PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

FACULDADE DE ODONTOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

MESTRADO EM PRÓTESE DENTÁRIA

CARLOS AUGUSTO ACCORSI RIBEIRO

ANÁLISE COMPARATIVA *IN VITRO* DA CITOTOXICIDADE DOS ÁCIDOS HIALURÔNICOS DE ALTO E BAIXO PESO MOLECULAR EM ENXERTIA ÓSSEA COM E SEM HIDROXIAPATITA E β-TRICÁLCIOFOSFATO.

Porto Alegre

2014

CARLOS AUGUSTO ACCORSI RIBEIRO

ANÁLISE COMPARATIVA *IN VITRO* DA CITOTOXICIDADE DOS ÁCIDOS HIALURÔNICOS DE ALTO E BAIXO PESO MOLECULAR EM ENXERTIA ÓSSEA COM E SEM HIDROXIAPATITA E β-TRICÁLCIOFOSFATO.

Dissertação apresentada como requisito parcial para a obtenção do título de Mestre em Odontologia, na área de Prótese Dentária, pelo Programa de Pós-Graduação da Faculdade de Odontologia, da Pontifícia Universidade Católica do Rio Grande do Sul.

Linha de Pesquisa: Técnicas e Aparelhos em Odontologia.

Orientador: Prof. Dr. Eduardo Rolim Teixeira

Porto Alegre
2014

CARLOS AUGUSTO ACCORSI RIBEIRO

ANÁLISE COMPARATIVA *IN VITRO* DA CITOTOXICIDADE DOS ÁCIDOS HIALURÔNICOS DE ALTO E BAIXO PESO MOLECULAR EM ENXERTIA ÓSSEA COM E SEM HIDROXIAPATITA E β-TRICÁLCIOFOSFATO.

Dissertação apresentada como requisito parcial para a obtenção do título de Mestre em Odontologia, na área de Prótese Dentária, pelo Programa de Pós-Graduação da Faculdade de Odontologia, da Pontifícia Universidade Católica do Rio Grande do Sul.

Linha de Pesquisa: Técnicas e Aparelhos em Odontologia.

Aprovada em 27 de março de 2014.

BANCA EXAMINADORA

Prof. José Antônio Poli de Figueiredo

Prof. Guilherme Genehr Fritscher

Prof. Marcel Fasolo de Paris

À minha esposa, Roberta Sartori, dedico esta pesquisa.

AGRADECIMENTOS

Agradeço à professora Profa. Dra. Denise Cantarelli Machado, por ceder o laboratório do IPB para a realização da pesquisa.

Agradeço especialmente ao biólogo Fágner Henrique Heldt pela incansável assistência e pela prestatividade sem fim.

À minha colega Thaísa Barbizan Bordin e a Daniel Gonçalves Boeckel, pela parceria na pesquisa, companheirismo e pelas produtivas discussões que enriqueceram o trabalho.

Ao professor Eduardo Rolim Teixeira, pela oportunidade de entrar no universo da pesquisa cientifica de base, pela orientação e pela amizade.

À CAPES, pela bolsa que permitiu a realização do mestrado.

RESUMO

A reposição de base óssea perdida é um fator de grande complexidade nas reabilitações orais envolvendo implantes osseointegrados. A engenharia tecidual tem evoluído nos últimos anos por apresentar-se como uma alternativa ao enxerto ósseo autógeno, não exigindo um sitio doador de tecido para tal. Dentre as principais técnicas e biomateriais utilizados em engenharia tecidual, o acido hialurônico (AH) apresenta-se como um composto promissor como veículo celular. Presente na matriz extracelular de todos os tecidos vivos, ele permite sua modificação estrutural, maximizando seu potencial como veículo celular em reparos ósseos. Dependendo do seu peso molecular, estudos mostram diferentes efeitos citotóxicos podem ser verificados. Desta forma, este estudo procurou avaliar, *in vitro*, a citotoxicidade do AH de alto e de baixo peso molecular, sobre células da linhagem NIH-3T3, segundo o teste padrão da norma ISO 10993-12 para analise de citotoxicidade. Para isso, um teste de MTT foi realizado com o seguinte arranjo de grupos: (G1) Células + AH de alto peso molecular; (G2) Células + AH de baixo peso molecular; (G3) Células + AH de alto peso molecular + Hidroxiapatita; (G4) Células + AH de baixo peso molecular + Hidroxiapatita; (G5) Células (controle positivo); (G6) Células + hipoclorito (controle negativo). Os resultados mostram que todos os grupos apresentam viabilidade superior ao controle negativo e inferior ao controle positivo exceto o grupo G1, onde verificou-se ausência de diferença estatística entre este e o controle positivo. Resultados preliminares apontam para o emprego do AH de alto peso molecular como compatível para veículo celular em engenharia tecidual.

Palavras-chave: Citotoxicidade. Ácido Hialurônico. Engenharia de Tecidos.

ABSTRACT

The reposition of a lost osseous structure is challenging in oral rehabilitations involving osseointegrated implants. Tissue engineering techniques aiming reposition of lost calcified tissues has evolved in the last years as an alternative to the autologous bone graft, without the need of a donor site. Among the required techniques and biomaterials, the hyaluronic acid (HA) presents itself as promising alternative for tissue engineering. HA is present in the extracellular matrix of all living tissues, and also allows modifications in its structure, serving as a carrier for several compounds utilized in bone repair. Depending on its molecular weight, studies show that different cytotoxic effects might be observed. Thus, the present study evaluated, *in vitro*, the cytotoxicity of high and low molecular weight HA on NIH-3T3 cells, following the ISO 10993-12 test standards for cytotoxicity analysis. A MTT test was conducted with the following groups: (G1) cells + high molecular weight hyaluronic acid; (G2) cells + low molecular weight hyaluronic acid; (G3) cells + high molecular weight hyaluronic acid + hydroxyapatite; (G4) cells + low molecular weight hyaluronic acid + hydroxyapatite; (G5) cells (positive control); (G6) cells+ hypochlorite (negative control). The results show that most groups presented higher cellular viability when compared to the negative control and lower than the positive control group ($p<0.05$). Group G1 presented no statistical difference when compared to the positive control (G5) ($p<0.05$), indicating that high molecular weight HA might be applicable as a cell carrier for tissue engineering.

Key-words: Cytotoxicity. Hyaluronic Acid. Tissue Engineering, Scaffold

LISTA DE TABELAS E ILUSTRAÇÕES

Tabela 1. Montagem dos grupos analisados com seus respectivos volumes de cada componente por poço.....**20**

Imagem 1. Disposição dos grupos na placa de 96 poços.....21

Tabela 2. Viabilidade celular de cada grupo, expressa em porcentagem.....24

Imagem 2. Porcentagem de viabilidade cellular de cada grupo e seu desvio padrão……..25

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

AH (HA)	Ácido Hialurônico (Hyaluronic Acid)
HMWHA	Ácido hialurônico de alto peso molecular (High Molecular Weight Hyaluronic Acid)
LMWHA	Ácido hialurônico de baixo peso molecular (Low Molecular Weight Hyaluronic Acid)
HP	Hidroxiapatita
kDa	Quilo daltons
MDa	Milhões de daltons
DMEM	Meio de Eagle modificado por *Dulbecco*
SFB	Soro fetal bovino
DPBS	Fosfato de *Dulbecco* tamponado salino
MTT	*Metil tetrazolium* 3-(4,5-dimetil-2-tiazolil)-2,5-difenil-2il-tetrazólico
β-TCP	Beta tricálcio fosfato
mg	Miligramas
ml	Mililitro
µl	Microlitros
ANOVA	Análise de variância múltipla
DMSO	Dimetil sulfóxido

Artigo escrito nas normas da revista Annals of Biomedical Engineering (http://www.springer.com/biomed/journal/10439).

SUMÁRIO

Página de título.....14

Resumo.....16

Introdução.....17

Materiais e Métodos.....19

Resultados.....23

Discussão.....25

Referências Bibliográficas.....30

Anexo A – Tables, Figures and Legends.....37

Anexo B - Alterações no Projeto de Pesquisa No. 0048/11.....41

Anexo C - Resposta Solicitação de Alteração em Projeto de Pesquisa..42

Anexo D - Orientações para publicação na revista Annals of Biomedical Engineering.....43

Anexo E – Introdução em português.....50

MTT Assay of High and low molecular weight hyaluronic acid with hydroxiapatite and β-tricalciumphosphate

Carlos Augusto Accorsi Ribeiro – Graduate Student, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil;

Thaisa Barizan Bordin – Graduate Student, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil;

Daniel Gonçalves Boeckel – Ph.D. Student, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil;

Rosemary Sadami Arai Shinkai – Professor, Post Graduate Program in Dentistry, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil;

Márcio Lima Grossi – Assistent Professor, Post Graduate Program in Dentistry, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil;

Eduardo Rolim Teixeira – Professor, Post Graduate Program in Dentistry, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil;

Corresponding address: Carlos Augusto Accorsi Ribeiro. Rua Julio de Castilhos, 403/7. Carlos Barbosa-RS-Brazil. CEP:95185-000. Phone +55(54)3461-1771. carlosribeiro_implant@hotmail.com

Declaration

This study was partially funded by the Ministry of Education and Culture of Brazil (MEC/CAPES).

MTT Assay of High and low molecular weight hyaluronic acid with hydroxiapatite and β-tricalciumphosphate

The reposition of a lost osseous structure is challenging in oral rehabilitations involving osseointegrated implants. Tissue engineering techniques aiming reposition of lost calcified tissues have evolved in the last years as an alternative to the autologous bone graft, without the need of a donor site. Among the required techniques and biomaterials, the hyaluronic acid (HA) presents itself as promising alternative for tissue engineering. HA is present in the extracellular matrix of all living tissues, and also allows modifications in its structure, serving as a carrier for several compounds utilized in bone repair. Depending on its molecular weight, studies show that different cytotoxic effects might be observed. Thus, the present study evaluated, *in vitro*, the cytotoxicity of high and low molecular weight HA on NIH-3T3 cells, following the ISO 10993-12 test standards for cytotoxicity analysis. A MTT test was conducted with the following groups: (G1) cells + high molecular weight hyaluronic acid; (G2) cells + low molecular weight hyaluronic acid; (G3) cells + high molecular weight hyaluronic acid + hydroxyapatite; (G4) cells + low molecular weight hyaluronic acid + hydroxyapatite; (G5) cells (positive control); (G6) cells+ hypochlorite (negative control). The results show that most groups presented higher cellular viability when compared to the negative control and lower than the positive control group ($p<0.05$). Group G1 presented no statistical difference when compared to the positive control (G5) ($p<0.05$), indicating that high molecular weight HA might be applicable as a cell carrier for tissue engineering.

Key-words: Cytotoxicity. Hyaluronic Acid. Tissue Engineering, Scaffold

INTRODUCTION

Dental implants have become a significant device in the treatment of patients with maxillary bone loss, by serving as a stable anchorage for dental, palate and cleft and also facial reconstructive prosthesis[1].

One of the basic requirements for implant placement is adequate bone volume[2, 3], and numerous techniques of bone grafting are nowadays available, with the autologous bone graft still being considered the one providing the best clinical results. However, limitations such as the need of a donor site, associated surgical morbidity and limited bone quantity, may sometimes impair its indication and/or restrict its results[4, 5]. Different techniques applying bone substitutes with distinct formations such as xenografts, alloplastic materials and allografts are currently available for grafting procedures, presenting mostly good clinical results[6, 7], but still presenting some limitations regarding their application[8, 9].

In the past decades, techniques involving tissue engineering, the field of medical science that combines the natural repair mechanisms of the living tissue with the principles of engineering, were significantly studied and developed to restore living tissues lost to trauma, diseases and/or natural resorption[10, 11, 12]. Tissue engineering basically combines *in vitro* cell cultures, firstly in bi-dimensional scaffolds, and later their application in tridimensional carriers with or without specific proteins for differentiation[13], forming grafting compounds that carry components to induce and/or enhance the natural tissue healing process[14, 15]. The hyaluronic acid (HA) has been suggested as a possible cell scaffold for tissue-engineered bone reconstruction[16]. HA is an organic acid present in the extracellular matrix of all living tissues[17, 18], that allows specific modifications in its molecular structure. Such modifications might be critical to lower its potential for allergenic and inflammatory

tissue reactions, as well as enhance its antibacterial and angiogenic properties[19, 20], and promote shock-absorbing features relevant during cell proliferation and differentiation[21].

Alloplastic materials such as Hydroxylapatite and β-tricalcium phosphate (HP-βTCP) have been extensively studied in the past decades and are currently being applied as bone substitutes to a wide range of different bone defects[22, 23] . HP-βTCP are ceramic biomaterials presenting well documented biocompatibility and osteoconductive properties[24, 25], promoting bone formation on its surface. Application of this type of biomaterials with cells cultured *ex vivo* for bone grafting has been suggested as a promising technique for bone regeneration[26].

Based on the controversy in the literature regarding the application of HA and its ideal molecular characteristics to act as a scaffold for tissue-engineered bone regeneration, this study evaluated the cytotoxicity of low (LMW-HA) and high molecular weight HA (HMW-HA) in cultured NIH-3T3 cells with and without HP-βTCP by the MTT (methylthiazol tetrazolium) method.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS), applying the ISO 10993-12 standards pertinent for an *in vitro* cytotoxicity analysis.

Cellular culture and sample preparation

An ampoule containing NIH-3T3 cell line was removed from liquid nitrogen and unfrozen in a water bath at 37 ºC. The cellular suspension was then transferred to a culture flask containing *Dulbecco's modified Eagle's medium* (DMEM) supplemented with 10% of bovine fetal serum (BFS) and 1% penicillin/streptomycin. When semi confluence of 70% was achieved, the cells were washed with *Dubelcco's phospate tamponated based saline* (DPBS), trypsinized and ressuspended in 1ml of DMEM. They were colored with trypan blue and counted in a Neubauer chamber. Then, the cells were cultivated in a 96-well plate (TPP, St. Louis, MO, EUA) in a density of 0,5 x 10^5 in 200 µl DMEM in each well. One plate for each evaluated period, meaning 24, 48, 72 and 96 hours was used. These plates were kept in an atmosphere at 37°C and with 5% CO_2 for 24 hours. After 24 hours, the medium was removed and the wells were washed with DPBS and, following the results of a pilot HA concentration study, control and test groups were assembled. Each group for each evaluated period was tested in quadruplicate. The plates were then returned to the same mentioned atmosphere (37°C and with 5% CO_2), where they remained until the MTT (Across Organics, New Jersey, EUA) viability test was performed. Each tested group (Table 1) along with both positive and negative control groups were run in quadruplicate for MTT assay.

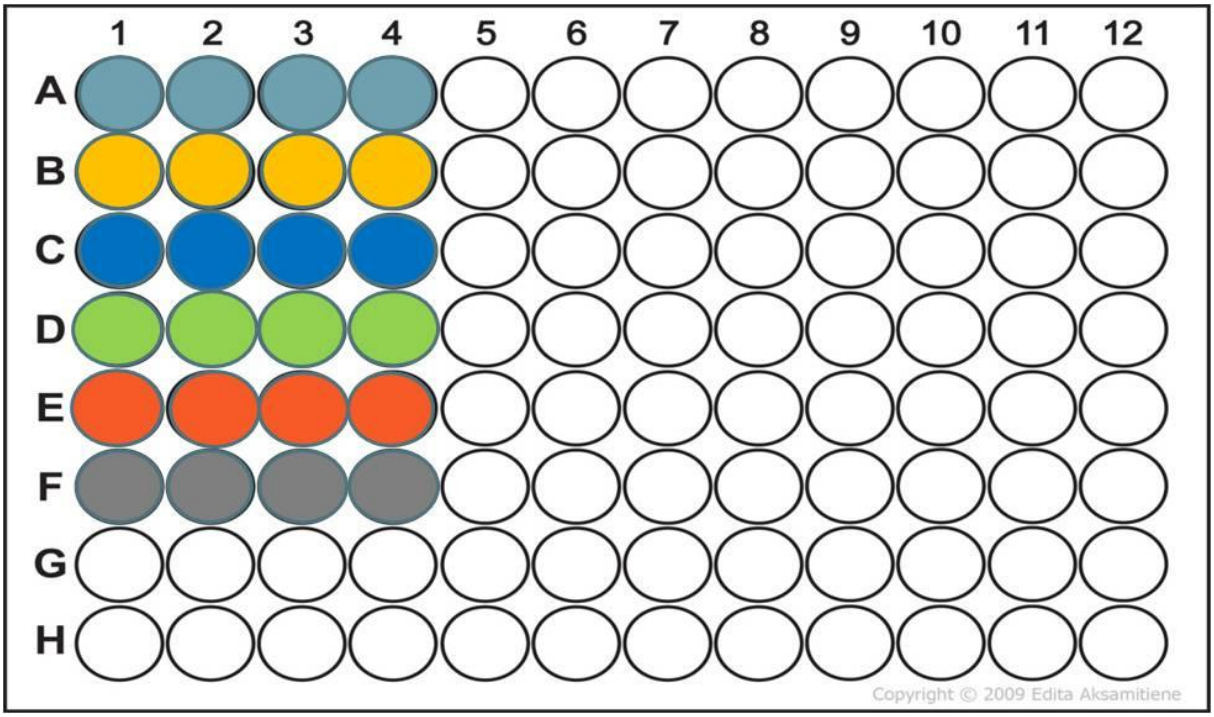
Table 1. Test and control groups and their respective components.

Group	Cellular density	DMEM	HMW-HA	LMW-HA	HP-βTCP	Hypochlorite
G1	$0,5x10^5$	184µl	16µl	------	------	------
G2	$0,5x10^5$	196µL	------	4µl	------	------
G3	$0,5x10^5$	184µl	16µl	------	4mg	------
G4	$0,5x10^5$	196µl	------	4µl	4mg	------
G5	$0,5x10^5$	200µl	------	------	------	------
G6	$0,5x10^5$	------	------	------	------	200µl

DMEM (*Dulbecco's modified Eagle's medium);* **HMW-HA** *(High Molecular Weight Hyaluronic Acid);* **LMW-HA** (Low Molecular Weight *Hyaluronic Acid);* **HP-βTCP** (Hydroxyapatite and β-tricalcium phosphate).

The disposition of each group in the 96 well plate is demonstrated bellow:

Image 1.Disposition of treatment group (in quadruplicate) in the 96 well plate



A = (G1) - NIH-3T3 cells and HMW-HA, **B = (G2)** - NIH-3T3 cells and LMW-HA; **C = (G3)** - NIH-3T3 cells, HMW-HAHP-βTCP; **D = (G4)** - NIH-3T3 cells, LMW-HA and HP-βTCP; **E= (G5)** - NIH-3T3 cells (Positive control); **F= (G6)** - NIH-3T3 cells and Hypochlorite (Negative control).

Application of High Molecular Weight Hyaluronic Acid

A total of 16µl of HMW-HA (molecular weight of 1000kDa) gel (Teosyal 30G Touch Up®, Teoxane, Geneve, Switzerland), containing 25mg/mL of HA in a pH of 7.3 was used in each culture well of groups G1 and G3. The applied HA concentration was determined based on the results of a pilot concentration test.

In 16µl of Teosyal a final concentration of 0,4mg/mL of HA was obtained and applied per well.

Application of Low Molecular Weight Hyaluronic Acid

A total of 4µl of LMW-HA (molecular weight of 500-730kDa) gel (Hyaloss®, Anika Therapeutics, Reggio Emilia, Italy), containing 25mg/mL of HA in a pH of 7.3 was used in each well in groups G2 and G4. HA concentration was also determined based on the results of a pilot concentration test.

In 4µl of Hyaloss a final concentration of 0,1mg/mL of HA was obtained and applied in each well.

Application of Hydroxiapatite and β-tricalcium phosphate (HP-βTCP)

A total of 4mg of HP-βTCP (Straumann® BoneCeramic™, Biora AB, Institute Straumann AG, Malmo, Sweden), a synthetic bone substitute with 60% hydroxyapatite and 40% β-tricalcium phosphate was previously weighted and later added to the compounds of groups G3 and G4.

Application of Sodium Hypoclorite

A total of 200µl of a 1% Sodium Hypochlorite solution was used as medium to establish the negative control in group G6.

Cell Viability Assay (MTT)

The MTT assay was conducted aiming to determine the ability of the cells to reduce 3-(4.5-dimethyl-2-thiazole)-2.5-diphenyl-2-2yl-tetrazolium bromide (Across Organics, New Jersey, USA) in insoluble violet *formazan* crystals.

After each cell group establishment, a 10% MTT solution (5mg/mL) diluted in PBS was added to each well. The cultures were immediately incubated for 2 hours at 37ºC and protected from light until the presence of the violet *formazan* crystals was observed. For *formazan* crystals solubilization, 100µl of DMSO (Dimethyl Sulfoxide) was added to each well. Later, the absorbance at a wavelength of 570nm (Microplate Reader, Molecular Devices, SpectraMax® M 5, USA) was recorded and analyzed using the Softmax Pro 5.2® program.

Statistical analysis

All obtained results were expressed as mean ± standard deviation (SD). A comparative analysis of the means was performed using the *student-T* test and Analysis of Variance (*ANOVA*). Also, a *Tuckey* Multiple Comparison test was performed. The significance level was set at $p < 0,05$.

RESULTS

Results of the MTT assay of each tested group were compared to the positive control (G5), which was considered 100% viable for analysis (Table 2 and Figure 1).

All experimental groups showed significantly higher cell viability compared to the negative control group (G6) ($p<0,05$). Group 1 was the only group presenting no

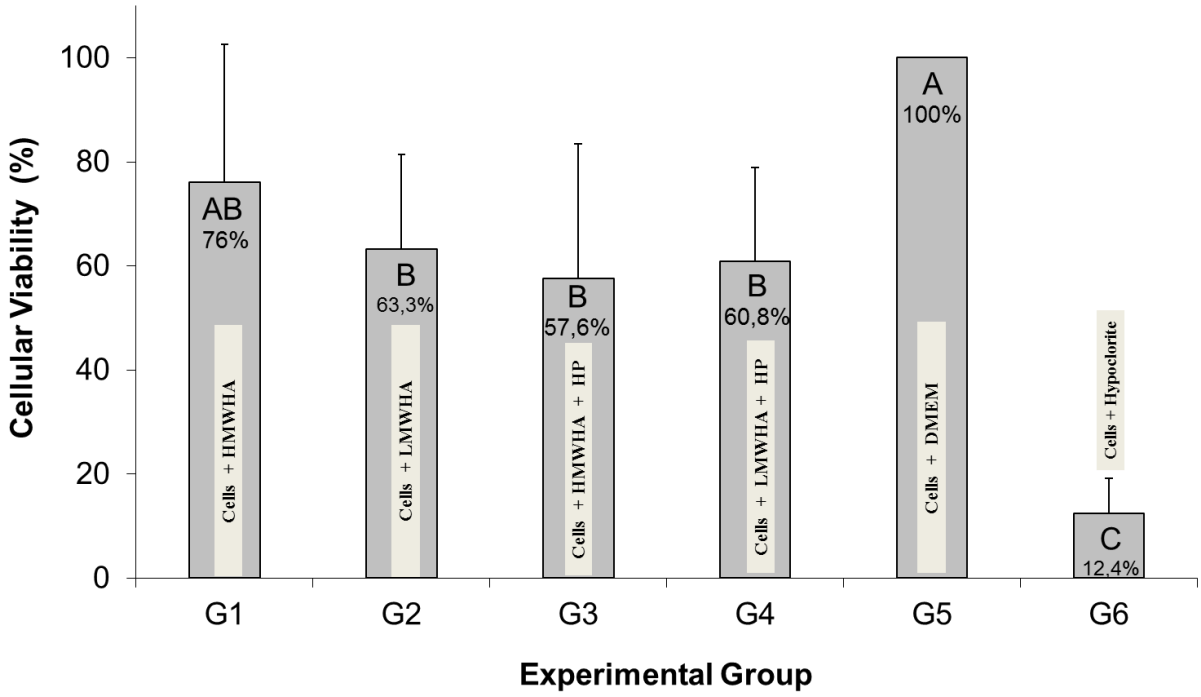
statistical differences to the positive control (G5) (p<0,05). All other tested groups presented significantly lower percentages of cell viability compared to the positive control group (G5) (p>0,05).

Table 2. Results of the MTT assay for each evaluated group.

	N	Mean	St.d Deviation	Std. Error	95% Confidence Interval		Minimum	Maximum
					Lower Bound	Upper Bound		
G1	16	1,45444	0,530858	0,132715	1,17156	1,73731	0,501	2,340
G2	16	1,19296	0,338835	0,84709	1,0111	1,37261	0,506	1,822
G3	16	1,08613	0,478396	0,119599	0,83121	1,34104	0,82	2,055
G4	16	1,14600	0,357528	0,89382	0,95549	1,33651	0,643	2,060
G5	16	1,90506	0,309629	0,77407	1,74007	2,07005	1,225	2,313
total	80	1,35674	0,503370	0,56278	1,24472	1,46876	0,082	2,340

Image 2. Percentage of cell viability and their standard deviation of each evaluated group. Different letters indicate statistical difference between groups

(p<0,05).



The HMW-HA group alone (G1) presented the highest percentage of cell viability (76%), while the remaining groups presented cellular viability percentages of 57,6% (G3), 60,8% (G4) and 63,3% (G2), differing statistically from the control group (G5). (p> 0,05)

A comparison between test groups suggested that the presence of HP-βTCP had no significant influence in cell viability. (p> 0,05)

DISCUSSION

The present *in vitro* study evaluated the cytotoxicity of high and low molecular weight HA with and without the presence of an inorganic ceramic substratum.

Results indicated statistical differences between most experimental and control groups, showing significantly lower percentages of cell proliferation for groups G2 (63,3%), G3 (57,6%) and G4 (60,8%) in comparison to the positive control group

(p<0,05), which was statistically similar to group G1 (p>0,05). These results suggested that, even though most experimental groups presented relevant percentages of cell proliferation, the HMWHA might provide better conditions for cell proliferation when acting as a carrier for tissue-engineered compounds.

Both LMWHA and HMWHA have been suggested to play different roles in the tissue wound healing process. While the first stimulates mostly macrophage phagocytosis, the later, having a greater presence in the wounded site, acts in the cell signaling mechanisms and thus might promote cell differentiation. It has been said that LMWHA and HMWHA might regulate human fibrocyte differentiation differently in wound repair[27].

The present findings go in accordance with Takeda et al.[28], which applied HMWHA as a scaffold for Brain-Derived Neurotrophic Factor in an animal model aiming periodontal tissue regeneration. They suggested that the application of HMWHA might increase the number of human periodontal ligament adherent cells and stimulate their proliferation, concluding that its properties could be advantageous for clinical applications involving periodontal ligament regeneration. Also, the use of HMWHA *in vivo* has been associated to the promotion of fibroblast proliferation, along with its molecular degradation in living tissues pointed to promote local angiogenic reactions[29, 30] and an anti-inflammatory effect as well[31].

In contrast to the present findings, other studies emphasized the possible benefits of the application of LMWHA as cell carrier for tissue engineered compounds based on its specific structural properties. Kim et al[32] applied LMWHA as cultured-cell carrier and justified as being highly hygroscopic and present viscous properties that might increase its solubility *in vivo* and diminish the ultimate viscosity of the final compound, thus resulting in a graft with easier clinical handling properties.

Results of our investigation indicated a 76% cell viability for the HMWHA group. The present results might be comparable to those of Kim et al[33], who presented a 72% of cell viability applying a LMWHA scaffold. Nevertheless, in their study, test groups receiving the addition of BMP-2 and also a synthetic PEG-tetra thiols as a HA cross-linker had their viability percentages enhanced to 81%. This cross-linker is believed to add resistance to the HA molecule against hyaluronidase degradation. Noteworthy, in a recent study, the same group of researchers applied a HMWHA based scaffold, and pointed that it might benefit tissue regeneration by reproducing the extracellular matrix biological degrability, achieving a cellular viability of +90%[30].

Concentrations of both HMWHA and LMWHA for the present investigation were established as 0,4mg/mL and 0,1 mg/mL, respectively, based on a concentration curve test. In a study by Huang et al[34], HA of different concentrations (0,5mg/ml, 1mg/ml, 2mg/ml) and different molecular weights (60 kDa, 900 kDa e 2300 kDa) were tested. From the obtained results, they concluded that the *in* vivo effects of HA on cell cultures are directly linked to the HA concentration, composition and molecular characteristics. In the present investigation, a cell viability of 76% obtained in the HMWHA group was considered applicable for future *in vivo* studies and thus justified the applied HA concentration.

The cell viability results obtained in groups 2 to 4 were significantly lower than the control group. This result might be associated with the anti-adhesive properties suggested for both LMWHA and HMWHA when in combination with other products[35]. It has been previously mentioned also that these HA anti-adhesive properties, derived from HA chemical composition and production, might negatively affect cell adhesion and proliferation[36]. In the present study, differences in cell viability between

groups 1 and 3 (HMWHA with and without HP-βTCP) could be explained based on this hypothesis, meaning an induced alteration of some HA properties and/or local pH-value in the presence of HP-βTCP that might negatively affected cell viability.

The synthetic bone substitute (HP-βTCP) was applied in groups 3 and 4 aiming volumetric and consistency improvement of the future bone grafting compound, and possibly an extension of the total substrate area for cellular adhesion and growth. It has been said that the osteoconductive properties combined with a bioactive surface present on the HP-βTCP crystals may positively influence osteoblastic cell behavior[37], though other authors refer a possible citotoxicity linked to increased pH-value derived from βTCP dissolution in organic fluids[38]. Results from the present investigation failed to demonstrate a negative correspondence between cell viability at early culture stages and the application of HP-βTCP.

The proposed methodology followed the ISO 10993-12 standards, which details specifies requirements on the procedures to be followed in the preparation of samples for medical device testing in biological systems. Application of NIH-3T3 fibroblastic cell lineage for the present investigation, along with the test sample preparation and selection of experimental controls followed the described protocols[39]. Also, the MTT assay used here has been characterized as a fast, inexpensive and practical test for citotoxicity analysis[40,41].

The results of this investigation suggest that HMWHA might be applicable as a cell scaffold for tissue engineering. The HA physiochemical properties and biological functions indicate a promising potential as a long-acting delivery mechanism for specific drugs and/or proteins at the grafted site[42], along with the possibility of

changes in the HA molecular structure to improve its properties to fit a specific application purpose[43].

Future *in vitro* and *in vivo* studies are needed to investigate the behavior of the HA-based graft compound concerning its ideal molecular weight, acid concentration, its possible degradation in presence of hyaluronidase in living tissues, and possible interactions with the degradation subproducts derived from combined materials used within the graft compound.

REFERENCES

1.Kim, D.D., Ghali, G.E, Dental implants in oral cancer reconstruction. Oral Maxillofacial Surg Clin N Am 23 (2011) 337–345

2.Aksu AE, Rubin JP, Dudas JR, Marra KG. Role of gender and anatomical region on induction of osteogenic differentiation of human adipose-derived stem cells. Ann Plast Surg. 2008 Mar;60(3):306-22.

3.Anitua E. Plasma rich in growth factors: preliminary results of use in the preparation of future sites for implants. Int J Oral Maxillofac Implants. 1999 Jul-Aug;14(4):529-35.

4.Brugnami, F., Caiazzo, A., Leone, C. Local intraoral autologous bone harvesting for dental implant treatment: alternative sources and criteria of choice. Keio J Med 2009; 58 (1):24－28

5.Cordaro, L., Torsello, F., Accorsi Ribeiro, C., Liberatore, M., Mirisola di Torresanto, V. Inlay-onlay grafting for three-dimensional reconstruction of the posterior atrophic maxilla with mandibular bone. Int J Oral Maxillof Surg; 39(4):350-7.

6.Nevins, M., Camelo,M., De Angelis, N. Hanratty, J.J., Khang, W.G., Kwon, J., Rasperini, G., Rocchietta, I., Schupbach, P., Kim, D.M. The clinical and histologic efficacy of xenograft granules for maxillary sinus floor augmentation. The International Journal of Periodontics & Restorative Dentistry,Volume 31, Number 3, 2011

7.Figueiredo, A., Coimbra, P., Cabrita, A., Guerra, F., Figueiredo, M. Comparison of a xenogeneic and an alloplastic material used in dental implants in terms of

physico-chemical characteristics and in vivo inflammatory response. Materials Science and Engineering C 33 (2013) 3506–3513

8.Gruskin, E., Doll, B. A., Futrell, W., Schmitz, J. P., Hollinger, J. O.Demineralized bone matrix in bone repair: History and use. Advanced Drug Delivery Reviews 64 (2012) 1063–1077

9. Mah, J., Hung, J., Wang, J., Salih, E. The efficacy of various alloplastic bone grafts on the healing of rat calvary defects, Eur. J. Orthod 26 (2004) 475-482.

10.Arosarena, O.Tissue engineering. Curr Opin Otolaryngol Head Neck Surg 2005, 13:233—241 9.Spagnoli, D.B., Marx, R.E. Dental Implants and the Use of rhBMP-2. Oral Maxillofacial Surg Clin N Am 23 (2011) 347–361

11.Spagnoli, D.B., Marx, R.E. Dental Implants and the Use of rhBMP-2. Oral Maxillofacial Surg Clin N Am 23 (2011) 347–361

12.Bose, S.; Roy, M.; Bandyopadhyay, A. Recent advances in bone tissue engineering scaffolds Trends biotechnol (2012) 30: 546 - 554).

13.Balazs EA, Bland PA, Denlinger JL, Goldman AI, Larsen NE, Leshchiner EA, Leshchiner A, Morales B. Matrix engineering. Blood Coagul Fibrinolysis. 1991 Feb;2(1):173-8.

14.Wang,W. J. H., van den Beucken, J. J.J.P., Yang, F., Walboomers, X. F., Leeuwenburgh, S., Jansen, J. A. Local delivery of small and large biomolecules in craniomaxillofacial bone. Advanced Drug Delivery Reviews 64 (2012) 1152–1164

15.Chang, P-C., Seol, Y-J., Cirelli, JA., Pellegrini, G., Jin, Q., Franco LM., Goldstein, SA., Chandler, LA., Sosnowski, B., Giannobile, WV. PDGF-B gene therapy accelerates bone engineering and oral implant osseointegration. Gene Therapy (2010) 17, 95–104.

16. Akizuki T, Oda S, Komaki M, Tsuchioka H, Kawakatsu N, Kikuchi A, Yamato M, Okano T, Ishikawa I. Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs. J Periodont Res 2005; 40: 245–251.

17. Laurent TC, Laurent UB, Fraser JR. Functions of hyaluronan. Ann Rheum Dis 1995;54:429–432

18. Allison DD, Grande-Allen KJ. Review. Hyaluronan: A powerful tissue engineering tool. Tissue Eng 2006;12:2131–2140

19. Hunt DR, Jovanovic SA, Wikesjö UME, Wozney JM, Bernard GW. Hyaluronan supports recombinant human bone morphogenetic protein-2 induced bone reconstruction of advanced alveolar ridge defects in dogs. A pilot study. J Periodontol 2001;72:651-658.

20. Creuzet C, Kadi S, Rinaudo M, Auzély-Velty R. New associative systems based on alkulated hyaluronic acid. Synthesis and aqueous solution properties. Polymer. 2006;47:2706-2713

21. Boeckel DG, Shinkai RSA, Grossi ML, Teixeira ER. In vitro evaluation of citotoxicity of hyaluronic acid as na extracelular matrix on OFCOL II cells by the MTT assay. Oral Surg Oral Med Oral Pathol Oral Radiol 2012 Nov 9. pii: S2212-4403(12)01016-4. doi: 10.1016/j.oooo.2012.07.486. [Epub ahead of print].

22. Dahlin C, Johansson A. Iliac crest autogenous bone graft versus alloplastic graft and guided bone regeneration in the reconstruction of atrophic maxillae: a 5-year retrospective study on cost-effectiveness and clinical outcome. Clin Implant Dent Relat Res. (2011);13(4):305-10.

23. Garlini G, Redemagni M, Donini M, Maiorana C. Maxillary sinus elevation with an alloplastic material and implants: 11 years of clinical and radiologic follow-up. J Oral Maxillofac Surg. 2010 May;68(5):1152-7.

24. Cordaro L, Bosshardt DD, Palattella P, Rao W, Serino G, Chiapasco M. Maxillary sinus grafting with Bio-Osss or Straumanns Bone Ceramic: histomorphometric results from a randomized controlled multicenter clinical trial. Clin. Oral Impl. Res. 19, 2008; 796–803

25. Covani U, Orlando B, Giacomelli L, Cornelini R, Barone A. Implant survival after sinus elevation with Straumann BoneCeramic in clinical practice: ad-interim results of a prospective study at a 15-month follow-up. Clin. Oral Impl. Res. 22, 2011; 481–484

26. Ueda M , Yamada Y , Ozawa R , Okazaki Y . Clinical case reports of injectable tissue-engineered bone for alveolar augmentation with simultaneous implant placement . Int J Periodontics Restorative Dent . 2005;25:129–137.

27. Maharjan AS, Pilling D, Gomer RH. High and low molecular weight hyaluronic acid differentially regulate human fibrocyte differentiation. PLoS One. 2011;6(10):e26078. Epub 2011 Oct 11.

28. Takeda, K., Sakai, N., Shiba, H., Nagahara, T., Fujita, T., Kajiya, M., Iwata, T., Matsuda, S., Kawahara, K., Kawaguchi, H., Kurihara, H. Characteristics of High-Molecular-Weight Hyaluronic Acid as a Brain-Derived Neurotrophic Factor Scaffold in Periodontal Tissue Regeneration TISSUE ENGINEERING: Part A Volume 17, Numbers 7 and 8, 2011

29. Price, R.D., Berry, M.G., Navsaria, H. A. Hyaluronic acid: the scientific and clinical evidence. Journal of Plastic, Reconstructive & Aesthetic Surgery (2007) 60, 1110e1119

30.Kim J, Kim IS, Cho TH, Kim HC, Yoon SJ, Choi J, Park Y, Sun K, Hwang SJ. In vivo evaluation of MMP sensitive high-molecular weight HA-based hydrogels for bone tissue engineering J Biomed Mater Res A. 2010 Dec 1;95(3):673-81.

31.Kato, Y., Nakamura, S., and Nishimura, M. Beneficial actions of hyaluronan (HA) on arthritic joints: effects of molecular weight of HA on elasticity of cartilage matrix. Biorheology 43, 347, 2006

32.Kim J, Park Y, Tae G, Lee KB, Hwang CM, Hwang SJ, Kim IS, Noh I, Sun K. Characterization of low-molecular-weight hyaluronic acid-based hydrogel and differential stem cell responses in the hydrogel microenvironments. J Biomed Mater Res A. 2009 Mar 15;88(4):967-75. doi: 10.1002/jbm.a.31947.

33.Kim J, Kim IS, Cho TH, Lee KB, Hwang SJ, Tae G, et al. Bone regeneration using hyaluronic acid-based hydrogel with bone morphogenic protein-2 and human mesenchymal stem cells. Biomater 2007; 28:1830-1837

34.Huang L, Cheng YY, Koo PL, Lee KM, Qin L, Cheng JC, Kumta SM. The effect of hyaluronan on osteoblast proliferation and differentiation in rat calvarial-derived cell cultures. J Biomed Mater Res A. 2003 Sep 15;66(4):880-4

35.Park YD, Tirelli N, Hubbell JA. Photopolymerized Hyaluronic Acid-Based Hydrogels and InterPenetrating Networks. Biomaterials. 2003 Mar; 24(6):893-900.

36.Nuttelman CR, Benoit DS, Tripodi MC, Anseth KS. The effect of thylene glycol methacrylate phosphate in PEG hydrogels on mineralization and viability of encapsulated hMSCs. Biomaterials 2006;27(8):1377–86

37. Manferdini C, Guarino V, Zini N, Raucci MG, Ferrari A, Grassi F, et al. Mineralization behavior with mesenchymal stromal cells in a biomimetic hyaluronic acid-based scaffold. Biomaterials. 2010 May;31(14):3986-96. doi: 10.1016/j.biomaterials.2010.01.148. Epub 2010 Feb 20

38. Zimmermann CE, Gierloff M, Hedderich J, Açil Y, Wiltfang J, Terheyden H. Biocompatibility of bone graft substitutes: effects on survival and proliferation of porcine multilineage stem cells in vitro. Folia Morphol (Warsz). 2011 Aug;70(3):154-60.

39. Retamoso, L.B., Luz, T.B., Marinowic, D. R., Machado, D.C., De Menezes, L.M., Freitas, M.P.M., Oshima, H.S.M. Cytotoxicity of esthetic, metallic, and nickel-free orthodontic brackets: Cellular behavior and viability. American Journal of Orthodontics & Dentofacial Orthopedics Volume 142, Issue 1 , Pages 70-74, July 2012.

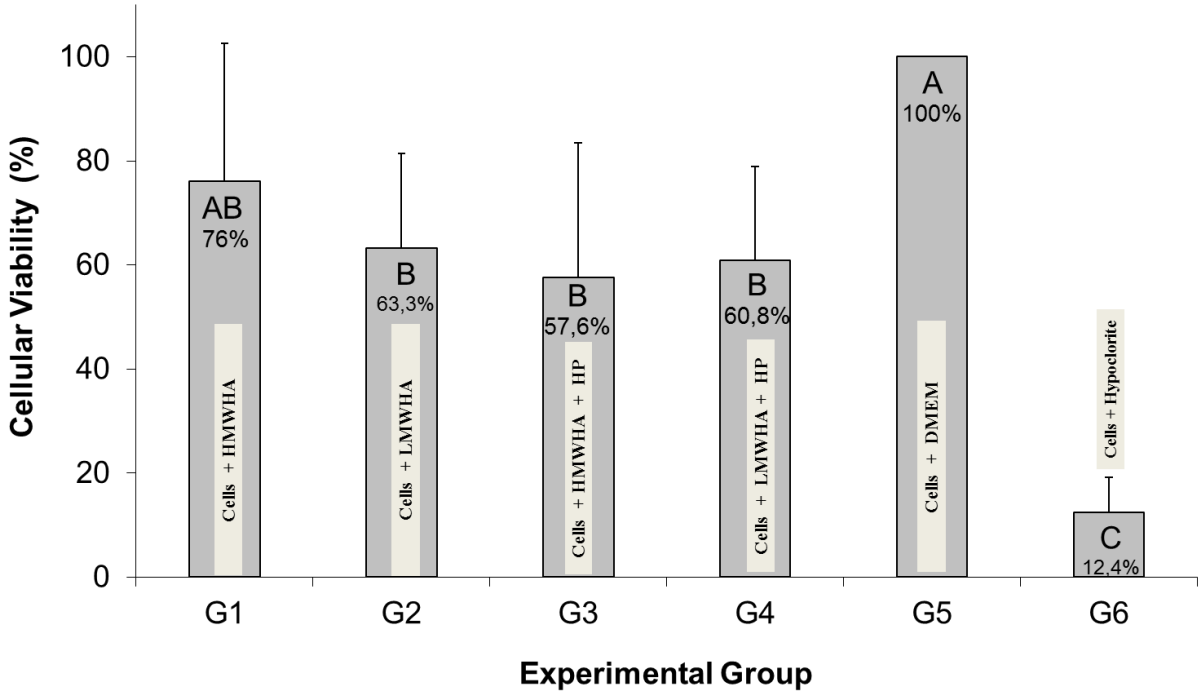
40. Riss TL, Moravec RA, Niles AL, Benink HA, Worzella TJ, Minor L. authors; Minor L, editor. Cell Viability Assays. 2013 May 1. In: Sittampalam GS, Gal-Edd N, Arkin M, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences. Available from: http://www.ncbi.nlm.nih.gov/books/NBK144065/

41. Wan H, Williams R, Doherty P, Williams DF. A study of the reproducibility of the MTT test. Journal Materials Science: Materials in Medicine 1994;5:154-159.

42. C.E. Schanté, Zubera, G., Herlinb, C., Vandammea, T.F. Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications Carbohydrate Polymers 85 (2011) 469–489.

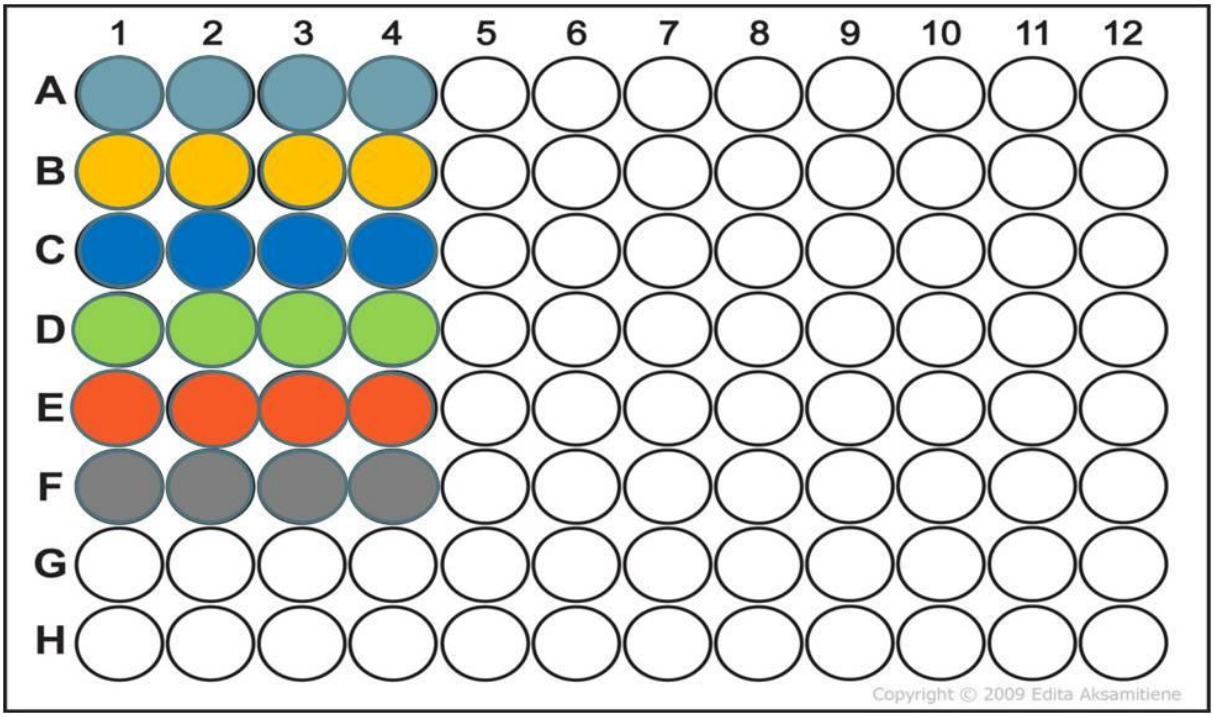
43. Oh EJ, Park K, Kim KS, Kim J, Yang JA, Kong JH, Lee MY, Hoffman AS, Hahn SK. Target specific and long-acting delivery of protein, peptide, and nucleotide therapeutics using hyaluronic acid derivatives. J Control Release. 2010 Jan 4;141(1):2-12. doi: 0.1016/j.jconrel.2009.09.010. Epub 2009 Sep 13.

ANEXO A - TABLES, FIGURE LEGENDS



Graphic 1. Cellular viability when compared to Positive Control. (G1) - NIH-3T3 cells and HMW-HA, (G2) - NIH-3T3 cells and LMW-HA; (G3) - NIH-3T3 cells, HMW-HAHP-βTCP; (G4) - NIH-3T3 cells, LMW-HA and HP-βTCP; (G6) - NIH-3T3 cells and Hypochlorite (Negative control).

Image 2.Disposition of treatment group (in quadruplicate) in the 96 well plate



A = (G1) - NIH-3T3 cells and HMW-HA, **B = (G2)** - NIH-3T3 cells and LMW-HA; **C = (G3)** - NIH-3T3 cells, HMW-HAHP-βTCP; **D = (G4)** - NIH-3T3 cells, LMW-HA and HP-βTCP; **E= (G5)** - NIH-3T3 cells (Positive control); **F= (G6)** - NIH-3T3 cells and Hypochlorite (Negative control).

Table 1. Treatments and respective volume per well.

Group	Cellular density	DMEM	HMW-HA	LMW-HA	HP-βTCP	Hypochlorite
G1	$0,5x10^5$	184µl	16µl	------	------	------
G2	$0,5x10^5$	196µL	------	4µl	------	------
G3	$0,5x10^5$	184µl	16µl	------	4mg	------
G4	$0,5x10^5$	196µl	------	4µl	4mg	------
G5	$0,5x10^5$	200µl	------	------	------	------
G6	$0,5x10^5$	------	------	------	------	200µl

Table 1. Table 1. Treatments and respective volume used per well.

DMEM (*Dulbecco's modified Eagle's medium);* **HMW-HA** *(High Molecular Weight Hyaluronic Acid);* **LMW-HA** (Low Molecular Weight *Hyaluronic Acid);* **HP-βTCP** (Hydroxyapatite and β-tricalcium phosphate). **(G1)** - NIH-3T3 cells and HMW-HA, **(G2)** - NIH-3T3 cells and LMW-HA; **(G3)** - NIH-3T3 cells, HMW-HAHP-BTCP; **(G4)** - NIH-3T3 CELLS, LMW-HA and HP-BTCP; **(G5)** NIH-3T3 CELLS (POSITIVE CONTROL); **(G6)** - NIH-3T3 cells and Hypochlorite (Negative control).

Table 2. Results of the MTT assay for each evaluated group.

	N	Mean	St.d Deviation	Std. Error	95% Confidence Interval		Minimum	Maximum
					Lower Bound	Upper Bound		
G1	16	1,45444	0,530858	0,132715	1,17156	1,73731	0,501	2,340
G2	16	1,19296	0,338835	0,84709	1,0111	1,37261	0,506	1,822
G3	16	1,08613	0,478396	0,119599	0,83121	1,34104	0,82	2,055
G4	16	1,14600	0,357528	0,89382	0,95549	1,33651	0,643	2,060
G5	16	1,90506	0,309629	0,77407	1,74007	2,07005	1,225	2,313
total	80	1,35674	0,503370	0,56278	1,24472	1,46876	0,082	2,340

ANEXO B - Alterações no Projeto de Pesquisa No. 0048/11

Porto Alegre, 28 de Setembro de 2012

Profa. Ana Maria Spohr
Comissão Científica e de Ética – CCE
Faculdade de Odontologia
Pontifícia Universidade Católica do Rio Grande do Sul – PUCRS

Ref.: Alterações no Projeto de Pesquisa No. 0048/11

Prezada Coordenadora:

Por meio desta, venho solicitar o registro das seguintes alterações no projeto de pesquisa aprovado segundo protocolo 0048/11, entitulado "O Ácido Hialurônico como Veículo de Células Osteoprogenitoras para Enxertia Óssea":

- Substituição do pesquisador associado Sabrina Rebollo Zani pelos pesquisadores associados Carlos Augusto Accorsi Ribeiro e Thaisa Barizan Bordin.

- Acréscimo de outro grupo experimental aos já existentes contendo ácido hialurônico de baixo peso molecular (Hyaloss™, Fidia Farmaceiutici S.p.A., Itália).

Saliento que a inclusão deste grupo experimental não acarretará na alteração e/ou acréscimo do número de animais previstos para uso no descrito experimento.

Cordialmente,

Prof. Eduardo Rolim Teixeira
Pesquisador Responsável
Depto. de Prótese Dentária – FO/PUCRS

ANEXO C – Resposta Solicitação de Alteração em Projeto de Pesquisa

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
Comissão de Ética no Uso de Animais

Ofício 141/12 CEUA/PRPPG

Porto Alegre, 22 de novembro de 2012.

Prezado Professor,

Em resposta a sua solicitação de alteração no projeto sob nº 11/00253 da CEUA, intitulado "Avaliação do potencial citotóxico do ácido hialurônico sobre células osteoprogenitoras diferenciadas a partir de células-tronco mesenquimais", informamos que o mesmo foi alterado. Portanto a continuidade de sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora da CEUA – PUCRS

Ilmo. Sr.
Prof. Dr. Eduardo Rolim Teixeira
FO
Nesta Universidade

PUCRS
Campus Central
Av. Ipiranga, 6690 – Prédio 60, sala 314
CEP: 90610-000
Fone/Fax: (51) 3320-3345
E-mail: ceua@pucrs.br

ANEXO D - Orientações para publicação na revista Annals of Biomedical Engineering

Manuscript Size and Format

Manuscripts that do not meet our author guidelines will be rejected without review.

Manuscripts must not exceed approximately 20 double-spaced pages, including references but not figures or tables. Upload the manuscript in a single MS Word file. The manuscript must have 1” margins and 12 point Times New Roman or Arial font. Authors should limit figures to a manageable number (6-8 should suffice). All text must be double-spaced, including, footnotes, references, legends, and tables. Tables and figures must be included at the end of the manuscript, not in the body text, and be referred to in the manuscript in a sequential manner.

Pages should be numbered consecutively beginning with the title page.

Each section should be clearly labeled. Pages must be arranged, and labeled, as follows:

- 1. Title page

  2. Abstract and key terms

     3. Introduction

     4. Materials and Methods

     5. Results

     6. Discussion

     7. Acknowledgments

     8. References

     9. Tables, figure legends

Each table should be typed on a separate page and double−spaced.

Figure captions should be typed double spaced on a separate page.

Figures should be identified with figure number and name of first author.

The text should be clear and concise, conforming to accepted standards of English.

References and Citation Format

References are limited to pertinent published works or papers that have been accepted for publication. Usually this is achieved with fewer than 30 references. An abstract may be cited only when it is the sole source.

References should be typed separately, double−spaced, arranged alphabetically by author, and numbered serially, with only one reference per number. The number appropriate to each reference should be superscripted at the proper point in the text. The formats are:

Journal articles. Last name of first author, followed by initials, initials and last names of each coauthor; title of article (first word only capitalized); name of journal (abbreviated as in Serial Sources for the BIOSIS Data Base, published by BioSciences Information Service), volume, inclusive pages, and year.

Example: 1. Haselton, F. R., R. E. Parker, R. J. Roselli, and T. R. Harris. Analysis of lung multiple indicator data with an effective diffusivity model of capillary exchange. J. Appl. Physiol. 57:98−109, 1984.

Book references. Author(s) as above; title of book (main words capitalized); city of publication; publisher; year and pages, e.g.,: Thompson, D. A. W. On Growth and Form. Cambridge: Cambridge University Press, 1961, 346 pp.

For chapter in an edited book: Glass, L. and A. Shrier. "Low dimensional chaos in the heart." In: Theory of Heart: Biomechanics, Biophysics, and Nonlinear Dynamics of Cardiac Function , edited by L. Glass, P. Hunter, and A. McCulloch. New York: Springer−Verlag, 1991, pp. 289−−312.

For full author instructions, please click on the links below:

Manuscript Size and Format

Suggesting Reviewers and Associate Editor

Submission

Research Articles

Revisions

References and Citation Format

Figures
Submission of Electronic Figures, General
Submission of Scanned Figures
Tables
Mathematical Formulas and Equations
Review Articles
Letters to the Editor
Auxiliary Materials
Unique Materials, Data and Computer Code
Experimental Procedures
Copyright
Permissions
Proofs
Reprints
Springer Open Choice
Examples of Areas of Interest
Guiding Principles for Research

- Manuscript Size and Format

1) Manuscripts must not exceed approximately 20 double spaced pages, uploaded in a single file. The manuscript must have 1” margins and 12 point Times New Roman or Arial font. The page count includes references and but not figures or tables. Authors should limit figures to a manageable number (6-8 should suffice). Authors may consider publishing illustrations in excess of 8 as Electronic Supplementary Material. Occasional exceptions to these guidelines will be made at the editor’s discretion. All copy must be double-spaced, including text, footnotes, references, legends, and tables. Tables and figures should be referred to in the manuscript in a sequential manner.

2) Pages should be numbered consecutively beginning with the title page. Each section should be clearly labeled. Pages should be arranged in the following order:

1. Title page

2. Abstract and key terms

3. Introduction

4. Materials and Methods

5. Results

6. Discussion

7. Acknowledgments

8. References

9. Tables, figure legends

3) Each table should be typed on a separate page and double−spaced.

4) Figure captions should be typed double spaced on a separate page.

5) Figures should be identified with figure number and name of first author.

6) The text should be clear and concise, conforming to accepted standards of English style and usage. Unfamiliar or new terms should be defined when first used.

- Title:

The title should be informative. It should contain no unnecessary words and should not exceed 116 characters including spaces between words. The title page should have the title of the article, author(s), department and institution in which the work was done with address, an abbreviated title for the running head (not exceeding 55 characters including spaces between words), name and address for correspondence, and a contact telephone number, FAX number, and email.

- Abstract:

  A one−paragraph abstract of not more than 200 words must accompany each manuscript. It should state concisely the reason for the study, what was done, what was found, what was concluded, and the relevance.

- Key Terms:

  After the abstract, list three to ten terms not included in the title.

- Abbreviations, symbols, and terminology:

  Include in the manuscript a list of new or special abbreviations used in the paper, with spelled−out form or definition. Frequently used abbreviations need only be defined at first mention. For commonly accepted abbreviations, word usage, symbols, etc., authors are referred to the CBE Style Manual (sixth edition, 1994). Chemical and biochemical terms and abbreviations should be in accordance with the recommendations of the IUPAC−IUB Combined Commission on Biochemical Nomenclature. Isotope specification should conform to the IUPAC system.

- Glossary of terms:

  When only a few symbols and terms are used, define each one when it is first introduced.

  The definition should include:

  1) the symbol (Roman or Greek),

  2) its name,

  3) a definition in words, and

  4) units.

- Units:

  Authors should use the International System of Units (SI) except where common usage contradicts. Authors may follow the SI units with the equivalent

value in common units (usually c.g.s. system) in parentheses. Units with more than two components should be written without slashes or dots, using superscripts, as in ml g −1 s −1 for flow per gram of tissue. Units such as ml/g/s are unacceptable.

- Spelling:

  Follow Webster's Third New International Dictionary for spelling, compounding, and word division.

- Drugs, Chemicals, and Trade Names:

  Proprietary (trademarked) names should be capitalized. Check spelling. The chemical or generic name should precede the trade name or abbreviation the first time it appears.

- Footnotes:

  Avoid footnotes. Use parenthetic statements in the text instead.

- Acknowledgments:

  At the end of the article one or more statements should specify

  (a) contributions that do not justify authorship;

  (b) technical help;

  (c) financial and material support, specifying the nature of the support;

  (d) financial relationships that may pose a conflict of interest.

Manuscripts should meet the requirements outlined above to avoid delay in review and publication. With the exception of the style and ordering of the references, these style requirements match the "Uniform Requirements" published by the International Committee of Medical Journal Editors (Ann. Intern. Med. 126:36−−47, 1997). An online version of the "Uniform Requirements" can be viewed at the end of the chapter.

ABME discourages submissions of routine computational simulations that produce easily anticipated results, lack experimental validation, or represent incremental advancements of understanding.

Papers must be submitted via upload in a word processing format, preferably in Microsoft Word. Authors can submit LaTex manuscripts as is through our online submission process. Our software can accept LaTeX manuscripts, and usually formats them properly when converting them to PDF. Errors that occur in the conversion to PDF will be fixed when the paper is being prepared for publication. However, Microsoft Word files are preferred for upload.

Persons who have contributed intellectually to the paper but whose contributions do not justify authorship may be named and their function or contribution described. For example, "scientific advisor," "critical review of study proposal," "data collection," or "participation in clinical trial" are appropriate. Such persons must have given their permission to be named. Authors are responsible for obtaining written permission from persons acknowledged by name.

Reviewers and Associate Editor

Authors must suggest at least one (1) Associate Editor to oversee their manuscript, and also suggest at least four (4) potential reviewers. Reviewer suggestions must include email addresses. However, there is no guarantee that the suggested associate editor or reviewers will be used. Authors must also select five (5) classifications for their manuscript. Authors should also add personal classifications if they like.

Anexo E – Introdução em português

Implantes dentários se tornaram importantes alternativas no tratamento de pacientes com perda óssea maxilo-mandibular, servindo de ancoragem para próteses dentárias, obturadoras de fendas palatinas e de próteses faciais[1].

Um dos fundamentos básicos para a colocação de implantes dentários é um adequado volume ósseo[2, 3], e inumeras técnicas de enxertia óssea estão disponíveis hoje, sendo o osso autólogo ainda considerado o padrão ouro e provendo com os melhores resultados clínicos. Entretanto, limitações como a necessidade de area doadora, associada a morbidade desse sítio, somada a eventual limitação de osso disponível, podem diminuir ou contra-indicar indicação dessa técnica[4, 5]. Diferentes técnicas, que utilizam substitutos ósseos de origens diversas, como xenoenxertos, materiais alloplasticos ou alloenxertos estão atualmente disponíveis, apresentando, na sua maioria, bons resultados clínicos[6, 7], mas ainda apresentam algumas limitações sobre a extensão de sua aplicabilidade clínica. [8, 9].

Nas últimas décadas, técnicas envolvendo engenharia tecidual, area da medicina que combina os mecanismos naturais de cicatrização dos tecidos vivos com princípios de engenharia, foram muito estudadas e desenvolvidas para repor tecidos vivos perdidos por trauma, doenças ou reabsorção natural[10, 11, 12]. A engenharia tecidual basicamente combina culturas celulares *in vivo*, inicialmente matrizes bidimensionais para posteriormente serem aplicadas em carreadores tridimensionais, com ou sem a incorporação de proteínas[13], formando compostos de enxertia tecidual que podem carregar drogas, genes ou outros materiais que possam induzir ou acelerar o processo natural de cicatrização[14, 15]. O ácido hialurônico (HA) tem sido sugerido como um possível carreador cellular para reconstrução tecidual

óssea atrravés da engenharia tecidual[16]. HA é um ácido inorganic e está presente na matriz extracellular de todos os organismos vivos[17, 18], e permite alterações em sua cadeia molecular. Estas alterações podem ser importantes para diminuir o potencial alergênico e respostas inflamatórias, bem como aumentar seu potencial antibacteriano e propriedades angiogênicas[19, 20], além de promover capacidades de absorção de impacto, relevantes na proliferação e diferenciação cellular [21].

Materiais aloplásticos como a hidroxiapatita com beta-tricálciofosfato (HP-βTCP) tem sido extensivamente pesquisadas nas últimas décadas e são atualmente aplicadas como substitutes ósseos emu ma ampla gama de defeitos ósseos[22, 23] . HP-βTCP são cerâmicas bioativas que apresentam bem documentadas propriedades osteocondutivas e biocompatibilidade[24, 25], promovendo formação óssea em sua superfície. A aplicação desse biomaterial em culturas celulares *ex vivo* para enxertia óssea tem sido sugerida como uma técnica promissora para regeneração óssea. [26].

Baseado na controvérsia da lieratura sobre o uso do HA e sobre as características ideais referents ao seu peso molecular ideal para server como um carreador cellular em engenharia tecidual óssea, esse estudo avalia a citotoxicidade do HA de baixo peso molecular (LMW-HA) e alto peso molecular (HMW-HA) em cultura cellular de fibroblastos da linhagem NIH-3T3, com e sem a presence de HP-βTCP, através do teste MTT (methylthiazol tetrazolium).