1, 3, 7, 10, 14 and 21 days. Bars with * of similar colors indicate a difference in significant ($p<0.05$) between them in the same period.

Because the Alamar Blue test was not toxic to the cells, the same samples were used during all the experiment. At the end of the Alamar Blue test, at 21 days, the discs were submitted to the alizarin red test.

3.7. Mineralization nodule formation

Calcium deposition activity was measured by colorimetric quantitative analysis at 550nm (Fig. 12). No statistically significant differences were found between the groups. For this test it was necessary to perform a negative control with discs without cells due to the possible interaction of reagent (alizarin red) and strontium.

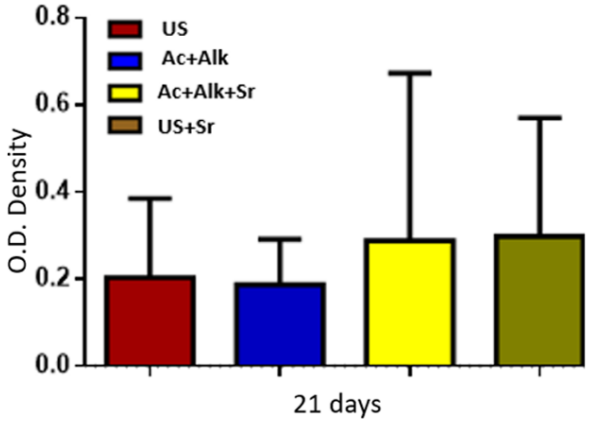


Fig. 12. Colorimetric analysis of calcium deposition on the surface at 550 nm. There were no statistically significant differences between the groups.

4. Discussion

In this study, the proposal to conciliate the chemical treatment with acid and alkali previously the addition of strontium by the hydrothermal method in discs of Ti-15Mo, allowed to take advantage of both treatments developing a surface of nanotopographic characteristics, with high surface free energy, greater resistance to corrosion, greater incorporation of strontium impacting directly on the high viability and proliferation of cells MC3T3-E1, their spreading and formation of mineralization nodule. Thus, as in previous studies, it was demonstrated that the characteristics of the implant surfaces such as chemical composition, roughness, hydrophilicity are parameters reported for interfering in the cellular aspects that contribute or not to the biological processes having an important influence on the osseointegration [38-40].

The use of titanium discs in 15% molybdenum in this study demonstrated a predominantly beta crystalline structure. Our results agree with several studies [31, 41-43] which indicate that from a concentration of 10% molybdenum there is no longer any alpha-phase titanium. Variations were found in the study of Martins et al. [29] in which alpha and beta phase were detected in Ti-15Mo. Researches that study the improvement of Ti alloys for Implant Dentistry demonstrate the several advantages of beta alloys, such as low modulus of elasticity, more similar to bone modulus and greater mechanical resistance [31]. In addition, the corrosion resistance presented by beta alloys is improved with the addition of molybdenum [44].

Our corrosion tests indicated that the surface treated samples stabilize the potential in the first hours of immersion probably due to the formation of a film on the

surface which gives stability to the system. While in the untreated group the open circuit potential probably oscillates due to the more active characteristic of the surface and the chloride ion attack on the metal matrix causing its dissolution at specific points creating localized pit corrosion and taking the observed oscillations. It was demonstrated by Kung et al. [45] that surfaces treated with strontium were more resistant to corrosion. In our results the superior resistance to corrosion was obtained in the group treated with acid/alkali and strontium (Ac+Alk+Sr) when submitted to the potentiodynamic polarization test.

Surfaces treated with acid followed by alkaline etching have been shown to produce nanotopographical surfaces in addition to an improvement in wettability [19]. In this study, the acid/alkali treated samples were the most hydrophilic among the groups. In general, hydrophilic surfaces represent better conditions for cellular adhesion than hydrophobic surfaces. Surface characterization studies attribute wettability a relevant role in the way osteoblasts adhere and proliferate in titanium [46]. Similarly, according to the concept of surface energy, the adhesion of one material to another will be all the better the greater the surface energies are. The addition of strontium on surfaces previously treated with acid/alkali (Ac+Alk+Sr) showed the best surface energy results together with the Ac+Alk group without showing significant difference between them.

According to Le Guéhennec et al. [39] surfaces can be divided into macro, micro and nanotopographic. Surfaces with roughness measurements below 1 μm are characterized as nanotopographic. These have the unique ability to directly affect molecular and cellular events that determine the global biological response to an implanted material, such as protein adsorption, cell adhesion and proliferation, among others [47]. In addition, rough surfaces contribute to the fixation and stability of implants [48]. It was observed that the groups with strontium had the highest values of roughness and that the chemical treatment prior to the addition of strontium influenced the decrease of these values.

The surfaces with strontium, in this study, showed to have a positive influence on cellular events and its characteristic of greater release of strontium in the first days was also verified in the study by Andersen et al. [49]. Park et al. [9] found that the addition of strontium on the surface significantly favored cell adhesion and viability. Zhang et al. [50] found improvement in cell viability on surfaces with strontium addition and its direct relation with strontium concentration applied. There is no consensus in

the literature on the ideal amount of strontium application to obtain good results. Previous studies have shown that low strontium concentrations are effective in the process of cell differentiation and that high concentrations can generate the reverse effect, damaging viability and proliferation [51].

In this study, among the treated surfaces, the addition of strontium favored for the best results of the Alamar Blue test allowing proliferation as much as the control group, demonstrating the effectiveness of the strontium concentration applied without generating cytotoxicity. Furthermore, our results showed cells with a more elongated shape and longer cytoplasmic extensions in the groups with strontium addition (Ac+Alk+Sr and US+Sr) already in the first periods.

Elongated morphology and cell contact intensification are effects related to the process of cell differentiation [52]. The shape of cells influences the regulation of their growth, their gene expression and differentiation. Cell morphology is directly related to the surface on which it adheres, influencing the expression of specific phenotypes. According to Rajaraman et al. [53] cell morphology may indicate the stage of adhesion and spraying being stage 1: round cells, 2: round cells with philopodia, 3: cells with cytoplasmic webbing, or 4: well flattened cells. A more rounded morphology with short cell extensions were found in the cells of the other groups and are characteristics of less rough topographies, which require greater cell effort to spread over the surface due to the absence of three-dimensional structures at the surface [54].

Regarding the results of alizarin red, mineralized nodules represent the capacity of osteogenic differentiation of the cells and surfaces with strontium has been highlighted in mineralization in several studies [7, 55, 56]. Although no significant differences were obtained in this study, a favorable effect to greater mineralization is observed in groups with strontium.

Finally, the hydrothermal method used in this study proved to be a simple and low-cost alternative for the addition of strontium on surfaces in accordance with previous studies [11, 57]. Future studies should evaluate other strontium incorporation methods and in vivo studies will be necessary to demonstrate the real effects of these benefits on osseointegration.

5. Conclusion

The hydrothermal method for the application of strontium on titanium surfaces presents itself as a simple and viable alternative that can have a positive impact on physicochemical aspects and cellular events. The results of this in vitro study demonstrated that the addition of strontium favored cellular responses in terms of proliferation/viability, adhesion and spreading. In addition, the previous acid/alkaline treatment and the use of Ti-15Mo alloy showed to contribute to the best results obtained.

Although future studies are necessary to elucidate in vivo interaction, these results indicate that surfaces with strontium incorporation by hydrothermal treatment can be effective in achieving an implant favorable to bone repair, improving the behavior of osteoblastic cells on their surfaces.

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3.2 Publicação 2*

Effect of two different methods of surface functionalization of Ti-15Mo alloy with strontium in the behavior of human periodontal ligament cells.

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Abstract

Osseointegrated implants are widely recognized as a therapeutic alternative for the loss of dental elements. However, current studies aim at developing implants that stimulate post-surgical bone repair around them, improving bone-implant contact and speeding up osseointegration. Titanium surface functionalization with strontium promotes both stimulation of bone formation and reduction of bone resorption and can be performed by different methods. The objective of this study was to compare, in vitro, Ti-15Mo alloy discs submitted to strontium application by the (1) magnetron co-sputtering and (2) hydrothermal methods. Both methods demonstrated to be effective in incorporating strontium on Ti-15Mo surfaces as detected in X-ray photoelectron spectroscopy (XPS) and produced different topographic characteristics as evidenced by scanning electron microscopy results. In addition, the response of periodontal ligament cells (PDLCs) to these surfaces revealed that both methods allowed cell viability. However, greater proliferation and spreading was observed in groups with magnetron co-sputtering treatment in the initial periods, demonstrating that different methods of strontium application influence the initial cellular processes.

Keywords: Dental Implants. Strontium. Periodontal Ligament.

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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-normalizacaoatualizado.pdf**

1 INTRODUCTION

Osseointegration is fundamental to the success of implants and is influenced by the characteristics of the material installed, both at a macroscopic level as the format of the implant, as well as the microscopic scale, corresponding to the alloys and surface treatments[1].

The superiority of mechanical properties compared to commercially pure titanium has led to the development of titanium alloys[2]. Ti-Mo alloys have been demonstrated to be a viable alternative to traditional Ti-6Al-4V alloys because they are more biocompatible, have high corrosion resistance, high mechanical performance and removal torque, in addition to chemical stability with Young's module reaching about 80 GPa, a reduced module when compared to Ti-6Al-4V (110 GPa) alloys[3,4].

Besides the composition of titanium with its alloys, the morphology and the physical-chemical properties of the surfaces are relevant aspects in the osseointegration of implants influencing the proliferation and activity of bone cells and consequently in the healing time[5,6]. With this knowledge, studies have been carried out aiming at treatments on implant surfaces in an attempt to optimize the osseointegration process mainly in patients with poor quality bones.

Many investigations have reported a significant improvement in osseointegration following surface modifications by laser irradiation[7], acid[8], alkaline[9]. The latter, acid and alkaline etching, show to positively influence the physicochemical properties of the surface such as wettability, surface energy, roughness, and when together, have shown to favor the cellular mechanisms for osteoblastic differentiation[10]. Although acid/alkaline treatment promotes good properties, it is still necessary to provide implants with early bone binding capabilities. The addition of elements such as magnesium and strontium on surfaces with previous chemical treatment has been reported to confer early bone binding capability through the release of ions of Sr and Mg[11].

The treatment of implant surfaces with strontium seeks the use of the positive properties of this element of both bone formation and decreased bone resorption[12,13]. The effects of strontium on the bone mechanism are already being taken advantage of in patients with severe osteoporosis, with a high risk of fracture and for secondary prevention through use of strontium ranelate[14]. The osteoinductive properties of strontium induce improvement of osseointegration in implants[15]. In addition, it

increases the osteogenic expression of markers such as Runx2, bone sialoprotein, osteocalcin and alkaline phosphatase (ALP) activity[16]. Strontium has been incorporated into the implant surface by means of methods such as: hydrothermal method[16,17] and by magnetron co-sputtering method[18].

The hydrothermal method is feasible for the incorporation of bioactive elements, such as calcium phosphate[19] silver[15] and strontium[20]. In addition to its low cost, recent studies have demonstrated the favorable effect on the proliferation of mesenchymal cells in cell culture after the deposition of strontium on the Ti surface by the hydrothermal method[21]. The magnetron co-sputtering method is a type of physical vapor deposition (PVD) and is used to produce implant materials consisting of a thin, homogeneous and adherent coating[22]. The surfaces of titanium modified with strontium by magnetron co-sputtering has elevated mechanical stability, releasing strontium in the local environment of osseointegrated implants[18], and demonstrating a significant increase in bone formation[23].

Undifferentiated mesenchymal cells are designated by their ability to characterize into several cell types[24]. Consisting of mesenchymal lineages, periodontal ligament cells (PDLC) can develop fibroblastic, adipocytic and osteoblastic properties[25-27]. Studies have shown that PDLCs have osteogenic potential, expressing alkaline phosphatase activity and other markers of osteogenesis such as osteonectin, osteopontin and are also able to promote mineralized nodule formation[25,28]. Previous studies that aim to improve dental and orthopedic implants have been using PDCLs in an attempt to stimulate osteoblastic differentiation through surface modifications such as chemical treatments in order to optimize osseointegration[21,29].

Considering the effect of strontium and the physical-chemical properties of surfaces on the osseointegration process, the objective of this study was to compare the influence of the method of strontium incorporation on implant surfaces in the in vitro behavior of human PDCL.

2 MATERIAL AND METHODS

Ti-15Mo samples preparation

Discs made from commercial Ti-15Mo rods (ATI Alvac, Monroe, NC, USA), 12.2 mm in diameter and 2mm thick, were used to characterize surface properties and for cell culture experiments. Ti-15Mo discs were wet-abraded to 1500 grit SiC abrasive

paper, and successively cleaned in acetone, alcohol, and deionized water and dried at room temperature (25°C).

Treatment of surfaces

The PVD coatings were produced by magnetron co-sputtering PVD (Cemecon). The Ti-15Mo discs were mounted on a rotating sample stage with a grade 1 Ti (purity of 99.5%) and a sintered composite $SrTiO_3$ target (purity of 99.9%), respectively. The size of the two targets were approximately 90mm x 500 mm. The background gas pressure for sputtering in the chamber was around 1 mPa of Argon.

For the hydrothermal treatment, a strontium solution was prepared by diluting the strontium hydroxide octahydrate $Sr(OH)_2.8H_2O$ (Sigma Aldrich, purity of 95%) in Milli-Q water to obtain the final concentration of 60 mM. The Ti-15Mo samples were placed in 100 mL of the previously prepared strontium solution, and the hydrothermal treatment were performed in an autoclave, with constant temperature and pressure (121°C and 1.0 to 1.5 Kgf/cm^2), during 1 hour. The other side of samples were carefully washed with Milli-Q water to remove the residual strontium and left to dry at room temperature.

One group received acid/alkaline treatment prior to the addition of strontium. For that, the Ti-15Mo discs were immersed in a solution of concentrated phosphoric-ortho acid (H_3PO_4 conc. 85%) at 80°C (± 5ºC) for 30 minutes. After, the samples were transferred to a polyethylene container containing 50 mL of NaOH at 60°C and kept for 24 hours. The group was submitted to functionalization of the surfaces with strontium by the hydrothermal method.

The samples were divided into 4 groups according to the surface treatments performed: Us - the samples were just polished without additional treatment (control group); Pvd.Sr – polished samples were functionalized with $SrTiO_3$ by PVD treatment; Us.Sr - polished samples were modified with $Sr(OH)_2.8H_2O$ by hydrothermal treatment; Ac.Alk.Sr – polished samples received acid/alkaline treatment on their surfaces prior to hydrothermal treatment with strontium.

Decontamination

Prior to analysis the samples were treated with UV ozone (BioForce, Nanosciences, IA) for 15 min at 50 mm distance for 1 h to remove organic contamination.

Surface characterization

The characterization of the surfaces was performed by evaluating the topography. For this analysis, scanning electron microscopy (SEM - Nova 600, FEI Company, Netherlands) was used with an accelerating voltage of 5 kV and a working distance of 4 mm and 5 mm. The chemical composition of the samples was determined using X-ray photoelectron spectroscopy (XPS- Kratos Axis UltraDLD, Kratos Analytical Ltd., UK)[30]. Three samples from each group were used for surface characterization.

Strontium washout

The washout was performed by placing the samples in a 24-well plate and adding 3 mL of Phosphate Buffered Saline (PBS) at 37°C with a pH of 7.4 under static conditions. For each group, 3 samples were used. The total volume of PBS was collected using a pipette and replaced by fresh PBS at intervals of 24 h, 3, 5, 7 and 14 days. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) (AMETEK Spectro Arcos, AMETEK, Germany) was used to quantify the amount of strontium released into the PBS.

In vitro biological analysis

Primary cell culture

For the accomplishment of this study, human PDLCs were used. The cells were cultured in MEM supplemented with 10% fetal bovine serum (Sigma-Aldrich) and antibiotics (25.000 IU/mL penicillin and 25 mg/mL streptomycin; DuraScan Medical Products, Odense, Denmark) at 37°C in an atmosphere of 100% relative humidity and 5% CO_2. Cell experiments (cell morphology assessment, cell proliferation and viability analysis) were undertaken with PDLCs in the third passage.

Cellular viability and proliferation

Proliferation and cell viability were evaluated after 1, 3, 7, 10 and 14 days of incubation using the Alamar Blue test (Molecular Probes, USA). The Alamar Blue Assay incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth and activity. The reduction reaction occurs in the cellular mitochondria and

converts the resazurin product (from blue color) to its reduced form of resofurin (of a red color).

Cells were seeded at a density of $5x10^4$ cells in discs surfaces and in each time point, they were incubated for 4 hours with 1 ml of work solution (MEM with 10% FBS, 25.000 IU/mL penicillin, 25 mg/mL streptomycin and 10% Alamar Blue). The working solution of Alamar Blue without cells were used as negative control. After, 150 µL of work solution from each sample were transferred to a 96 well-plate and read on a spectrophotometer (Spectramax M, Molecular Devices) at wavelengths of 570 and 600 nm and analyzed with the equipament's software (SoftMx Pro, Molecular Devices). The number of viable cells were associated with the reduction level and expressed as percentage of reduction of Alamar Blue. The percent reduction of Alamar Blue were calculated using the formula suggested by the kit manufacturer. Three samples were used for each group and the experiment was repeated twice at different times.

Cellular adhesion and spreading

For this analysis three samples from each group were used in each period. Cells were seeded at a density of $5x10^3$ cells in discs and allocated into 24-well plates. The cells were fixed with 10% formalin solution (4% formaldehyde) after 1, 3, and 7 days of culture. The nucleus was stained using DAPI nucleic acid stain (D9542, Sigma-Aldrich). The F-action in the cytoskeleton was 50 stained with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Eugene, OR, USA). Prior to staining the cells were washed in PBS, followed by permeabilization with T-PBS (0.1% Triton X-100 in PBS; Sigma-Aldrich) for 15 min. The T-PBS were removed and the cells were incubated with DAPI and Alexa Fluor 488 overnight, and there after washed twice with T-PBS.

Olympus cellSens dimension software was used to operate the Olympus BX 61 microscope (Olympus, Tokyo, Japan), and the nucleus and cytoskeleton of the cells adhered to the surface of the discs were visualized.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 6.0 for Windows (GraphPad Software Inc., La Jolla, USA).

Parametric or non-parametric tests for independent data and more than two samples were used (ANOVA or Kruskal-Wallis test, respectively, in case of normal

sample distribution and homogeneity of variances), followed by multiple comparison tests, when applicable. The minimum statistical significance was established in $P \leq 0.05$.

3 RESULTS

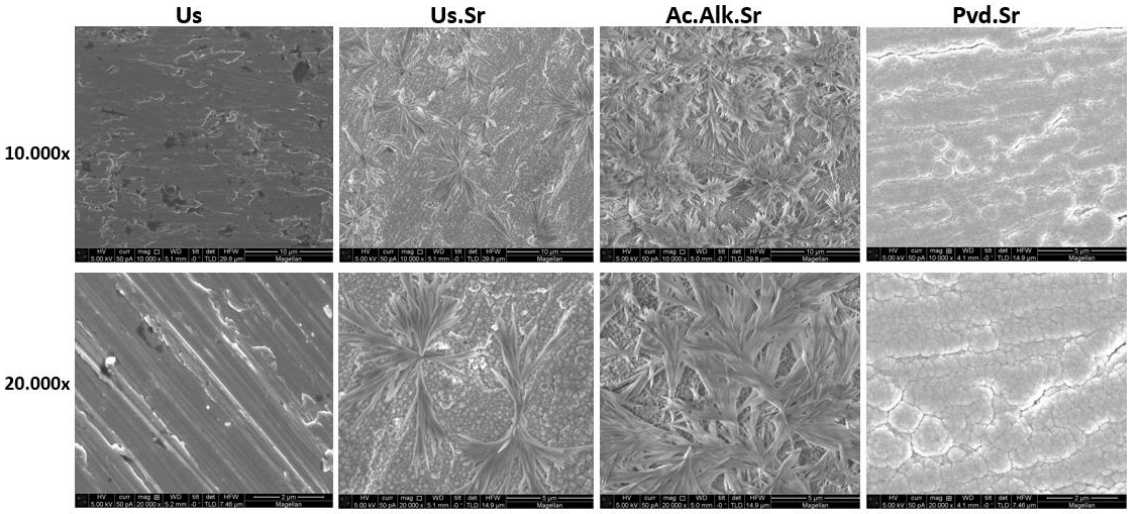
Surface characterization

Figure 1 shows the different surface morphologies of the samples analyzed by SEM (Nova 600, FEI Company, Netherlands). The samples from the US group show oriented lines induced by sandpaper. It is possible to note that the cells have a tendency to follow the orientation of these lines during their adhesion and proliferation (Figure 6)

The groups with hydrothermal treatment (Ac.Alk.Sr and US.Sr) showed a homogeneous distribution of structures with crystallized appearance on their surface. Similar structures were observed in the results of recent studies carried out by our group, in which the X-ray Dispersive Energy Spectroscopy test demonstrated to be strontium.

It can be observed that the acid/alkaline treatment before surface functionalization with strontium (Ac.Alk.Sr group) favored a greater deposition of strontium on the surface when compared to the US.Sr group. Although the XPS results did not show significant differences between these groups, our previous studies showed greater deposition of strontium on surfaces that combined chemical treatment with acid and alkali before the addition of strontium from ICP results.

Figure 1 - Scanning Electron Microscopy (SEM) of the different surfaces in 10000x and 20000x of the magnification



Source: own elaboration.

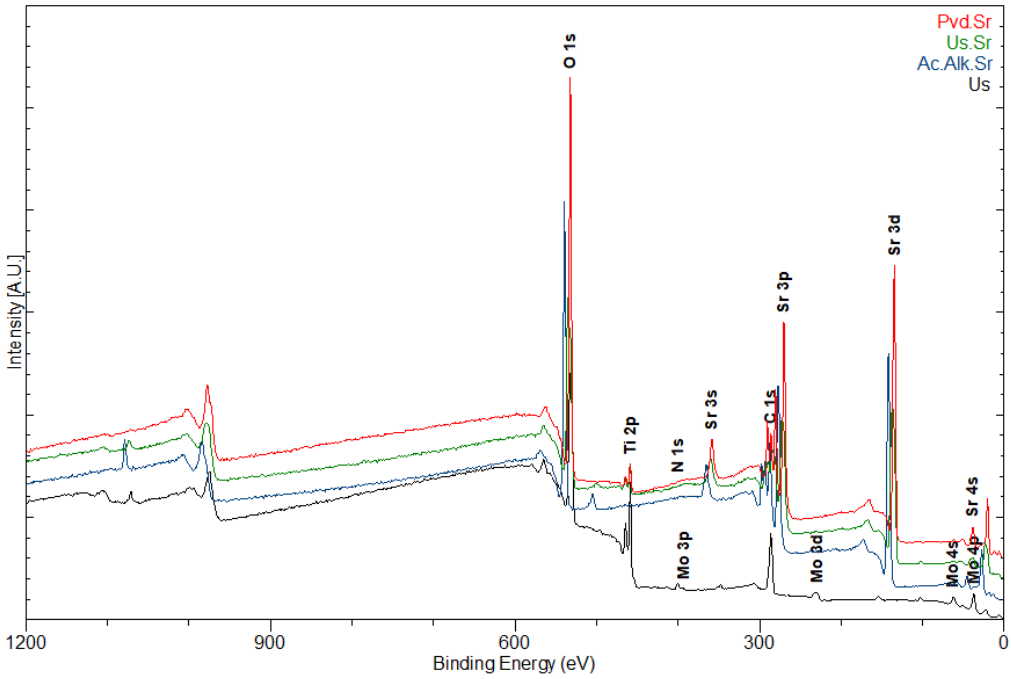
It is clearly notable distinct characteristics of the surfaces under different methods of strontium application and chemical forms of strontium ($SrTiO_3$ and $Sr(OH)_2.8H_2O$). The strontium in the hydrothermal treatment appears as crystal, in aspect of flowers. The surface of the PVD treatment shows a more solid and regular surface.

The chemical composition of the coatings was analyzed using XPS test. In Figure 2, four XPS spectra obtained from a Ti-15Mo substrate are shown. XPS was used to determine the surface content of Sr, Ti and O in the coatings. It was possible to detect molybdenum in untreated samples (US group). A low carbon contamination is also detected, most likely from hydrocarbons.

As the size of the peaks is not proportional to the quantity of the chemical element, the quantification of the elements was obtained using the CasaXPS software. In the US group it was found 9.88% of Ti, 43.3% of O and, as expected, no strontium was detected. In the Pvd.Sr group there was 9.64% of Sr, 1,46% of Ti and 35,33% of O. In the groups with hydrothermal treatment it was found 11,34% of Sr, 2,22% of Ti and 46,60% of O in the US.Sr and 10,50% of Sr, 38,36% of O in the Ac.Alk.Sr group. No titanium was detected in this last group. Samples from the Ac.Alk.Sr and Us.Sr groups were submitted to 3 days of wash out, with PBS changes on the first and third day. The XPS test was repeated and the results (Figure 3) demonstrated an increase

in the percentage of Ti in the Us.Sr group (3,56% Ti), but still no Ti detection in the Ac.Alk.Sr group.

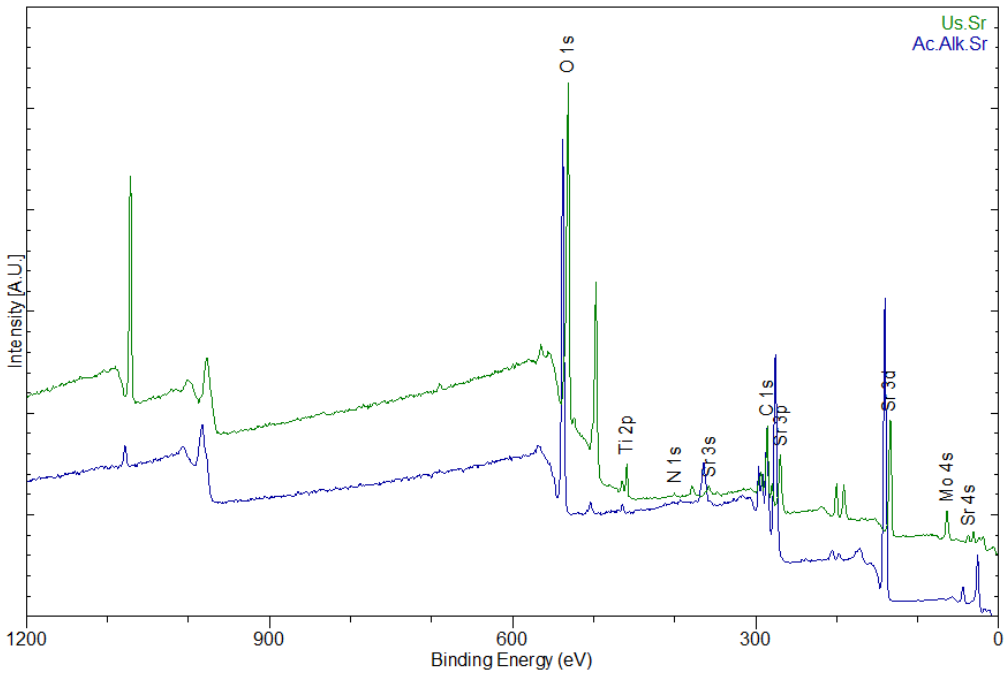
Figure 2 - Elementary analysis of the Ti-15Mo surfaces of the groups Us, Pvd.Sr, Us.Sr and Ac.Alk.Sr by X-ray photoelectron spectroscopy (XPS)



Source: own elaboration.

The surface chemical composition is dependent of coating thickness. The alkaline and acid treatment prior to the hydrothermal treatment with strontium increases strontium adhesion on the surface, and thus, in 3 days of wash out there was still enough strontium on the surface not allowing the detection of titanium by XPS (Figure 4).

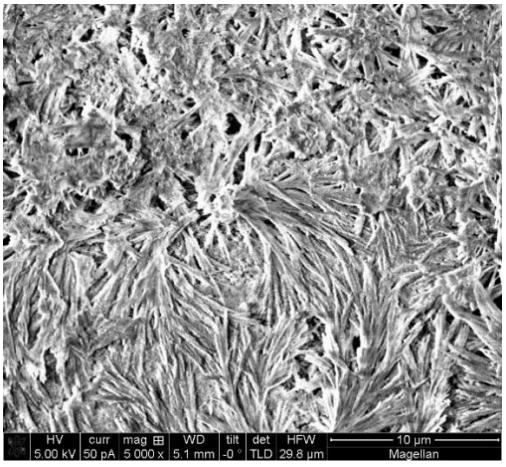
Figure 3 - Elementary analysis of the Ti-15Mo surfaces of the groups Us.Sr and Ac.Alk.Sr by X-ray photoelectron spectroscopy (XPS) after 3 days of wash out



Source: own elaboration.

In both, Figures 2 and 3, the blue line (group Ac.Alk.Sr) is slightly shifted to the left and its peaks do not coincide with the other groups. This is because of the surface charging. It is possible to observe that there was some surface charging when the blue line does not end at point zero, but a small amount before zero. This small variation has no influence on the results.

Figure 4 - Scanning Electron Microscopy (SEM) of the Ac.Alk.Sr surface in x5000 of the magnification after 3 days of the wash out with PBS

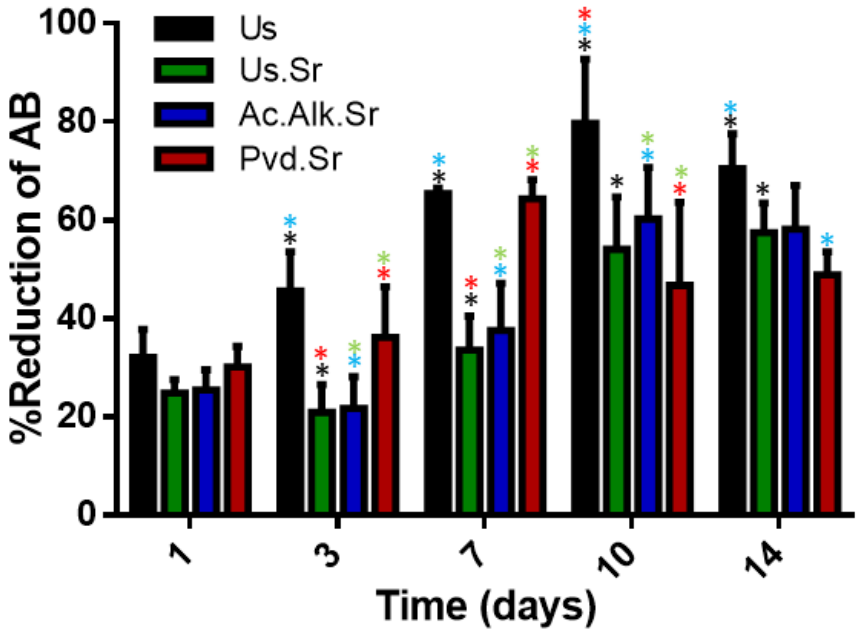


Source: own elaboration.

Biological analysis

Cell viability and proliferation were evaluated by the Alamar Blue test after 1 to 14 days of cell culture, being an indicator of cell growth based on the detection of the cellular metabolic activity. As demonstrated in Graphic 1, the control group showed significantly greater proliferation from day three. There was no significant difference in the effect of chemical treatment with acid and alkali previously the addition of strontium in most periods between the groups with hydrothermal treatment. In general, all the groups were viable for cell proliferation.

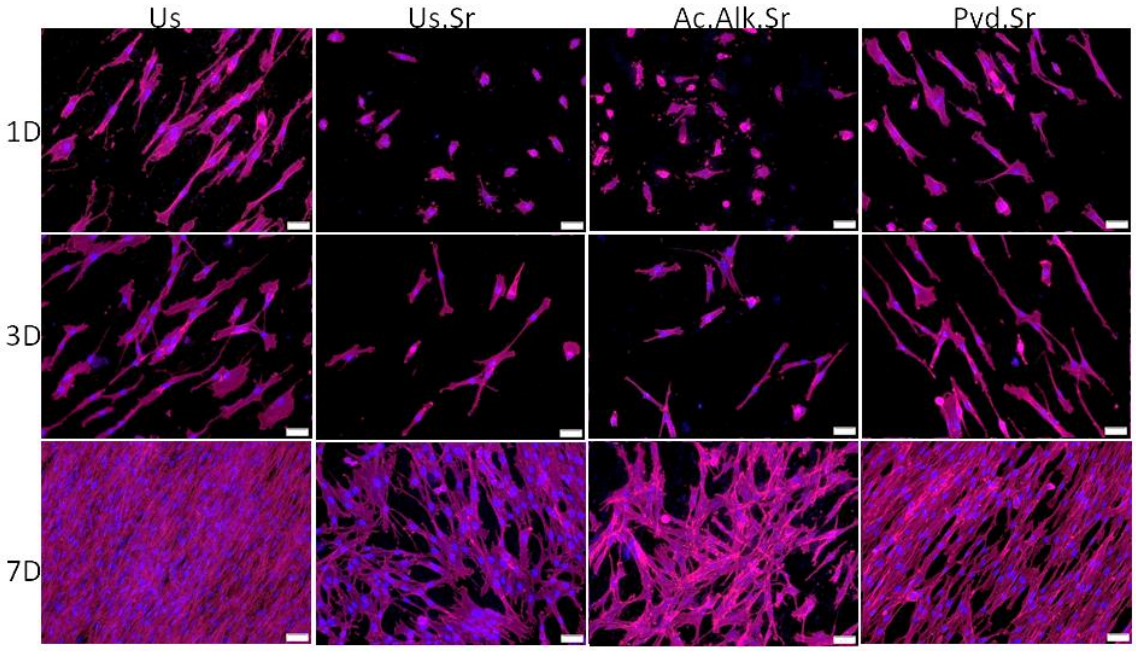
Graphic.1 - Results of the cellular viability test (Alamar Blue - AB) in periods of 1, 3, 7, 10 and 14 days. Bars with * of similar colors indicate a difference in significant ($p<0.05$) between them in the same period



Source: own elaboration.

For analysis of cell adhesion and spreading fluorescence test was used (Fig. 5).

Figure 5 - Periodontal ligament cells marked with DAPI (blue) and Alexa Fluor 488-conjugated phalloidin (pink). The images show cell adhesion after the incubation period of 1, 3 and 7 days of the different groups. Magnification 20x, scale 50µm



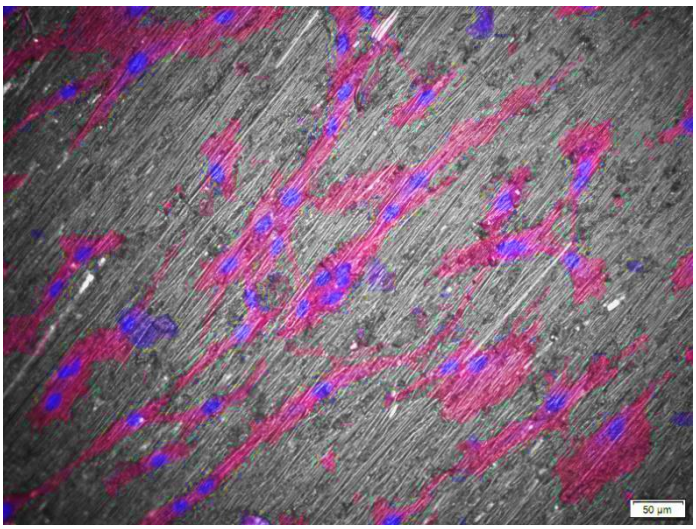
Source: own elaboration.

The PDL cells cultivated on the surface of the discs of the Us and Pvd.Sr groups showed similar cellular spreading between them in periods of 1, 3 and 7 days. It is possible to observe that among the groups with treatment, the Pvd.Sr group promotes cell spreading already in the first periods. The groups with strontium addition by the hydrothermal method (Us.Sr and Ac.Alk.Sr) had similar cellular spreading characteristics in all the analyzed periods.

Regarding the morphology, the groups Us and Pvd.Sr exhibit cells with elongated shape, flattened aspect, emitting their first prolongations, denominated philopodias representing a more advanced stage of development already in the first period. While the groups Us.Sr and Ac.Alk.Sr the cells are in the initial stage of cellular adhesion, with small and rounded format. After 3 days, some cells show an elongated format, emitting their first prolongations. In the period of 7 days, the cells already show themselves in greater number and more prolongations in all the groups.

Figure 6 shows that the cells follow the orientation of the polishing lines in the US samples, demonstrated by an overlay of the images obtained from the SEM and fluorescence analyses.

Figure 6 - Periodontal ligament cells marked with DAPI (blue) and Alexa Fluor 488-conjugated phalloidin (pink). The figure shows the cells in 3 days on the surface of the Us disc. Magnification 20x, scale 50µm



Source: own elaboration.

4 DISCUSSION

Studies have shown that strontium acts both to promote bone formation and to inhibit its resorption by interfering with the RANK/RANKL/OPG pathway and Wnt signal pathways[31,32]. In vivo, strontium has been shown to increase the peri-implant bone volume, contributing to the bone anchorage of implants[33]. Methods for strontium incorporation in implant surfaces have been developed[17,34-36], but the properties of this element (Sr) need to be conciliated with a form of application that is feasible and capable of reproducibility, considering in addition to effectiveness, technical complexity and costs. This study evaluated the hydrothermal and PVD magnetron co-sputtering methods for strontium addition on Ti-15Mo discs surfaces. Both proved to be effective for the incorporation of strontium in the samples and for cell viability. However, the difference in techniques demonstrated interference in the initial cellular responses and the hydrothermal method showed less proliferation and cellular spreading in the initial periods.

The PVD treatment by magnetron co-sputtering method for strontium deposition on the implant surface presents promising results in vitro and in vivo[18,33,37]. The hydrothermal method has been used to apply several elements such as P, Ca, Ag and Sr on implant surfaces demonstrating positive effects on osteogenic activities[15,19,20].

In the present study, the morphological analysis of the surfaces of Ti-15Mo coated by $SrTiO_3$ by PVD treatment (Pvd.Sr group) and $Sr(OH)_2.8H_2O$ by hydrothermal treatment (Us.Sr and Ac.Alk.Sr group) was performed from images obtained in SEM. Images of SEM show the top view of the surfaces. In this study the PVD magnetron co-sputtering treatment presented a homogeneous and adherent surface, characteristic of this treatment[22], but the appearance of cauliflower found in previous studies[37] was not clearly observed. Most likely, the polishing of the discs prior to the treatment may have influenced the direction of the $SrTiO_3$ coating, as it is possible to observe lines caused by polishing in the SEM images of the Us group. In groups with hydrothermal treatment, strontium is visualized on the surface as organized flower-like crystallized structures, characteristic already reported in previous studies of our group.

XPS test showed results in accordance with previous studies on the chemical composition of surfaces with PVD magnetron co-sputtering treatment demonstrating their reproducibility[18]. The hydrothermal treatment allowed more strontium deposition in the sample. The acid/alkaline treatment prior to the hydrothermal treatment with strontium contributed to greater adherence of the strontium, demonstrated on SEM images (Fig. 1) and SEM images after the wash out tests (Fig.4).

The combination of other treatments such as acid and alkaline etching with the addition of strontium stimulates the functioning of the implants, contributing to the improvement of the surface properties, as shown in previous studies[11]. Phosphoric acid treatments have shown improvement in wettability and roughness of surfaces[38]. The use of NaOH has been shown to contribute to the precipitation of hydroxyapatite[39], and together they induce a more pronounced osteoconductive behavior[10]. The addition of strontium by the hydrothermal method plus the previous modification with acid and alkali showed, in our previous study, to be effective in cellular events and physical-chemical characteristics, improving even the resistance to corrosion.

With potential for osteoblastic differentiation, periodontal ligament cells are of great importance in regeneration of lost alveolar bone tissue[40]. Thus, the evaluation of the effects of the studied surfaces on these cells is warrantable.

Cellular viability tests are fundamental in the evaluation of surface biocompatibility. In this study, all surfaces allowed viable cellular proliferation. The group with PVD magnetron co-sputtering treatment demonstrated high cell proliferation comparable to the control group. As demonstrated in previous studies, the PVD magnetron co-sputtering treatment in implants has biocompatible physical-chemical

characteristics[36,41,42]. Moreover, the addition of strontium on the surfaces by this method allows the use of the qualities of this element (Sr), without generating cytotoxicity and by having local release, does not generate adverse effects systematically[23].

The groups with hydrothermal treatment showed less proliferation in the initial period of cell culture, and the increase in proliferation was directly related to the decrease of strontium on the surface. Biological analyses showed that both morphology and proliferation were affected in the first period. Zhang et al.[43] in their study on the effect of strontium on biomaterials, observed that there is a direct relationship between viability and strontium concentration. Higher concentrations negatively affect cell viability. In addition, the release of strontium hydroxide contributes to an alkaline environment. Shen et al.[44] and Shen et al.[45] studied the effects of ph on bone regeneration. Although alkaline ph is favorable for an osteoblastic response, ph above 8.5 may be prejudicial. The present study, however, did not provide sufficient data to state that the smallest proliferation is linked to cytotoxicity, since this association is not necessarily logical.

Despite the initial influence on cell proliferation and spread, within 3 days it is possible to observe the beginning of cell spreading in groups with hydrothermal treatment (Us.Sr and Ac.Alk.Sr). In addition, throughout the periods, cell proliferation results were similar to those of the other groups (US and Pvd.Sr).

5 CONCLUSION

Strontium has been shown to contribute to the process of osseointegration assisting in the osteoblastic response. This study revealed that different ways of deposition and the chemical form in which strontium is presented, affected the initial biological response of human periodontal ligament cells.

Even with the limitations of this study it was possible to observe that both PVD and hydrothermal techniques were able to incorporate strontium and generate surfaces with physical-chemical characteristics favorable to cell proliferation.

Although the hydrothermal method has advantages as low cost and simplicity, in this study the initial response, mainly regarding proliferation and spreading stages were affected. In order to evaluate the long-term effects, additional studies are needed.

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4 CONSIDERAÇÕES FINAIS

As evidenciações sobre a influência das características macro e microscópicas dos implantes no processo de osseointegração tem impulsionado cada vez mais pesquisas que buscam aperfeiçoar as propriedades dos implantes no intuito de otimizar o tempo de reparação principalmente em pacientes que possuem alterações no processo de cicatrização e reparo.

O desenvolvimento de materiais com boas propriedades físico-químicas e biocompatíveis são características almejadas para que haja o sucesso a curto e longo prazo dos implantes. O estudo de aperfeiçoamento da composição do titânio em implantes tem demonstrado que as ligas de Ti-15Mo apresentam qualidades físico-químicas comparáveis a das tradicionais ligas de Ti-6Al-4V, com superior biocompatibilidade.

Métodos para modificações das superfícies dos implantes também têm sido desenvolvidos para alcançar os objetivos almejados. Tratamentos com ácido e base são relatados por influenciar positivamente as propriedades físico-químicas como rugosidade, molhabilidade e energia de superfície impactando diretamente na melhoria dos eventos celulares. Da mesma forma, a funcionalização das superfícies de titânio com elementos bioativos como o estrôncio tem proposto aproveitar as propriedades moleculares desse elemento de favorecer a formação óssea e de prevenir a perda óssea. A conciliação de boas técnicas permite reunir as qualidades de cada método desenvolvendo uma superfície promissora a osseointegração.

No presente trabalho, ligas de ti-15Mo foram submetidas a diferentes tratamentos de superfícies e analisadas quanto a sua influência nas propriedades físico-químicas e biológicas por meio de avaliações in vitro. Diante dos resultados apresentados, superfícies com adição de estrôncio mostraram contribuir para os eventos celulares, como adesão, proliferação, espraiamento e formação de nódulo de mineralização. Além disso, a conciliação de tratamentos prévios à adição de estrôncio com uso de ácido e base e o uso de liga de titânio em 15% em molibdênio promoveram melhorias nas propriedades físico-químicas dessas superfícies, como melhora na energia de superfície e resistência a corrosão.

Estudos futuros sugerem observar as vantagens dessas modificações em modelos in vivo para estabelecer e provar a eficácia na osseointegração.

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APÊNDICE A - Materiais e métodos

PUBLICAÇÃO 1

Preparação das amostras de Ti-15Mo:

Barras comerciais de Ti-15Mo (ATI Alvac Monroe, NC, EUA) foram seccionadas para a obtenção de discos de 12.2 mm de diâmetro e 2 mm de espessura. Para o polimento das superfícies, foi utilizada uma politriz elétrica, com série gradual de lixas de carbeto de silício de granulação 600, 1200 e 1500. Após isso, as amostras foram lavadas numa sequência de banhos com acetona, álcool etílico e água deionizada, sob vibração do ultrassom (Ultramet 2003 Sonic Cleaner da Buehler) durante 10 minutos em cada solução. Depois as amostras foram secadas em temperatura ambiente e armazenadas em embalagens de autoclave. Para esse estudo, 4 grupos foram obtidos de acordo com o tratamento recebido: polido (sem tratamento), ácido/alcalino, ácido/alcalino e hidrotermal com estrôncio e, por fim, um grupo apenas com tratamento hidrotermal com estrôncio.

Tratamento das superfícies:

O tratamento ácido foi realizado com ácido fosfórico-orto concentrado (H_3PO_4 conc.) a 85,0%, transferido para um béquer de 300 mL e mantido à temperatura de 80º C (± 5º C). Os discos foram mergulhados na solução e mantidos por 30 minutos. O tratamento alcalino consistiu em preparar 50 mL de solução de NaOH e transferí-la para um frasco de polietileno com tampa. As amostras submetidas anteriormente ao ácido foram mergulhadas nessa solução e mantidas a uma temperatura de 60ºC durante 24 horas.

Funcionalização das superfícies com estrôncio:

<u>Tratamento hidrotermal</u>

Uma solução de estrôncio foi preparada por meio da diluição de hidróxido de estrôncio octahidratado ($Sr(OH)_2.8H_2O$) em água Milli-Q, para se obter a concentração final de 60 mM. As amostras Ti-15Mo foram colocadas em 100 mL da solução de

estrôncio e autoclavadas com temperatura e pressão constantes (121°C e 1,0 a 1,5 Kgf/cm^2), durante 1 hora.

Desinfecção dos espécimes:

Todas as amostras foram desinfectadas em capela de fluxo laminar por irradiação ultravioleta (Veco VLFS-12) a uma distância de 50 mm por 1 hora cada lado.

Caracterização físico-química das superfícies:

A caracterização das superfícies foi feita através da avaliação da topografia e energia livre de superfície (molhabilidade) das diferentes superfícies. Para isso os testes realizados foram: Microscopia eletrônica de varredura (MEV) associada à Espectroscopia por energia dispersiva de raios X (EDS), Difração de Raios X (DRX), Microscopia confocal a laser e Ângulo de contato, além de ensaios eletroquímicos para análise da corrosão.

Microscopia eletrônica de varredura (MEV) associada à Espectroscopia por energia dispersiva de raios X (EDS)

A MEV foi realizada com um microscópio eletrônico de varredura (JEOL JSM-6610V, JAPAN) equipamento multiusuário da Faculdade de Odontologia de Araraquara e acoplado a ele um sistema de EDS, o qual possibilitou a determinação da composição química qualitativa e semi quantitativa das amostras, a partir da emissão de raios X característicos.

Difração de Raios X (DRX):

A difração de Raios X (DRX) foi realizado no DEMa/UFSCar (Departamento de Engenharia de Materiais – Universidade Federal de São Carlos) utilizou-se a faixa de 5 a 90° em 2 theta (x) usando como fonte a radiação CuKα (λ = 15,405 nm) a uma taxa de 2 minutos e permitiu a avaliação da estrutura cristalina do material.

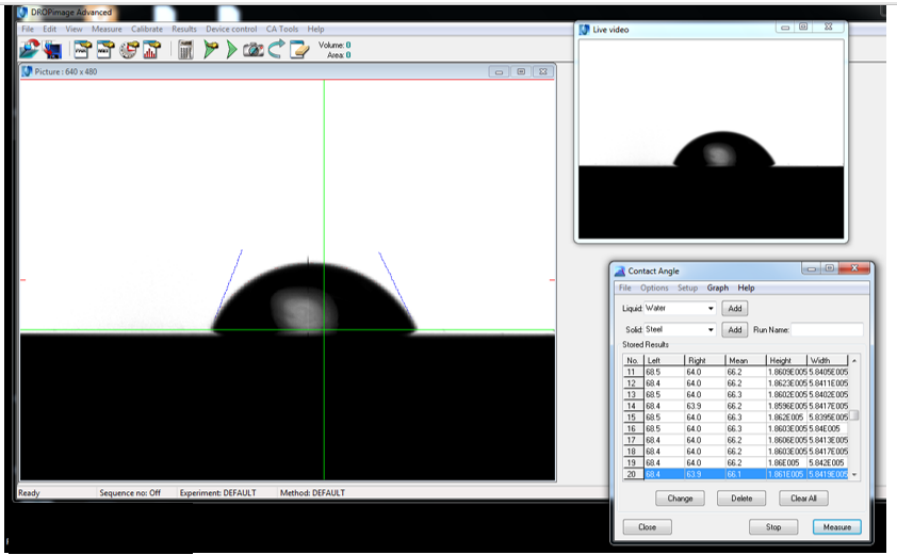
Confocal a laser:

As medidas de rugosidade sobre as superfícies das amostras foram realizadas com auxílio de um microscópio confocal a laser da marca OLYMPUS – LEXT OLS 4000 no DEMa. Além dos valores de rugosidade, foram obtidas imagens das superfícies estudadas no formato 3D.

Ângulo de contato:

Para essa análise, as amostras foram avaliadas quanto sua hidrofilicidade e hidrofobicidade utilizando-se de água destilada e diiodometano como líquidos de medida à temperatura ambiente. Uma gota de 3 µL foi dispensada sobre a superfície de Ti-15Mo, e o ângulo de contato formado foi determinado por meio de 20 mensurações realizadas a cada 0,01 s. Este procedimento foi repetido 7 vezes. O ângulo de contato e energia livre de superfície foi obtida pelo software DROPimage CA, Ramé-Hart, EUA (Foto 1).

Foto 1- Software DROPimage em análise do ângulo de contato de uma gota de água sob a superfície de uma amostra do grupo polido



Fonte: Arquivo pessoal do autor.

Ensaios Eletroquímicos

Os testes eletroquímicos ocorreram no Laboratório de Ensaios Mecânicos da Faculdade de Odontologia de Araraquara. Uma solução de NaCl a 0,9% foi utilizada para realização dos testes de potencial de circuito aberto e respostas potenciodinâmicas nas amostras. Um eletrodo de trabalho composto do material em

estudo, um contra eletrodo de platina e um eletrodo de referência de prata cloreto de prata (Ag/AgCl KCl 3mol/L) constituíam a célula eletroquímica (Foto 2).

O potencial de circuito aberto foi utilizado para mostrar, qualitativamente, a resistência à corrosão dos materiais ao meio e foi medido e registrado por tempo de 1,5 h durante a estabilização das amostras no eletrólito. A resposta potenciodinâmica das amostras foram realizadas a uma taxa de varredura de 1 mV/s na faixa de -0,3 a 5,0 V e então avaliadas em MEV e EDS para análise das modificações de superfície após corrosão.

Foto 2 – Célula eletroquímica utilizada para realizar as análises de corrosão

Fonte: Arquivo pessoal do autor.

<u>Determinação da liberação de estrôncio</u>

Para esse teste as amostras foram alocadas em placas de 24 poços e adicionados 3 mL de solução salina tampão fosfato (PBS) a 37°C com um pH de 7,4 sob condições estáticas. Nos intervalos de 1, 3, 5, 7 e 14 dias o volume total de PBS era coletado usando uma pipeta e armazenado em tubos falcon de 15 mL. Os discos recebiam PBS fresco e o procedimento era repetido em cada intervalo de tempo. Para quantificação total de estrôncio nas superfícies, as amostras foram submetidas a 10mL de ácido nítrico 30%. As soluções de PBS e ácido nítrico contendo Sr liberado foram analisadas na UFSCar pelo Grupo de análise instrumental aplicada (Gaia)

através de espectrometria de emissão atômica por plasma indutivamente acoplado (Thermo Fisher Scientific iCAP 7000, EUA) (Foto 3).

Foto 3 - Espectrômetro de emissão por plasma indutivamente acoplado utilizado para os testes de liberação de estrôncio

Fonte: Arquivo pessoal do autor.

Análise biológica in vitro:

Células MC3T3-E1

Os testes biológicos foram realizados utilizando células de linhagem MC3T3-E1 de camundongo (Sigma Aldrich) cultivadas e mantidas em meio α-MEM suplementado com 10% de soro fetal bovino (FBS), 100 U/mL de penicilina, 100 µg/mL de estreptomicina e alocadas em estufa a 37°C e 5% de CO_2. Além disso, no meio α-MEM foi adicionado indutores osteogênicos com 2 mmol/L de ácido ascórbico e 10 mM/L β-glicerofosfato.

Adesão, Morfologia e Espraiamento celular

Para avaliação da adesão, morfologia e espraiamento celular foi realizada análise em MEV (JEOL JSM-6610LV, JAPAN). Nos períodos de 1 e 3 dias. As células foram cultivadas sobre os discos sob uma densidade de 2.10^4, e em cada período foram lavadas em PBS três vezes e fixadas com glutaraldeído 2% em α-MEM puro durante 1 hora, lavadas em PBS três vezes e desidratadas em concentrações crescentes de etanol (25%, 50%, 70%, 95% e 100%). Após secagem em um dissecador a vácuo, as amostras receberam deposição de ouro (Denton Vaccum Desk V, 120 segundos, corrente 20 mAmps).

Como análise complementar do espraiamento celular, as células foram avaliadas em 1, 3 e 7 dias por fluorescência direta utilizando os marcadores: faloidina conjugada com Alexa Fluor 488 (Molecular Probes, Eugene, OR, EUA), para marcação do citoesqueleto, e de 4',6'-diamino-2-fenil-indol (DAPI, Molecular Probes) para marcação nuclear. Para esse teste, as células foram lavadas com PBS a 37°C e aplicados formaldeído 4% por 10 minutos em temperatura ambiente. Após isso, foram lavadas novamente com PBS e adicionados Triton X-100 por 5 minutos e então fixados com Alexa Fluor e DAPI. Ambos os testes foram realizados em duplicata para cada período de tempo.

Viabilidade e Proliferação Celular

O teste de Alamar Blue (*Molecular Probes,* EUA) foi utilizado para avaliação da viabilidade e proliferação após 1, 3 ,7 ,10 ,14 e 21 dias de cultivo celular.

As células semeadas nos discos foram alocadas em uma placa de 24 poços e incubadas com meio de cultura e Alamar Blue a 10% do volume total de cada poço durante 4 horas. Após o tempo de incubação, alíquotas de 150 µL foram coletadas de cada amostra e transferidas para uma placa de 96 poços para a leitura em um espectrofotômetro em comprimentos de onda de 570 e 600 nm. O número de células viáveis está relacionado com o nível de redução de corante, cuja viabilidade é expressa em percentual de redução de acordo com o protocolo do fabricante. Duas amostras de cada grupo foram utilizadas e o experimento foi repetido três vezes em momentos diferentes.

Formação de nódulos de mineralização

Ao término de cada teste de Alamar Blue, as mesmas amostras eram utilizadas para análise da formação de nódulos de mineralização no 21° dia de cultivo celular. As amostras foram fixadas em álcool etílico a 70% a 4ºC por 1 hora. Após isso, foram lavadas em PBS e água destilada e coradas com vermelho de Alizarina (Sigma), à temperatura ambiente por 5 minutos. O excesso de reagente foi removido com água destilada. 1 ml de solução de cloreto de cetilpiridínio a 10% (Sigma-Aldrich) foi aplicado nas amostras, à temperatura ambiente e 150 µL da solução de cada amostra foram transferidos para uma placa de 96 poços e lidos em um espectrofotômetro (Spectramax M, Molecular Devices) em comprimentos de onda de 550nm para quantificação dos nódulos mineralizados.

Análise estatística:

As análises estatísticas foram realizadas com significância mínima estabelecida em $P \leq 0,05$. Foram utilizados teste paramétrico ou não paramétrico, para dados independentes e mais de duas amostras (ANOVA ou teste de Kruskal-Wallis, respectivamente, no caso de haver ou não distribuição amostral normal e homogeneidade de variâncias), seguido de teste de comparações múltiplas, quando aplicável.

PUBLICAÇÃO 2

Nesse estudo, são idênticos da publicação 1 os materiais e métodos correspondentes a: Preparação das amostras de Ti-15Mo; Tratamento das superfícies (ácido e base); Funcionalização das superfícies com estrôncio (Tratamento hidrotermal); Desinfecção dos espécimes; Determinação de liberação de estrôncio; Portanto, estes tópicos estão apenas citados ao longo do texto a seguir.

As amostras desse estudo foram divididas em 4 grupos de acordo com o tratamento de superfície: sem tratamento, amostras com adição de estrôncio pelo método hidrotermal, amostras com adição de estrôncio pelo método *magnetron co-sputtering* e ácido/alcalino com adição de estrôncio pelo método hidrotermal.

Preparação das amostras de Ti-15Mo

Tratamento das superfícies

Funcionalização das superfícies com estrôncio:

Tratamento hidrotermal

Deposição física a vapor *magnetron co-sputtering*

O tratamento das amostras pelo método *magnetron co-sputtering* foi feito no Instituto Tecnológico Dinamarquês, Aarhus, Dinamarca. Os discos de Ti-15Mo foram alocados em uma amostra rotativa composta por *targets* de titânio grau 1 e $SrTiO_3$ sob pressão de 1 mPa de gás de argônio. Cada *target* possuía o tamanho aproximado de 90mm x 500 mm (Foto 4).

Foto 4 – *Targets* utilizados para aplicação de estrôncio nas amostras pelo método *magnetron co-sputtering*

Fonte: Arquivo pessoal do autor.

Desinfecção dos espécimes

Caracterização físico-química das superfícies:

Microscopia eletrônica de varredura (MEV)

A MEV (Nova 600, FEI Company, Netherlands) foi realizada na Universidade de Aarhus, Dinamarca, e avaliou a topografia gerada após os tratamentos de superfícies realizados.

Espectroscopia fotoelétrica de raios X

O teste de espectroscopia fotoelétrica de raios X (Kratos Axis UltraDLD, Kratos Analytical Ltd., UK) foi realizada na Universidade de Aarhus, para avaliação da composição química dos revestimentos das superfícies.

Determinação da liberação de estrôncio

Análise Biológica in vitro:

Cultura de células primárias

Para a realização nas análises biológicas deste estudo, foram utilizadas células humanas do ligamento periodontal e cultivadas em MEM suplementados com 10% de soro fetal bovino (Sigma-Aldrich) e antibióticos (25.000 UI/mL de penicilina e 25 mg/mL de estreptomicina; DuraScan Medical Products, Odense, Denmark) a 37°C em uma atmosfera de 100% de umidade relativa e 5% de CO_2.

Aderência e espraiamento celular

Para avaliação da aderência e espraiamento celular $5x10^3$ células foram semeadas nos discos e avaliadas após 1, 3 e 7 dias de cultura celular. Em cada período, as células foram fixadas com 10% de solução de formalina (aproximadamente 4% de formaldeído). As células foram lavadas uma vez com PBS e permeabilizadas com T-PBS (0,1% Triton X-100 em PBS; Sigma-Aldrich) por 15 minutos. O núcleo celular foi corado com DAPI (D9542, Sigma-Aldrich) e o citoesqueleto com Falloidina Conjugada Alexa Fluor 488 (Sondas Moleculares, Eugene, OR, EUA). As células com DAPI e Alexa Fluor foram incubadas durante a noite e então novamente lavadas com T-PBS. Após isso, foram analisadas em microscopia de fluorescência (Olympus BX 61). Três amostras de cada grupo foram avaliadas em cada período.

Viabilidade celular e proliferação

As células humanas do ligamento periodontal foram avaliadas quanto a viabilidade e proliferação pelo teste de Alamar Blue, como realizado na publicação 1. As células foram semeadas sob os discos a uma densidade de $5x10^4$ células e avaliadas após 1, 3, 7, 10 e 14 dias. Para esse teste três amostras de cada grupo foram utilizadas e o experimento foi repetido duas vezes.

Análise estatística:

A análise estatística foi realizada utilizando o GraphPad Prism 6.0 para Windows (GraphPad Software Inc., La Jolla, EUA).

Foram utilizados testes paramétricos ou não paramétricos para dados independentes e mais de duas amostras (teste ANOVA ou Kruskal-Wallis,

respectivamente, em caso de distribuição normal da amostra e homogeneidade das variâncias), seguidos de testes de comparação múltipla, quando aplicável. A significância estatística mínima foi estabelecida em $P \leq 0,05$.

Araraquara, 02 de Março de 2020.

Flávia Gomes Matos