FACULDADE SETE LAGOAS – FACSETE

ODONTOLOGIA

WALLISON DANIEL TAVARES DE OLIVEIRA

CARACTERIZAÇÃO BIOLÓGICA DO IONÔMERO DE VIDRO MODIFICADO POR NANOPATICULAS DE FOSTATO DE CÁLCIO

TRABALHO DE CONCLUSÃO DE CURSO

SETE LAGOAS, MG

2019

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CARACTERIZAÇÃO BIOLÓGICA DO IONÔMERO DE VIDRO MODIFICADO POR NANOPATICULAS DE FOSTATO DE CÁLCIO

Trabalho de Conclusão de Curso apresentada como requisito à obtenção do título de Bacharel em Odontologia, pela Facsete.

Orientador: Prof. Dr. Vítor Cesar Dumont

Co-orientador: Prof. Dr. Adriana Gonçalves Silva.

Professor da Disciplina de TCC II: Fabrício Tinoco Alvim de Souza.

SETE LAGOAS, MG

2019

OLIVEIRA, Wallison Daniel Tavares

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1. ART. 2. Cimento Ionômero de Vidro. 3. Nanotecnologia. 4. Biocomposto. 5. Fosfáto de Cálcio. I. Dumont, Dumont, Vitor Cesar. II. Silva, Silva, Adriana Gonçalves. III. Título.

Sumário

SIGLAS

CIV – Cimento de ionômero de vidro

CTMH – Células tronco masenquimais humanas

nCaP/CIV – Cimento de ionômero de vidro modificado por nano-partículas de fosfato de cálcio

CaP – Fosfato de cálcio

ART – Técnica restauradora atraumática

DMEM - Dulbecco Modified Eagle

UFMG – Universidade Federal de Minas Gerais

FBS – Soro fetal bovino

HCl – Ácido clorídrico

CARACTERIZAÇÃO BIOLÓGICA DO IONÔMERO DE VIDRO MODIFICADO POR NANOPATICULAS DE FOSTATO DE CÁLCIO

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RESUMO

O tratamento restaurador atraumático previne e controla o desenvolvimento de lesões cariosas, sendo o material mais indicado o Cimento de Ionomero de Vidro (CIV) devido as propriedades físicas e biológicas. O propósito deste trabalho é avaliar a citotoxicidade de células tronco mesenquimais humanas (CTMH) em contato direto com as amostras de CIV e nCaP/CIV utilizando os ensaios de MTT e LIVE/DEAD. Os resultados do ensaio de MTT registraram um aumento de 6,50% da viabilidade celular, após o contato de CTMH com as amostras de nCaP/CIV quando comparado ao grupo controle CIV. Além disso, quando compara - se o comportamento das células CTMH em contato com os dois biomateriais observamos diferença significativa de 22,00%. Os resultados do ensaio por LIVE/DEAD confirmam a biocompatibilidade dos biomateriais desenvolvidos, visto que, as células CTMH em contato com a amostra de nCaP/CIV apresentaram a similaridade da fluorescência mais evidente ao ser comparado com a amostra de CIV.

Palavras chave: ART, Cimento Ionômero de Vidro, Nano tecnologia, Biocomposto, Fosfato de Cálcio.

INTRODUÇÃO

O Tratamento Restaurador Atraumático (ART) foi reconhecido pela Organização Mundial de Saúde em 1994 e preconiza como material restaurador a utilização do cimento de ionômero de vidro (CIV) de alta viscosidade fornecendo resultados confiáveis para restaurações de uma superfície em dentes posteriores decíduos e permanentes (1,2).

A técnica de ART é fortemente recomendada para o manejo de pequenas lesões cariosas oclusais sem comprometimento pulpar em dentes deciduos e permanentes (3, 4) e consiste na remoção da dentina desmineralizada e infectada e na manutenção da dentina afetada, usando apenas instrumentos cortantes manuais priorizando a máxima preservação das estruturas dentárias (5,6).

O ART é um método econômico e eficaz para prevenir e controlar o desenvolvimento de lesões cariosas, além de causar menos desconforto e ansiedade odontológica aos pacientes, quando comparado a técnica de preparo cavitário que utiliza os instrumentos rotatórios convencionais (7, 8).

O CIV tornou-se um material de destaque na aplicação clínica por agregar propriedades físicas e biológicas satisfatórias, como adesão à estrutura dentária minimizando a microinfiltração na interface dente/restauração, ação anticariogênica caracterizado pela liberação de flúor que atua na remineralizarão da dentina afetada e no controle da recidiva de cárie e biocompatibilidade (9-11).

Limitações do CIV estão associadas a baixa resistência mecânica, friabilidade e deteriorização em pH ácido, tornando-o mais susceptível às falhas clínicas (11).

A incorporação de cristais de apatita de tamanho nanométrico não apenas aumenta as propriedades mecânicas dos CIV convencionais, mas também pode aumentar a liberação de flúor e a bioatividade. São amplas as possibilidades de indicação para o CIV, desde restaurações em dentes posteriores permanentes ou decíduos, restaurações de dentes

anteriores, preferencialmente decíduos, em lesões não cariosas em região cervical, como forramento ou base de preparos cavitários para posterior restauração permanente, cimentação de pinos intra canais e coroas total ou parcial, vedamento de fossas e fissuras oclusais e vedação em perfuração radicular (14 - 18).

OBJETIVO GERAL

Comprovar a viabilidade de Células Tronco Mesenquimais Humanas (CTMH) em contato direto com as amostras de CIV e nCaP/CIV e realizar testes de viabilidade e atividade celular com diferentes linhagens celulares, utilizando-se dos ensaios de MTT e LIVE/DEAD.

OBJETIVO ESPECÍFICO

Utilizar o nCaP/CIV como material restaurador em dentes decíduos, quando a lesão cariosa cavitada, comprometer uma ou duas faces, utilizando a técnica ART.

METODOLOGIA

Ensaio de citotoxicidade por (3- (4, 5-dimetiltiazol-2-il) 2, 5-difenil tetrazólio) (MTT)

As células foram cultivadas em meio de Dulbecco Modified Eagle (DMEM) com 10% de soro fetal de bovino (FBS), penicilina G de sódio (10units.mL^{-1}), sulfato de estreptomicina (10mg.mL^{-1}), e 25µg.mL^{-1} anfotericina-b (todos da Gibco BRL, Nova Iorque, EUA) numa

atmosfera humidificada de 5% de CO_2 a (37±1) °C. As células foram utilizadas para as experiências de passagem de doze. Todos os testes biológicos foram conduzidos de acordo com as normas ISO 10993-5: 1999 (Avaliação biológica de dispositivos médicos; Parte 5: testes de citotoxicidade in vitro). As amostras do produto compósito nCaP/CIV com 10mm de diâmetro de cada biomaterial foram colocadas em 24 poços de placa. Em seguida, as células foram plaqueadas com CTMH (3 × 104 células) em cada material de amostra. O método de esterilização foi a radiação UV durante 60min num fluxo estéril e lavados em solução salina tamponada com fosfato (PBS), gelado. As amostras foram esterilizadas por radiação UV durante 60min num fluxo estéril. Os controles foram criados usando as células e meio DMEM (10%); Triton X-100 (1%; Sigma-Aldrich, St. Louis, MO, EUA) foi usada como um controle positivo, e tiras de polipropileno estéril (1mg.mL^{-1}; Eppendorf, Hamburgo, Alemanha) foram utilizadas como um controle negativo. Depois de 24h, todos os meios foram aspirados e substituídos por 210uL de meio de cultura com soro. MTT (170uL, 5mg.mL^{-1}; Sigma-Aldrich, St. Louis, MO, EUA) foi adicionado a cada poço e incubou-se durante 4h, seguida por incubação de 16h com SDS / 4% de HCl. Subsequentemente, 100mL foi removida de cada poço e transferidos para um plano de 96 poços, e a absorvância foi quantificada utilizando um leitor de Varioskan (Thermo Scientific) com um filtro de 595nm. Os valores obtidos foram expressos como a percentagem de células viáveis de acordo com a equação (Eq.1).

$$Viabilidade\ celular(\%) = \frac{Absorção\ de\ amostras\ e\ células\ SAOS \times 100}{Absorção\,(controle)} \quad \text{(Eq.1)}$$

Software Prism (GraphPad Software, San Diego, CA, EUA) foi utilizado para a análise estatística. A significância estatística foi testada utilizando One-way ANOVA

seguido por teste de Bonferroni. Um valor de p <0, 05 foi considerado estatisticamente significativo. Todas as experiências foram realizadas em triplicado (n = 3).

Ensaio de citotoxicidade por LIVE/DEAD

Células humanas mesenquimais da medula óssea estaminais (CTMH) da passagem 4 foram plaqueadas (3 x 105 células / poço) em placas de 96 poços. As populações celulares foram sincronizadas em meio de soro durante 24h, após este período, o meio foi aspirado e substituído por meio contendo 10% de FBS e extrato do compósito nCaP / CIV. Foram utilizados como controle de referência DMEM com 10% de PBS. Após 24h, todo o meio foi aspirado e os poços lavados por duas vezes com 10mL de solução salina tamponada com fosfato (PBS) da (Gibco BRL, Nova Iorque, EUA). As células CTMH foram tratadas durante 30min com o kit Live / Dead (Life Technologies do Brasil Ltda, São Paulo), de acordo com as especificações do fabricante. As imagens foram obtidas com um microscópio óptico invertido fluorescente (Leica DMIL LED, Alemanha), as emissões de fluorescência foram adquiridas em separado, calceína a 530 ± 12,5nm, e EthD-1 a 645 ± 20nm.

RESULTADOS

Ensaio de toxicidade celular MTT

Este teste é usado especificamente para avaliar a função mitocondrial e a viabilidade celular. Ao analisar o comportamento das células CTMH em contato com a amostra CIV, não houve diferença significativa na viabilidade quando comparado ao grupo controle. No entanto, ao analisar as células em contato com a amostra nCaP/CIV, houve um aumento significativo na viabilidade de 12, 00 ± 6, 50% quando comparado ao grupo controle. Além disso, quando comparamos o comportamento das células CTMH em

contato com os dois biomateriais observamos diferença significativa de 22,00% na amostra nCaP/CIV em relação à amostra do CIV (figura 1).

Figura 1

Ensaio LIVE / DEAD

Os resultados mostrados na Figura 2 confirmam a biocompatibilidade dos biomateriais desenvolvidos. Pode ser visto que as células CTMH em contato com ambas as amostras apresentaram padrões de fluorescência semelhantes quando comparados com o grupo de controle não tratado, sendo, fluorescência verde elevada (células viáveis) e pouca ou nenhuma fluorescência vermelha (células mortas). A similaridade da fluorescência é mais evidente nas células em contato com a amostra nCaP/CIV.

Figura 2

CONCLUSÃO

O biocompósito apresentou resultados satisfatórios para os ensaios de citotoxicidade por MTT e LIVE / DEAD evidenciando a biocompatibilidade do nCaP/CIV após o contato direto com CTMH. Sugere-se a realização de uma nova etapa de análises *in vivo* para possível aplicação clínica utilizando - o como material restaurador após o ART.

AGRADECIMENTOS

Os autores agradecem o apoio da CAPES, FAPEMIG e CNPq.

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LEGENDAS DAS FIGURAS

Figura 1 - Resposta de viabilidade de células-tronco mesenquimais de medula óssea humana pelo ensaio MTT após 72 horas de incubação em contato com o CIV e nCaP/CIV.

Figura 2 - O ensaio LIVE / DEAD com células CTMH após 72 horas de contato direto. Células vivas (A), (verdes) e células mortas (B), (vermelhas) nas amostras controle, CIV e nCaP/CIV (barra = 100µm; 200X).

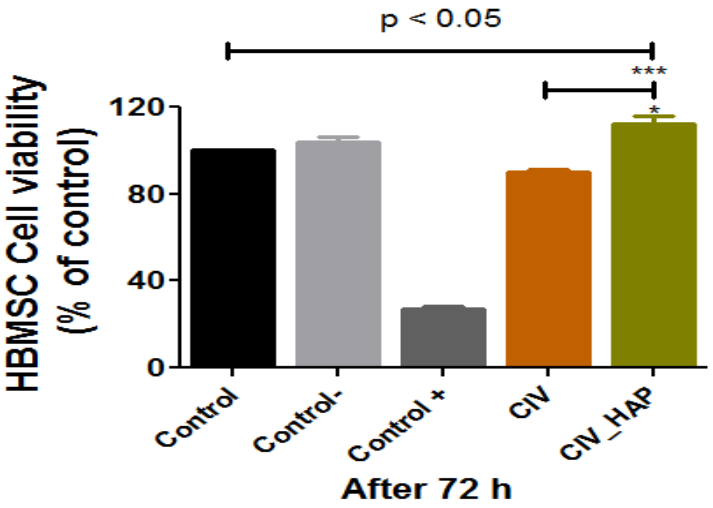
p < 0.05
***
*
HBMSC Cell viability
(% of control)
120
80
40
0
Control
Control-
Control +
CIV
CIV_HAP
After 72 h

Figura 1

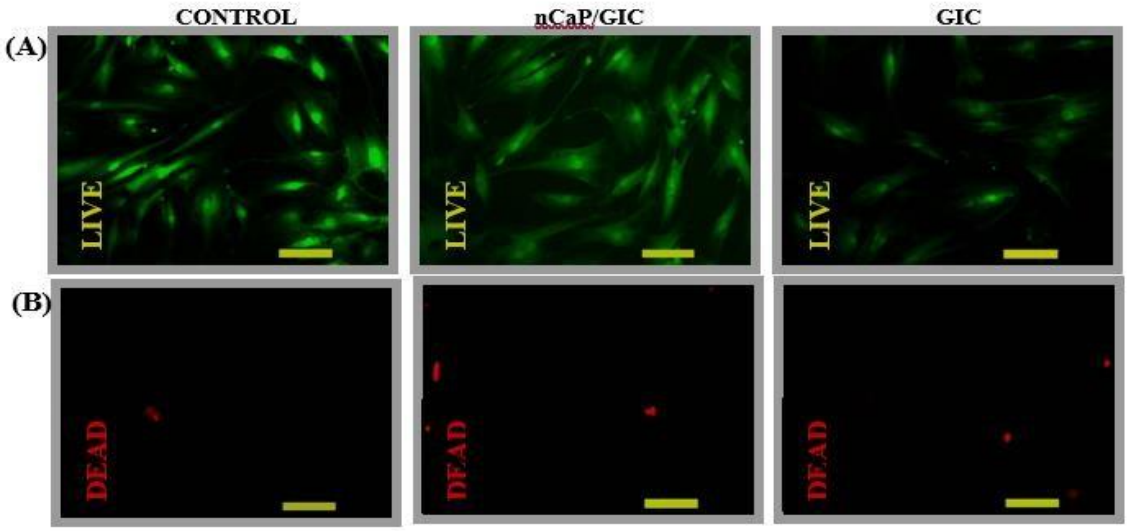

Figura 2

APÊNDICE A – FICHA DE ACOMPANHAMENTO

Orientador: Vítor Cesar Dumont	
Aluno: Wallison Daniel Tavares de Oliveira	
TÍTULO DO TCC: CARACTERIZAÇÃO BIOLÓGICA DO IONÔMERO DE VIDRO MODIFICADO POR NANOPATÍCULAS DE FOSTATO DE CÁLCIO .	
Telefone: (31)9 87789235	E-mail: daniel7l@hotmail.com

Descrição de atividades/ DATA: 22/06/18

Escolha do tema, elaboração do título e início do recrutamento de artigos científicos com os temas ART e CIV.

Retorno marcado para o dia: 17/08/18

Visto do aluno: _____

Data do retorno: 17/08/18

Elaboração dos critérios de avalição e controle periódico dos pacientes para realização do tratamento restaurador atraumático, utilizando o nCaP/CIV como material restaurador.

Retorno marcado para o dia: 21/09/18

Visto do aluno: _____

Data do retorno: 21/09/18

Acompanhamento: Apresentação dos artigos escolhidos com os temas ART e CIV da parte escrita.

Retorno marcado para o dia: 16/10/18

Visto do aluno: _____

Data do retorno: 16/10/18

Acompanhamento: Recrutamento de artigos científicos com tema nanotecnologia em biomateriais.

Retorno marcado para o dia: 8/11/18

Visto do aluno: _____

Data do retorno: 8/11/18

Acompanhamento: Mudança do tema e análise do novo tema proposto.

Retorno marcado para o dia: 21/02/19

Visto do aluno: _____

Data do retorno: 21/02/19

Acompanhamento: Recrutamento de artigos científicos para elaboração da introdução.

Retorno marcado para o dia: 07/03/19

Visto do aluno: _____

Data do retorno: 07/03/19

Acompanhamento: Apresentação da introdução e desenvolvimento da parte escrita com ênfase na metodologia.

Retorno marcado para o dia: 11/04/19

Visto do aluno: _____

Data do retorno: 11/04/19

Acompanhamento: Desenvolvimento parte escrita com ênfase nos resultados e conclusão.

Visto do aluno: _____

Retorno marcado para o dia: 09/05/19

Data do retorno: 09/05/19

Acompanhamento: Conclusão TCC.

Visto do aluno: _____

_____

Assinatura do Orientador

APÊNDICE B – AUTORIZAÇÃO DO ORIENTADOR PARA ENTREGA DO TCC

Eu, Vítor Cesar Dumont, orientador do (s) aluno (a) do curso de Odontologia da FACSETE, autorizo a entrega ao Coordenador do Curso, o Trabalho intitulado CARACTERIZAÇÃO BIOLÓGICA DO IONÔMERO DE VIDRO MODIFICADO POR NANOPATÍCULAS DE FOSTATO DE CÁLCIO, para avaliação da Banca Examinadora, conforme regulamento interno desta Faculdade.

Informo, ainda, que acompanhei o TCC, conforme cronograma abaixo:

Meses	Dias de orientação		Ass. do orientador
Junho	22		
Agosto	17		
Setembro	21		
Outubro	16		
Novembro	8		
Fevereiro	21		
Março	7		
Abril	11		
Maio	15		

Parecer do Orientador:

_____

_____

_____

_____.

_____

Assinatura do Orientador

APÊNDICE C – CRONOGRAMA DE EXECUÇÃO DO PROJETO

	AGO/ 2018	SET/ 2018	OUT/ 2018	NOV/ 2018	DEZ/ 2018	FEV/ 2019	MAR/ 2019	ABR/ 2019	MAI/ 2019	JUN/ 2019
Apresentação ao orientador	X									
Escolha do tema	X									
Elaboração e entrega do projeto		X								
Leitura Exploratória	X	X								
Mudança do tema				X						
Início da redação do TCC		X								
Avaliação do andamento do TCC			X	X	X		X		X	
Elaboração do TCC						X				
Digitação Provisória						X				
Conclusão e revisão do TCC								X		
Digitação das eventuais correções									X	
Entrega do TCC para avaliação da Banca Examinadora										X

APÊNDICE D – TERMO DE RESPONSABILIDADE DO ALUNO

Declaro, para os devidos fins que se fizerem necessários, que assumo total responsabilidade pelo conteúdo apresentado neste Trabalho de Conclusão de Curso, isentando a FACSETE e o Orientador de toda e qualquer representação contra o TCC, estando ciente da regulamentação institucional de TCC da Instituição.

Estou informado de que poderei responder administrativa, civil e criminalmente em caso de cópia encontrada no trabalho apresentado para correção.

Sete Lagoas/MG, 06 de junho de 2019

_____

Wallison Daniel Tavares de Oliveira

APÊNDICE E – ARTIGO SUBMETIDO A REVISTA INTERNACIONAL

Manuscript Details

Manuscript number	MRB_2019_1227
Title	ENHANCING GLASS IONOMER CEMENT FEATURES BY USING THE CALCIUM PHOSPHATE NANOCOMPOSITE
Article type	Research Paper

Abstract

This study is showed the synthesis of Glass ionomer cements (GIC) modified with calcium phosphate nanoparticles (nCaP). The nCaP/GIC were submitted to mechanical compression and diametral tensile tests, The biocomposite were characterized by scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD) and fourier transform infrared spectroscopy (FTIR). Cytotoxicity and cell viability tests were performed on the human bone marrow mesenchymal stem cells using a 3-(4,5-dimethylthiazol-2yl)2,5-diphenyl-tetrazolium-bromide assay and LIVE/DEAD assays. Statistically significant differences were observed for mechanical properties (Kruskal-Wallis, $p<0.001$), nCaP/GIC showed higher resistance to compression and diametral traction. The SEM analyses revealed a uniform distribution nCaP in the ionomer matrix. The EDX and XRD results indicated that hydroxyapatite and calcium β-triphosphate phases. The FTIR spectra revealed the asymmetric band of v3PO43- between 1100-1030cm-1 and the vibration band associated with v1PO43- in 963cm-1 associated with nCaP. The nCaP/GIC presented response to adequate cell viability and non-cytotoxic behavior.

Keywords	Ceramics; Composites; Chemical Synthesis; Electron Microscopy
Taxonomy	Materials Application, Materials Characterization, Materials Processing, Materials Science Engineering, Materials Property, Applied Sciences
Manuscript category	Electroceramics, structure analysis, other
Corresponding Author	Vitor Dumont
Corresponding Author's Institution	FACSETE
Order of Authors	Ana Caroline A. da Silva Duarte, Rafael Mendes, Rodrigo Pereira, SANDHRA MARIA DE CARVALHO, Wallison Tavares, Cintia Pimenta de Araujo, Adriana Silva, João Drumond, Vitor Dumont
Suggested reviewers	Monize Carvalho, Maria Santos, Talita Martins

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Highlights

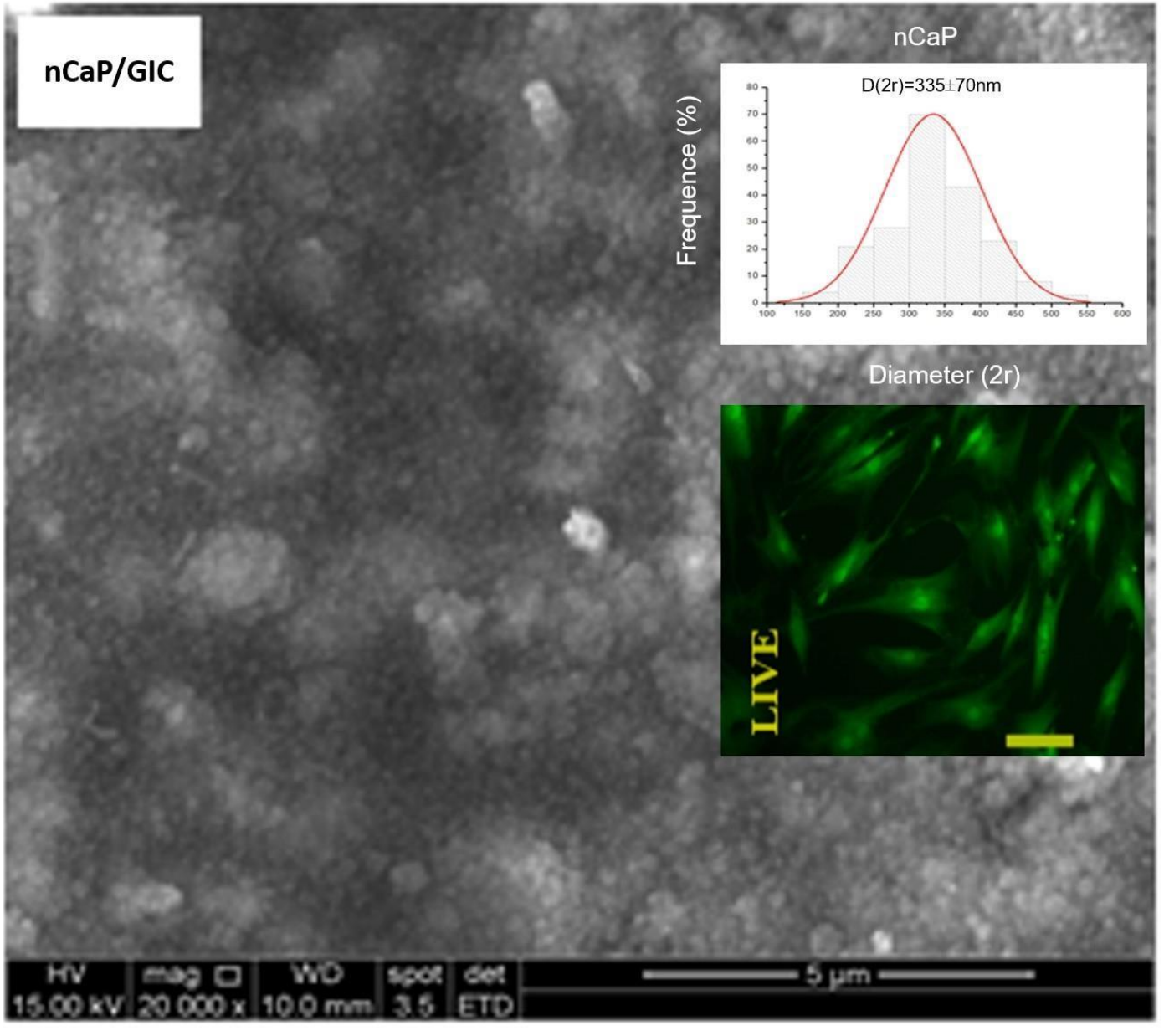
- The nCaP have shown proven results in resistance of composites.

- The addition of the calcium β-triphosphate nanoparticles in GIC hasn’t been reported.

- Glass ionomer cement with nanoparticles improved its mechanical properties.

- The restorative cement was modified with simple and low cost technique.

- The composite showed interaction between nanoparticles and ionomer matrix.


nCaP/GIC
nCaP
D(2r)=335±70nm
Frequence (%)
Diameter (2r)
LIVE
HV
15.00 kV
mag
20 000 x
WD
10.0 mm
spot
3.5
det
ETD
5 µm

ENHANCING GLASS IONOMER CEMENT FEATURES BY USING THE CALCIUM PHOSPHATE NANOCOMPOSITE

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1. Introduction

Glass ionomer cements (GIC) have become a prominent material in dentistry being widely used for aggregating satisfactory physical and biological properties. Adhesion to tooth structure characterized by the chemical interaction of carboxyl groups of polyacids to calcium ions of dental tissues, low contraction and expansion during prey reaction and thermal expansion coefficient similar to that of tooth structure minimize microleakage at the tooth / restoration interface [1-4]. Its anti-cytogenetic nature associated with biocompatibility and fluoride release act on the remineralization of dental tissues and on the control of caries recurrence, discarding the need for total removal of infected and softened dentin to control the progression of dental caries [2-5].

The use of GIC as a direct restorative material presents some limitations associated with its low mechanical resistance (abrasion and flexural), friability, high modulus of elasticity and deterioration in acidic pH, being therefore fragile and prone to fracture [2,3,6].

Modifications of the glass ionomer cements with metals, polymers and ceramics in different metric scales have been proposed with the aim of improving the mechanical and biological properties [3,4,7-12].

In this context, the use of calcium phosphate (CaP) biomaterials are of particular interest in improving mechanical properties, incorporating topographic features at the nanoscale that mimic the natural tooth nanostructure and establishing an intimate and functional relationship with adjacent tissue [13-18]. The amount of calcium phosphate incorporated requires compatibility between the nanoparticles and the polymer matrix, significantly influencing the wettability and viscosity of the composite [19].

Numerous studies with calcium phosphate particles added to composites demonstrated improvements in mechanical properties [1,3-12,19,20]. However, no study has been reported in the literature involving the hydroxyapatite and β-TCP phases of calcium phosphate in the modification of glass ionomer cement for the improvement of mechanical properties and decrease of cytotoxicity.

This study is reported to biocomposites synthesis based on arrays of glass ionomer cement (GIC) modified with calcium phosphate nanoparticles (nCaP). The biocomposites were characterized by scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR). Cytotoxicity and cell viability tests were performed on the human bone marrow mesenchymal stem cells (HBMSC) using a 3-(4,5-dimethylthiazol-2yl) 2,5-diphenyl tetrazolium bromide (MTT) assay and LIVE/DEAD assays.

2. Experimental procedure

2.1 Materials

All of the reagents and precursors, phosphoric acid (Sigma-Aldrich, USA, 85%, H_3PO_4), calcium hydroxide (Sigma-Aldrich, USA, ≥96%, $Ca(OH)_2$) and ammonium hydroxide (Synth, Brazil, 30%, NH_4OH) were used as received. Ionomer glass cement (GIC) (FGM, Brazil, Maxxion R) was modified. Deionized water (Millipore Simplicity™) with a resistivity of 18MΩ cm was used in the preparation of all solutions.

Potassium bromide (Sigma-Aldrich, USA, ≥99%, KBr), suitable for spectroscopy, was used to prepare the FTIR pellets.

2.2 Synthesis of calcium phosphate nanoparticles (nCaP)

The nCaP particles were synthesized by aqueous precipitation route, at (25±2)°C (Figure 1) [21]. The precursors were prepared as follows: 0.6ml of H_3PO_4 was slowly added to 89.4ml of deionized water under magnetic stirring for 15min. This phosphate precursor solution was referred to as "SOL_1".

Approximately 1.1g of $Ca(OH)_2$ powder was added to 10mL of deionized water and under vigorous stirring for 15min. This calcium suspension was referred to as "SUS_1". Then "SUS_1" was added slowly to "SOL_1" for the synthesis reaction and this mixture ("SUS_2") was magnetically stirred for 1h. Subsequently, SUS_2 was allowed to stand for 24h at (25±2)°C. The supernatant was decanted from the solid material. The precipitate was vacuum filtered using custom filter paper on a Büchner funnel, 3 washes were performed with deionized water and filtered again. The material retained was subjected to drying at a temperature of (25±2)°C for 96h. The chemical reaction of the formation of calcium phosphate is represented in the equation 1:

(Eq. 1) $$6H_3PO_{4(aq)} + 10\,Ca(OH)_{2(sus)} \rightarrow Ca_{10}(PO_4)O_6(OH)_{2(s)} + 18\,H_2O_{(l)}$$

2.3 Biocomposite synthesis (nCaP/GIC)

The nCaP/GIC was obtained by the addition of 1.1g of nCaP which were weighed and added to 10g powder of the GIC. The agglutination of the material followed the standards required by the manufacturer in room temperature (25±2)°C.

2.4 Mechanical tests

Test specimens (cps) (n = 20) of GIC and nCaP/GIC were made in a teflon matrix with 4mm in diameter and 8mm in length, resting on a glass plate. The cement was inserted into the matrix under pressure through a specific syringe (Centrix, DFL Ind., São Paulo, SP, Brazil) to minimize the formation of bubbles in the cement body. After complete filling of the matrix, a polyester strip was pressed on the surface of the cement under a weight of 500g until reaching its setting time in order to obtain adequate flow and surface smoothness of the material. After 24h of storage in distilled water, at (37±1)°C, cps (n = 10) of G1-GIC and G2- nCaP/GIC were subjected to the compressive strength test in a universal test machine EZ Test (Shimadzu , Japan) with a load cell of 200kgf at a speed of 1mm/min, with its long axis in the vertical

position, until its fracture. For the diametral tensile strength test, cps (n = 10) were submitted to the same load cell, but with a velocity of 0.5mm/min and with its long axis in the horizontal position.

The results were submitted to the normality test (Shapiro-Wilk), then a parametric statistical test (ANOVA) was applied to verify differences between the groups using the Statistical Package for Social Sciences (SPSS for Windows, version 17.0, SPSS Inc., USA). Statistical analysis of the data was performed with a level of significance of 95%.

2.5 Characterizations of the biocomposite (nCAP/GIC) and precursors

2.5.1 Scanning electron microscopy (SEM) and energy dispersion X-ray spectroscopy analysis (EDX).

The morphologies glass ionomer cement (GIC) modified with calcium phosphate nanoparticle (nCaP) were evaluated using a scanning electron microscope (SEM, FEI-INSPECTTM S50) coupled with energy dispersion X-ray spectroscopy (EDX, EDAX GENESIS). Before examination, the samples were coated with a thin carbon film via sputtering using a low deposition rate, cooling the substrate, and ensuring the maximum distance between the target and the sample to avoid sample damage. Images of secondary electrons (SE) were obtained using an accelerating voltage of 15kV.

The nCaP particles sizes and size distribution data were obtained based on the SEM images by measuring at least 100 randomly selected nanoparticles using an image processing program (ImageJ, public domain software, version 1.44, National Institutes of Health).

2.5.2 X-ray diffraction (XRD)

The crystallinity of the phases presents in the biocomposites (nCaP/GIC) was assessed based on the X-ray diffraction (XRD) patterns recorded using a PANalytical X'Pert diffractometer (Cu-Kα radiation with λ=1.5406Å). Measurements were performed in the 2θ range of 15° to 75° with steps of 0.06°.

2.5.3 Fourier transformed infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) was performed in the range of 650 to 4000cm $^{-1}$ (Fischer Thermo Nicolet 6700) using the transmission mode. The nCaP and nCaP/GIC were placed in a sample holder and scanned immediately (16 scans) with a resolution of 2cm $^{-1}$ background subtraction

2.6 Cytotoxicity assay

Culture of cells.

Human bone marrow mesenchymal stem cells

The Human bone marrow mesenchymal stem cells (HBMSC) were kindly Profa: Maria de Fátima Leite of department of physiology and biophysics, UFMG. The cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) penicillin G sodium (10units.mL $^{-1}$), streptomycin sulfate (10mg.mL $^{-1}$) and amphotericin-b (0.025mg.mL^{-1}) all from Gibco BRL (NY, USA) in a humidified atmosphere of 5% CO_2 at (37±1)°C. The cells were used for experiments on passage 5. Toxicity assay by resazurin and MTT: All biological tests were conducted according to ISO standards 10993-5:1999 (Biological evaluation of medical devices; Part 5: tests for in vitro cytotoxicity and Part 12-7; Sample preparation of extracts of test materials)

2.6.1 (3-(4,5-dimethylthiazol-2yl) 2,5-diphenyl tetrazolium bromide) MTT assay

HBMSC cells were plated (3×10^4 cells/well) in 96-well plates. Cell populations were synchronized in serum-free media for 24h. After this period, the medium was aspirated and replaced with medium containing 10% FBS. Samples of GIC and nCaP/GIC (5mg.mL $^{-1}$) were added to individual wells. Controls were used with the cells and DMEM with 10% FBS, the positive control Triton x-100 (1% v/v in phosphate buffered saline, PBS, Gibco BRL, NY, USA) and, as a negative control, chips of sterile polypropylene Eppendorf tubes (1mg.mL $^{-1}$, Eppendorf, Hamburg, Germany). After 72h, the medium was aspirated and replaced with 60µL of culture medium with serum in each well. Next, 50µL of MTT medium (5mg.mL $^{-1}$) (Sigma-Aldrich, MO, USA) was added to each well and was incubated for 4h in an oven at (37±1)°C and 5% CO_2. Subsequently, 40µL of the SDS solution/4% HCL was placed in each well and incubated for 16h in an oven at (37±1)°C and 5% CO_2. Then, 100µL was removed from each well and transferred to a 96-well plate to quantify the absorbance (Abs) using Varioskan Reader (Thermo Scientific) with a 595-nm filter. The values obtained were expressed as percentage of viable cells according to the following formula: Cell viability (%) = (absorbance samples and cells x 100) / absorbance (control). Assume the values of controls (wells with cells, and no samples) as 100% cell viability.

Prism software (GraphPad Software, San Diego, CA, USA) was used for data analysis. Statistical significance was tested using One-way ANOVA followed by Bonferroni test. A p value < 0.05 was considered statistically significant. Experiments were performed with n = 3.

2.6.2 Live / dead assay

HBMSC cells were plated (3×10^4 cells/well) in 96-well plates. Cell populations were synchronized in serum-free media for 24h. After this period, the medium was aspirated and replaced with medium containing 10% FBS. Samples of GIC and nCaP/GIC (5mg.mL $^{-1}$) were added to individual wells. After 72h, all media was aspirated, washed with PBS for two times with 10mL of phosphate buffered saline (PBS) from (Gibco BRL, NY, USA). The HBMSC cells were treated for 30min with the kit LIVE / DEAD Viability / citotoxicity from (Life Technologies of Brazil Ltda, São Paulo) according to manufacturer's specifications. Images were obtained with a inverted optical microscope (Nikon, Japan,), the fluorescence emissions should be acquired separately as well, calcein at 530 ± 12.5nm, and EthD-1 at 645 ± 20nm.

3. Results and discussion

3.1 Mechanical tests

There was a statistically significant difference between GIC and nCaP/GIC for the compressive strength test and the diametral tensile strength test (table 1).

The results of the nCaP/GIC in the mechanical assays may be associated with a strong influence of the increase of the calcium concentration in the prey steps of the material. In the initial stage during the agglutination of the powder and liquid, the hydrogen promotes the displacement of calcium and aluminum ions that react with the fluoride forming calcium and aluminum fluorides. As the pH of the system decreases the dissociation of these fluorides occurs which react with the copolymers forming more stable complexes. It is suggested that the increase in calcium concentration by the incorporation of nCaP displaces the chemical reaction a favor of the formation of these more stable compounds.

Another step that can be influenced by the incorporation of nCaP is the formation phase of the polyacid matrix. At this stage, the release of calcium occurs with greater velocity due to its cationic character interacting with the aqueous chains of polyacids forming crosslinks, forming the gel matrix allowing

hardening of the material. Thus, the high concentration of calcium can accelerate the prey of the material minimizing the influence of the medium, sinerese and imbibition, predisposing the nCaP/GIC to present better results in the mechanical tests.

3.2 Characterizations of the biocomposite (nCAP/GIC) and precursors

3.2.1 Scanning electron microscopy (SEM) and energy dispersion X-ray spectroscopy analysis (EDX).

Morphological evaluations of nCaP show considerable heterogeneity in the form of the synthesized particles (Figure 2(a)). The characteristic EDX spectra are shown in Figure 2(b) showing peaks associated with Ca and P elements, and a Ca / P ratio equal to 1.8, suggesting the precipitation of the hydroxyapatite phase. The synthesis process allowed the formation of particles in the gauge scale (Figure 2(c)).

The modification of the GIC allowed the synthesis of homogeneous biocomposites with greater surface roughness (Figure 3(a)). The EDX spectra showed peaks of Ca and P elements attributed to nCaP as shown in Figure 3(b). In addition, the Ca-Kα mapping analyzes revealed that the particles of nCaP are uniformly dispersed in the composite matrix without detecting any segregation (Figures 3(c) and 3(d)).

3.2.2 X-ray diffraction (XRD)

The standard calcium phosphate (nCaP) XRD, GIC and nCaP/GIC are shown in figure 4. The figure 4(a) showed characteristic peaks of calcium phosphate particles (International Centre for Diffraction Data, JCPDS 86-1203). The XRD spectra of nCaP (Figure 4(b)) showed major peaks characteristic of HA in 2 theta equal to 31,7º (2 1 1) 32,8º (3 0 0) 32,2º (1 1 2), and 25,9º (0 0 2) , and other smaller peaks with intensities associated β - tricalcium phosphate (β -TCP) phase (28.0◦, 31.2◦, and 34.5º). The XRD spectra of nCaP/GIC (Figure 4(c)) showed of the halo characteristic of polymers with amorphous appearance. X-ray diffraction also certifies that the GIC overlaps the characteristic peaks of β-TCP, evidencing only the peaks associated with hydroxyapatite (Figure 4(d)).

3.2.3 Fourier transformed infrared spectroscopy (FTIR)

The figure 5 show the spectra of nCaP/GIC. The FTIR results shown the asymmetric band of ν3 PO_4^{3-} between 1100-1030cm $^{-1}$ and the vibration band associated with ν1 PO_4^{3-} in 963cm^{-1} associated with the phases of calcium phosphate.

3.3 Cytotoxicity assay

In the current study the viability of HBMSC cells in direct contact with GIC and nCaP/GIC samples were analyzed by MTT assay. This test is specifically used to evaluate mitochondrial function and cell viability. When analyzing the behavior of HBMSC cells in contact with the GIC sample showed no significant difference in viability when compared to the control group. However, when analyzing the cells in contact with the nCaP/GIC sample there was a significant increase in viability of 12.00 ± 6.50% when compared to the control group. Moreover, when we compare the behavior of HBMSC cells in contact with the two biomaterials observed significant difference of 22.00% in nCaP/GIC sample compared to GIV sample (Figure 6).

The results shown in Figure 7 confirm the biocompatibility of the biomaterials developed. It can be seen that the HBMSC cells in contact with both samples showed similar patterns of fluorescence when compared to the untreated control group, ie, high green fluorescence (viable cells) and little or no red fluorescence (dead cells). The similarity of the fluorescence is more evident in cells in contact with the nCaP/GIC sample.

4. Conclusion

This study demonstrates for the first time that the incorporation of nanoparticles of calcium phosphate, hydroxyapatite and BTCP in nanometric scale affect the GIC prey phases. The results showed that nCaP was evenly distributed in the ionomer matrix, and provided improvements in the mechanical properties of the material. Regarding cytocompatibility in vitro, no toxicity was observed for any of the groups tested. The nCaP/GIC biocomposites are more promising for potential application in dentistry by favoring the repair and replacement of bone tissue in vivo assays.

Acknowledgments

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220

221**5. References**

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FIGURE CAPTIONS

Figure 1 – Synthesis of calcium phosphate nanoparticles (nCaP).

Figure 2 – Morphological analysis of nCaP ((A) SEM image). Chemical analysis: EDS spectra (B) and histogram of the mean size of nCaP (C).

Figure 3 – Morphological analysis of nCaP/GIC ((A) SEM image). Chemical analysis: EDS spectra (B) and mapping of Ca Kα (C) and P Kα (D) elements.

Figure 4 – XRD spectra of the reference CaP (ICDD-96-900-3549) (A), nCaP (B), GIC (C) and nCaP/GIC (D).

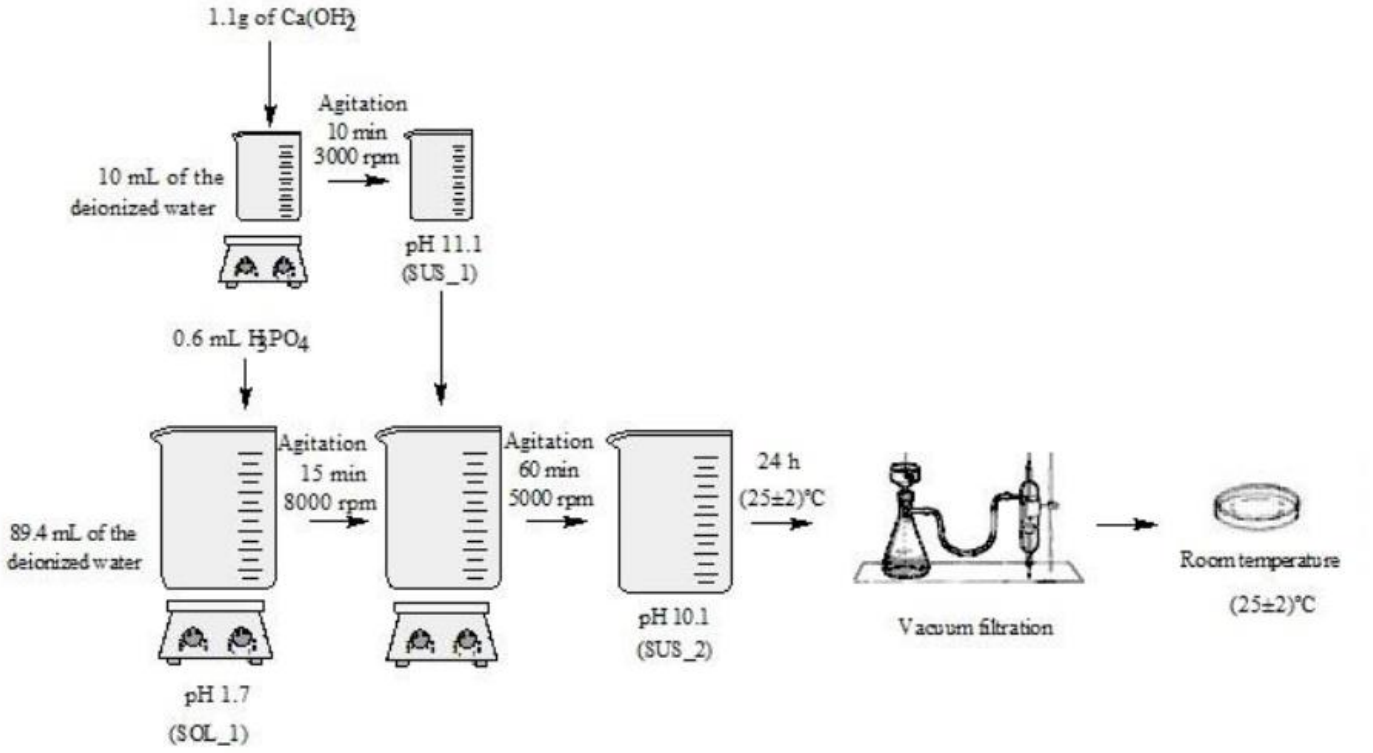
Figure 5 – FTIR spectra of the nCaP.

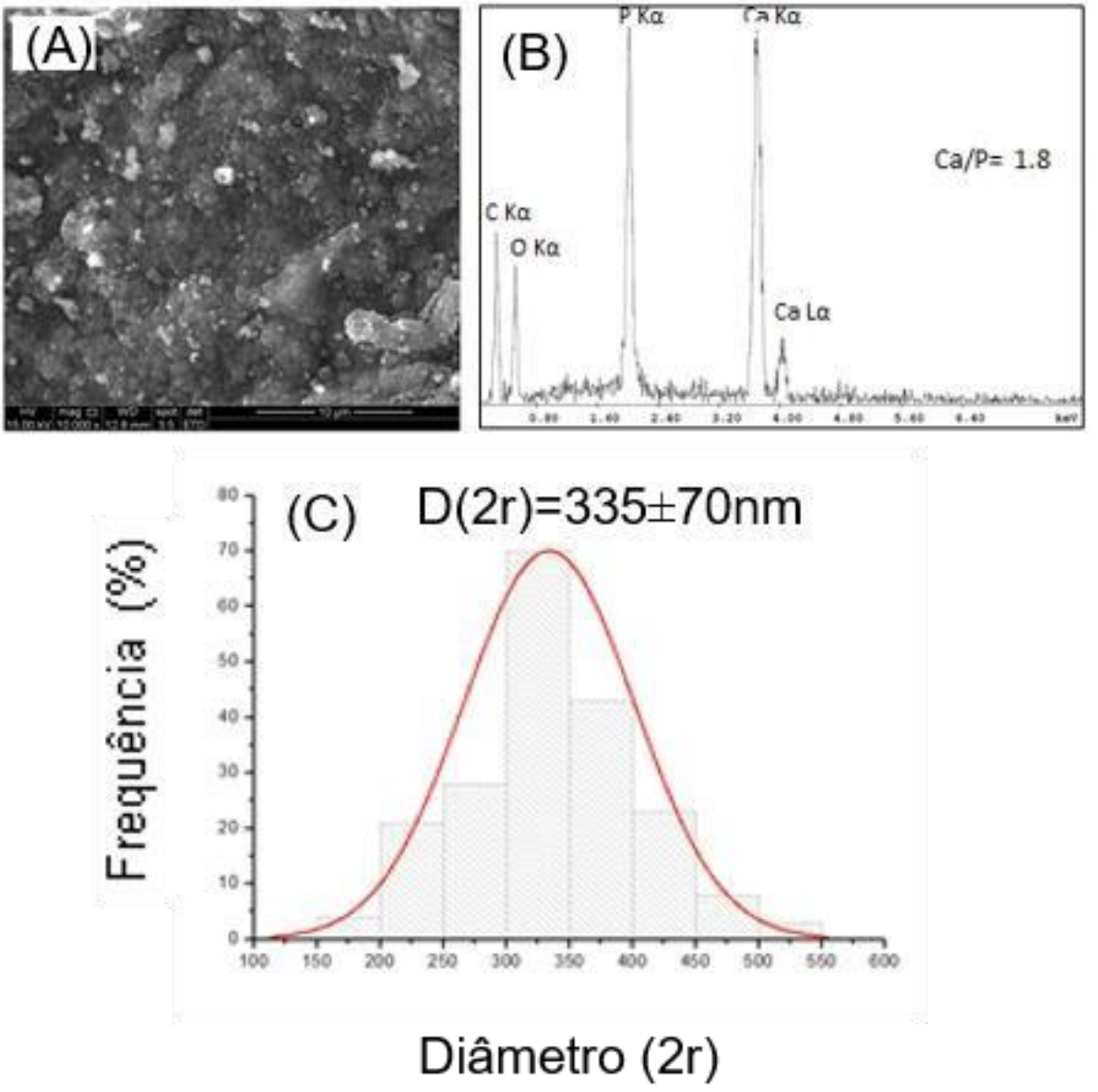
Figure 6 - MTT assay after 24h incubation direct contact with mesenchymal stem cells from bone marrow.

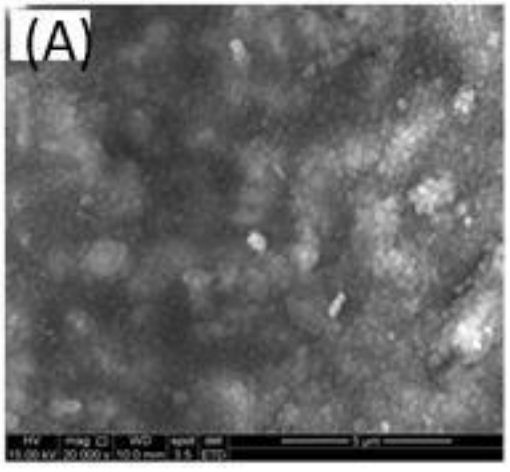
Figure 7 - Live/Dead assay with HBM cells after 72h of direct contact. In the control, in the GIC and nCaP/GIC samples (bar = 100 μm, 200x), live cells ((A), green) and dead cells ((B), red).

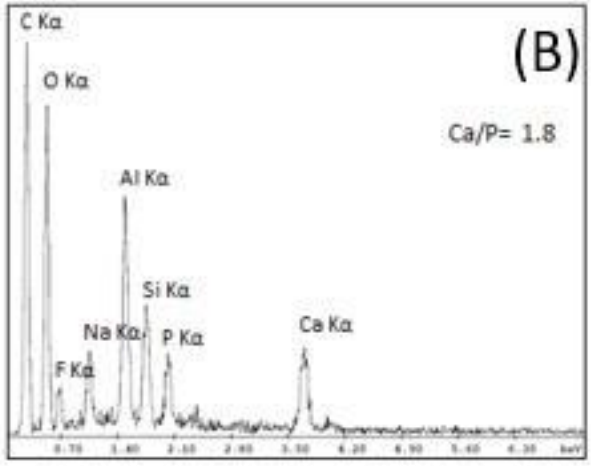
TABLES CAPTIONS

Table 1 - Mean values (MPa) of the compressive strength (CS) and diametral tensile strength (DTS).


1.1g of Ca(OH)2
Agitation
10 min
3000 rpm
10 mL of the
deionized water
pH 11.1
(SUS_1)
0.6 mL H3PO4
Agitation
15 min
8000 rpm
Agitation
60 min
5000 rpm
24 h
(25±2)°C
89.4 mL of the
deionized water
pH 1.7
(SOL_1)
pH 10.1
(SUS_2)
Vacuum filtration
Room temperature
(25±2)°C


(A)
(B)
P Kα
Ca Kα
Ca/P= 1.8
C Kα
O Kα
Ca Lα
(C)
D(2r)=335±70nm
Frequência (%)
Diâmetro (2r)


(A)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O Kα
(B)
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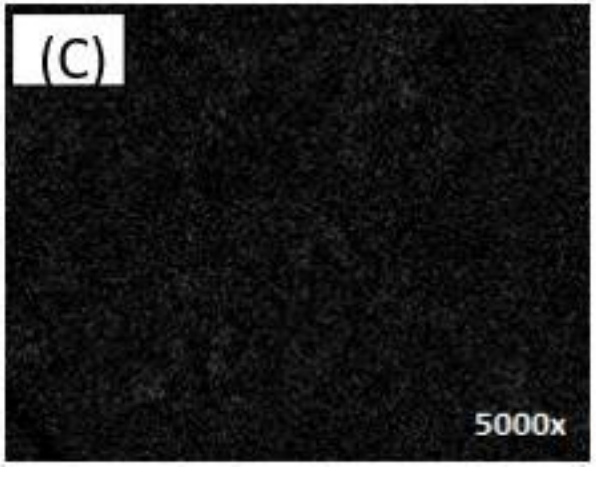
Si Kα
Na Kα
P Kα
Ca Kα
F Kα


(C)
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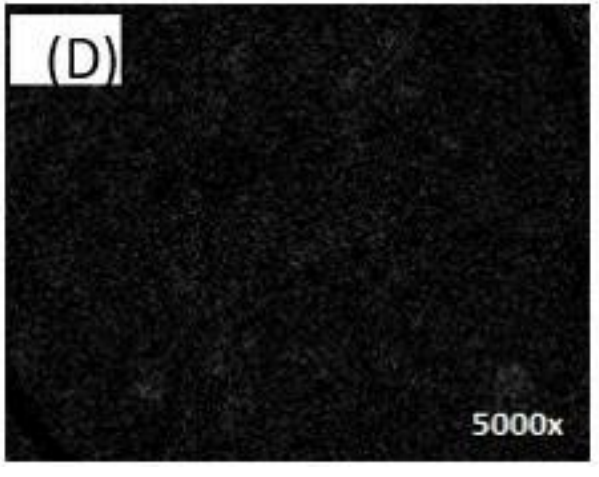
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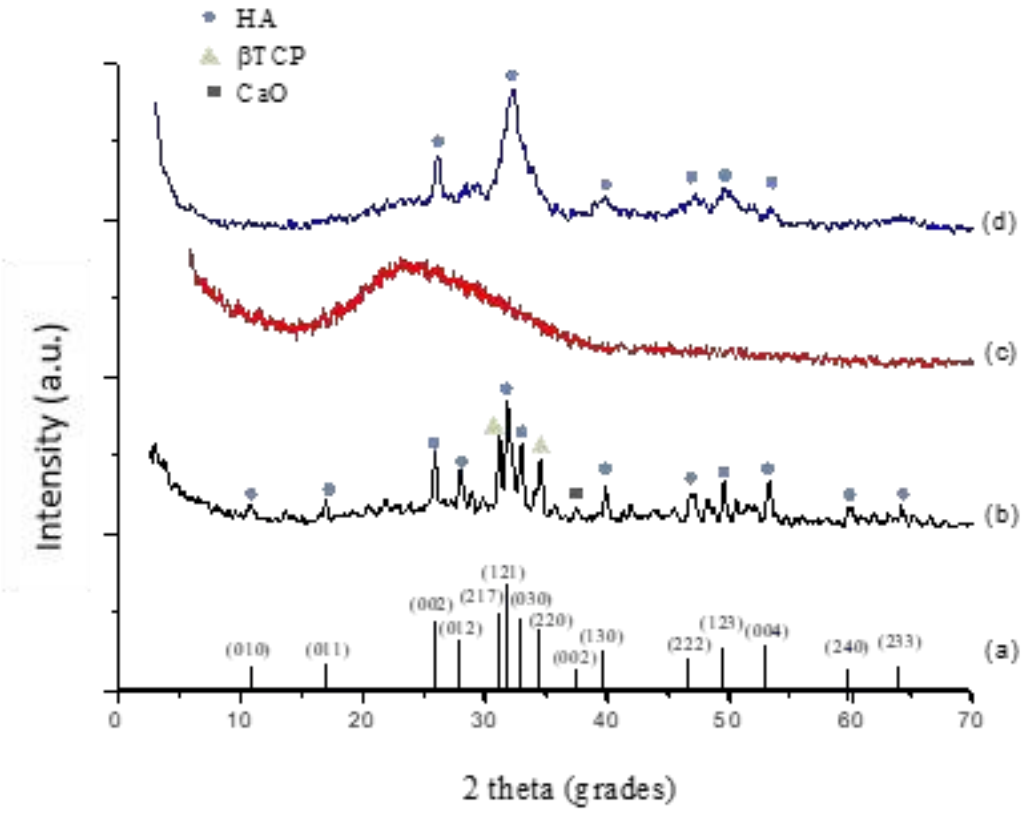
HA
βTCP
CaO
Intensity (a.u.)
(d)
(c)
(b)
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(011)
(002)
(012)
(121)
(217)
(030)
(220)
(002)
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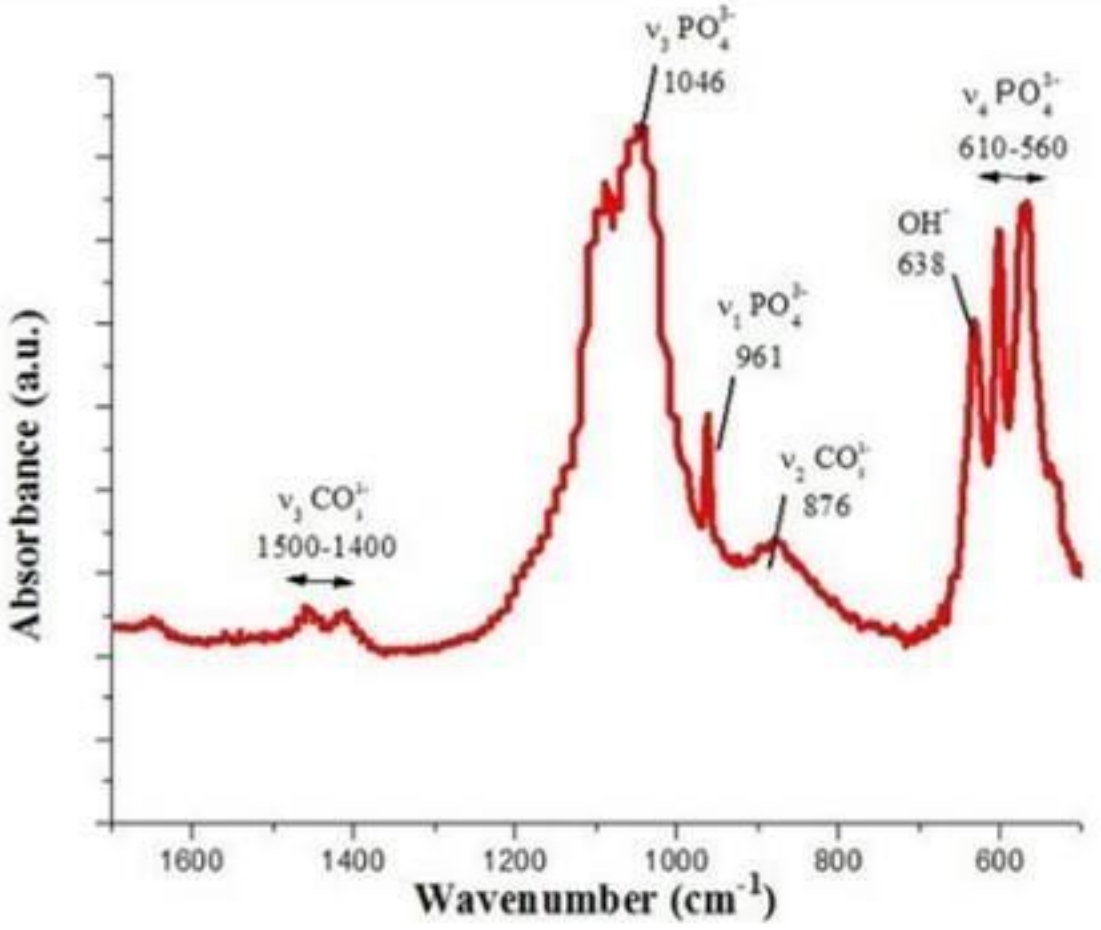
30
40
50
60
70
2 theta (grades)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$\nu_1 PO_4^{3-}$
961
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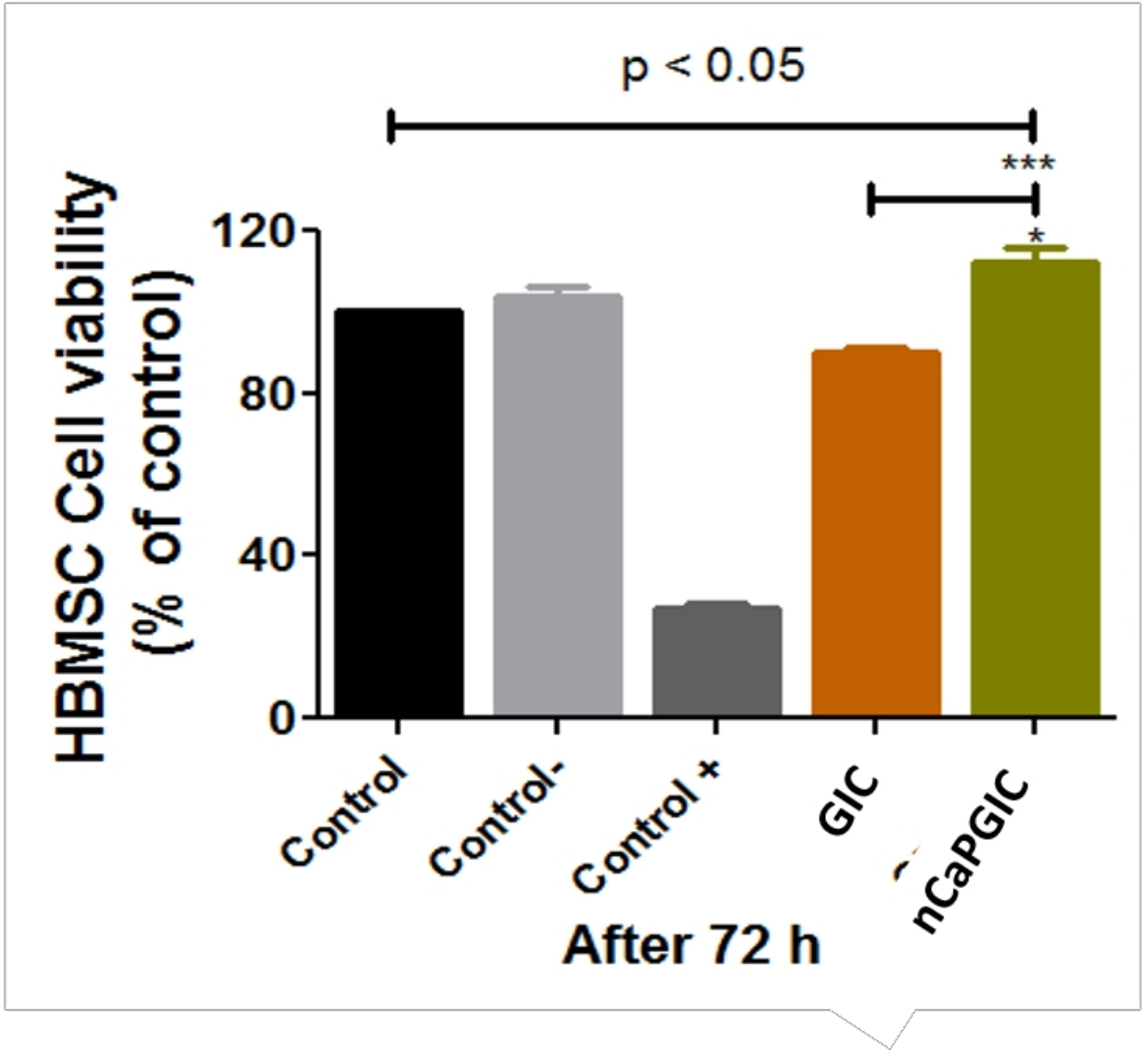
1600
1400
1200
1000
800
600
Wavenumber (cm^{-1})


p < 0.05
***
*
HBMSC Cell viability
(% of control)
120
80
40
0
Control
Control-
Control +
GIC
nCaPGIC
After 72 h

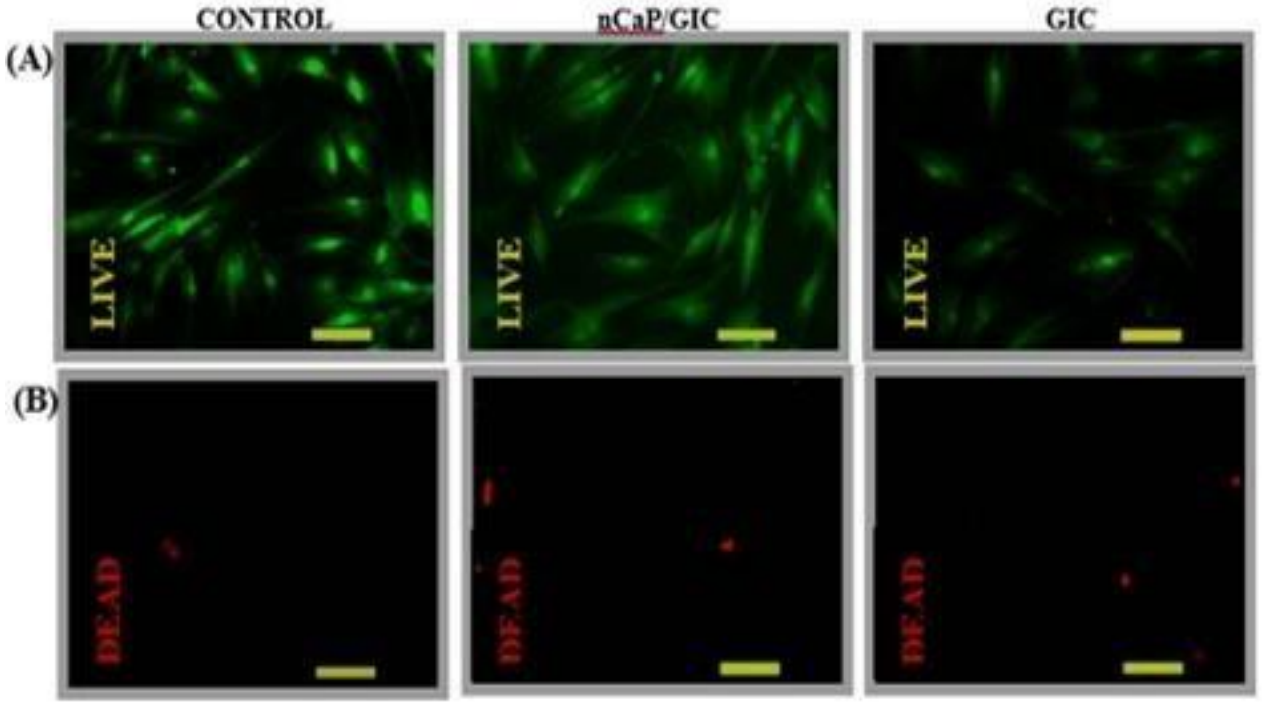

CONTROL
nCaP/GIC
GIC
(A)
LIVE
LIVE
LIVE
(B)
DEAD
DEAD
DEAD

Table 1

Specimens	CS	*P**	DTS	*P**
GIC	21,8±6	p=0,002	24,4±7	p<0,001
nCaP/GIC	39,2±4		47,1±4	

*Anova assay