In vitro evaluation of cytotoxicity of hyaluronic acid as an extracellular matrix on OFCOL II cells by the MTT assay

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Objective. To evaluate the cytotoxicity of hyaluronic acid (HA) on a tissue-engineered compound for bone grafting containing osteoblastic cells (OFCOL II), platelet-rich plasma (PRP) with or without thrombin (Thr), and hydroxyapatite (HP) by the MTT assay.

Study Design. Studied groups were formed as follows: (A) Cells + HA + PRP with Thr + hydroxyapatite (HP); (B) Cells + HA + PRP + HP; (C) Cells + HA + HP; (D) Cells + HP; (E) Cells + HA; (F) Cells + PRP with Thr; (G) Cells + PRP; and (H) Pure Dulbecco’s modified Eagle’s medium (DMEM) with 15% fetal bovine serum. A 2-way ANOVA and Tukey’s test were applied for statistical analysis (P < .05).

Results. Results of cell viability for each group were as follows: A: 79%, B: 67%, C: 68%, D: 99%, E: 74%, G: 89%, F: 90%, and Group H: 100%.


The restoration of large bone defects constitutes a significant clinical challenge for orthopedic and dental surgeons.1 More than 2.2 million bone graft procedures are performed annually around the world in dental, neurosurgical, and orthopedic fields.2 The popularity of dental implants has prompted an even higher demand for bone reconstruction, especially in the dentoalveolar region.3

One of the prerequisites for oral rehabilitation involving dental implants is the presence of an adequate alveolar process, which might allow the correct placement of implants to support a functional prosthesis for a long time.4,5 Aiming to improve situations in which this bone structure is compromised, several different biomaterials and surgical techniques have been tested as alternatives for alveolar bone reconstruction.2,6,7

Several disadvantages associated with the use of some currently available biomaterials for bone grafting have been suggested, including some related to autogenous bone,5 even though it has been widely considered the biomaterial of choice for bone grafting because of its osteoconductive and osteogenic properties.2 The indication of autogenous bone as the material of choice for graft surgeries should be addressed carefully, however, as aspects, such as donor site morbidity and limited material availability, may frequently constitute a clinical inconvenience.5 Described as an attempt to overcome these disadvantages, bone reconstructive alternatives involving tissue-engineered compounds have been considered as a promising treatment option with great potential for clinical application in dentistry.8

Tissue engineering is the general term used to describe the production of living tissues through the application of principles of biology and engineering, involving mainly cell-culture techniques and porous matrices.9 Different types of cells, including osteoblasts, may proliferate and maintain their phenotypes when cultured first in bidimensional substrates, and later inserted into porous matrices or tridimensional gels,9,10 maintaining consistency and dimensional stability during early phases of bone graft healing particularly to avoid collapsing of surrounding soft tissues.

As tissue engineering evolves, many biomaterials have been evaluated during the search for the ideal matrix for tissue reconstruction.11 A biocompatible ma-

Statement of Clinical Relevance

Results of the present investigation suggest that the HA matrix evaluated here might negatively influence bone cell viability in tissue-engineered grafting compounds, indicating the need to further investigate the ideal structural characteristics of HA to be used for this purpose.

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Hyaluronic acid (HA) has been considered a possible alternative biomaterial for tissue engineering. It constitutes the basic component of extracellular matrix in some organic tissues, and has been indicated as a possible cell carrier to be applied for tissue-engineered bone reconstruction. Its reported shock-absorption properties and low potential for allergic and/or immunogenic reactions also endorse its application for this purpose, although not supported by some studies that indicated a decrease in cellular viability when HA is applied as a matrix, in contrast to these findings, other reports have presented even a possible increase in cell viability associated with its use. Based on this controversy in the literature, the present study was conducted to evaluate in vitro the possible effects of HA as a matrix for a tissue-engineered compound of cultured osteoblastic cells associated with platelet-rich plasma (PRP) and synthetic hydroxyapatite (HP) by the methylthiazol tetrazolium (MTT) assay.

**MATERIAL AND METHODS**

This study was approved by the Research Ethics Committee of the Faculty of Dentistry of the Pontifical Catholic University of Rio Grande do Sul (PUCRS).

**Cell culture and sample preparation**

An ampule containing the OFCOL II cell line was removed from liquid nitrogen and unfrozen in a water bath at 37°C. The cellular suspension was transferred to a culture flask containing Dulbecco’s modified Eagle’s medium (DMEM) with 15% fetal calf serum and 10 μg/mL gentamicin in the flow hood. After adhesion of the cells to the culture flask, the medium was changed to eliminate residues from the freezing medium. These remaining cells were then rinsed with dimethyl sulfoxide (DMSO) (Henrifarma, São Paulo, São Paulo, Brazil). The OFCOL II cell cultures were incubated and kept in a humidified atmosphere of 95% air and 5% CO2 at 37°C to maintain a 7.4 pH. Once semiconfluence was achieved, the cells were washed with a buffered saline solution without calcium or magnesium (CMF-PBS) (Henrifarma, São Paulo, Brazil), trypsinized, and counted in a Neubauer chamber. Next, the cells were cultivated in a 96-well plate (TPP, St. Louis, MO) at a density of 5 × 10³ cells in 15% DMEM in a volume of 21 μL in each well. The experimental and control groups were run in quadruplicate. The cells were seeded 24 hours before the experiment in each well, when they reached semiconfluence (80% growth). The MTT assay was conducted after 48 hours of cell culture added with HA, HP, and PRP according to the groups’ division for cellular proliferation analysis.

As shown in Table 1, I control and 7 experimental groups were run in quadruplicate for cellular viability analysis by the MTT assay. Each experiment was repeated 3 times.

**PRP preparation**

For the PRP preparation, approximately 15 mL of human blood was collected in 3 different commercially available 5-mL tubes containing 3.2% sodium citrate (BD Vacutainer/Buff Na. Citrate—ref: 369,714; BD Vacutainer; Sao Paulo). Using a double centrifugation technique, the first one was performed at 400g for 10 minutes to separate the blood plasma. After this centrifugation, the supernatant was removed using a serologic pipette. The supernatant was placed in a second Falcon-type tube, which was then centrifuged at 800g for 10 minutes to concentrate the platelets. After this second centrifugation step, the supernatant was completely removed and part of it was returned to the tube, so that the material contained within the tube was 10% of the original collected volume. The entire procedure,

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells</th>
<th>HA</th>
<th>PRP</th>
<th>HP</th>
<th>DMEM</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>21 μL with 5 × 10³</td>
<td>25 μL</td>
<td>12.5 μL</td>
<td>4 mg</td>
<td>149 μL</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>Group B</td>
<td>21 μL with 5 × 10³</td>
<td>25 μL</td>
<td>12.5 μL</td>
<td>4 mg</td>
<td>151 μL</td>
<td>—</td>
</tr>
<tr>
<td>Group C</td>
<td>21 μL with 5 × 10³</td>
<td>25 μL</td>
<td>—</td>
<td>4 mg</td>
<td>164 μL</td>
<td>—</td>
</tr>
<tr>
<td>Group D</td>
<td>21 μL with 5 × 10³</td>
<td>25 μL</td>
<td>—</td>
<td>4 mg</td>
<td>189 μL</td>
<td>—</td>
</tr>
<tr>
<td>Group E</td>
<td>21 μL with 5 × 10³</td>
<td>25 μL</td>
<td>—</td>
<td>—</td>
<td>164 μL</td>
<td>—</td>
</tr>
<tr>
<td>Group F</td>
<td>21 μL with 5 × 10³</td>
<td>—</td>
<td>12.5 μL</td>
<td>—</td>
<td>174 μL</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>Group G</td>
<td>21 μL with 5 × 10³</td>
<td>—</td>
<td>12.5 μL</td>
<td>—</td>
<td>176 μL</td>
<td>—</td>
</tr>
<tr>
<td>Group H (Positive control)</td>
<td>21 μL with 5 × 10³</td>
<td>—</td>
<td>—</td>
<td>189 μL</td>
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</table>

**Table 1.** Tested groups and the respective volumes of each component per well

**DMEM,** Dulbecco’s Modified Eagle’s Medium; **HA,** hyaluronic acid; **HP,** hydroxyapatite; **PRP,** platelet-rich plasma; —, indicates that these components are not part of the respective experimental group.
starting with 15 mL of blood, resulted in a final volume of 1.5 mL of PRP.

To obtain the autologous thrombin, we transferred 1.2 mL of the upper portion of the blood plasma column after the first centrifugation in a sterile 15-mL Falcon tube. Next, 0.3 mL of 10% calcium gluconate was added to the 1.2 mL of plasma with platelets and then placed in water bath at 37°C for 15 minutes to set the plasma into a gel state. Another centrifugation step of 800g was performed for 10 minutes, resulting in separation of thrombin in the tube.

The PRP gel was finally obtained through the addition of 2.5 μL of autologous thrombin to 12.5 μL of PRP in a ratio of 1:5. A 1-minute waiting period was necessary for the formation of a PRP gel in the A, B, F, and G test groups. The volume of 12.5 μL was selected based on previous study. 20

Application of HA
A total of 25 μL of HA gel (Teosyal 30G Touch Up, Teoxane, Geneva, Switzerland) containing 25 mg/mL of HA with a pH of 7.3 was used in each culture well for the A, B, C, and E groups. HA concentration was determined based on the results from a concentration curve test done in a pilot study. After an initial dilution of 25 μL of Teosyal in 100 μL of 15% DMEM, we obtained a final concentration of 2.97 mg/mL of HA applied per well.

Application of HP
A total of 4 mg of commercially available synthetic HP (Straumann BoneCeramic Biora AB, Malmö, Switzerland) composed of 60% HA and 40% β-tricalcium phosphate (β-TCP), presenting a pore size of 100 to 500 μm was weighed and placed in each culture well (TPP) of the A, B, C, and D groups. The HP content was exposed to ultraviolet light for 20 minutes before its addition to the cultures for sterilization.

Cell viability assay (MTT)
The MTT assay was conducted here to assess cell viability, aiming to evaluate the ability of live osteoblastic cells to reduce 3-(4,5-dimethyl-2-thiazole)-2,5-diphenyl-2-yl-tetrazolium bromide (Acros Organics, Morris Plains, NJ) and form insoluble violet formazan crystals. After each cell group establishment, a 10% MTT solution (5 mg/mL) diluted in PBS was added to each well. The cultures were immediately incubated for 2 hours at 37°C and protected from light until the presence of the violet formazan crystals was observed. For formazan crystals, solubilization, 100 μL of DMSO was added to each well. Later, the absorbance at a wavelength of 570 nm (Microplate Reader, SpectraMax M 5, ‘EUA; Molecular Devices, Sunnyvale, CA) was recorded and analyzed using the Softmax Pro 5.2 program.

Statistical analysis
All results were expressed as mean ± SD. A comparative analysis of means was performed using the analysis of variance (ANOVA) and Tukey’s multiple comparisons test (P < .05).

RESULTS
The optical density (OD) values indicated a percentage of viable cells compared with the control group (H), which was considered 100% viable.

According to these results, a significant difference was observed between groups B and C in comparison with group H (P < .05). No significant difference was observed between groups A, D, E, F, and G in comparison with group H (P > .05).

Group B (with 67% viability) and group C (with 68% viability) were the groups with HA that exhibited the lowest viabilities. The viabilities of these 2 groups were similar but differed significantly from that of group D (cells + HP), which presented the greatest viability at 99%. Group A (cells + HA + PRP with thrombin + HP) with 79% cellular viability, group E (cells + AH) with 74% viability, group F (cells + PRP with thrombin) with 90% viability, and group G (cells + PRP) with 89% viability did not differ statistically from each other (P > .05) (Fig. 1).

DISCUSSION
As significant differences in cell viability were verified among the studied groups, it might be important to analyze individually the possible influence of HA, PRP, human thrombin, and HP on the obtained cell viability results.

To our knowledge, studies evaluating the use of HA as a matrix applicable to bone grafting involving tissue-engineering techniques are limited. Therefore, the present study aimed to evaluate the viability of osteoblastic cells in the presence of this material. Also, it is clear that there is no consensus in the current literature regarding the ideal formulation of HA as to its structural constitution and/or ideal concentration. Some studies reported a decrease in cellular viability when HA was used,16,17 whereas others indicated opposite results.18,19 Previous studies involving bone tissue engineering suggested the need for a material that might act as a vehicle capable of transporting and maintaining bone cells to the grafted site, ensuring the tissue volumetric increase necessary for bone reconstruction.12 Because of its characteristics, HA may act as a plasticizing agent for tissue-engineered graft compounds by combining mineral matrices and cultured cells into a gel-like composite easily handled during surgeries, and keep its con-
sistency and dimensional stability postoperatively to avoid collapsing of surrounding tissues during early stages of bone healing. Although not evaluated here, these advantages might help to achieve a better clinical response of tissue-engineered bone grafts compared with the traditional techniques currently available.

The cytotoxicity of the HA, teosyal, which has been said to present similar composition as the HA in living tissues, was tested on OFCOL II osteoblastic cells. The term cytotoxicity is used here to describe the chain of molecular events that interfere in cell molecular synthesis, causing cellular dysfunction and/or its structural damage. The present investigation aimed to verify if HA might constitute an applicable vehicle with some required characteristics desired to biocompatible matrices, such as being synthetic, resorbable, fully biocompatible, nonallergenic, and nonimmunogenic. The decrease in cellular viability verified in the presence of HA might indicate a possible inhibitory effect on the cells of the studied groups. Based on these results, it might be speculated that the cell viability reduction verified here could be related not to HA chemical composition, but to the molecular weight of the applied acid.

Some studies applying HA with high molecular weight (>1000 kDa) at concentrations ranging between 50 μg/mL and 1 mg/mL reported a clear stimulation of chondrocyte, fibroblast, and melanoma production in vitro. Others, though, have reported that application of high molecular weight HA might inhibit proliferation of fibroblasts, keratinocytes, ovarian cells, tendon cells, and macrophages in cultures.

Similar to other in vitro studies that investigated osteoblastic cells and high molecular weight HA (>1000 kDa), the present study also suggested a possible inhibition of cell proliferation, which was not observed in other investigations that analyzed low molecular weight HA (<50 kDa).

Furthermore, an HA concentration of 2.97 mg/mL was applied to all HA groups, which was determined based on results of a concentration curve test done in an earlier pilot study. This concentration was higher than those recommended by Kawasaki et al., Yoneda et al., and Ahrens et al. Although the HA concentration applied here was determined by a concentration curve in a pilot study, it differed from the recommendations of these studies that failed to describe specifically how it was calculated. Further investigations might be necessary to establish the ideal HA concentration to be applied in tissue-engineered graft compounds in vivo and its possible effects on bone cell proliferation and differentiation.

The HA influence on bone cells cultured in the absence of hyaluronidase enzyme is another relevant issue present here to be discussed. This absence could lead to some inconveniences to the cell metabolism, allowing the HA molecule in its natural state to act in close contact with cells for a longer time, and most likely inhibiting the natural processes of cell proliferation and differentiation. With the HA degradation through the addition of hyaluronidase to the experiment, smaller acid molecules would be in contact with the cells and possibly induce a more favorable environment for cell division and differentiation as well as a possible activation of the cell-signaling chain linked to inflammatory response.

In the present study, human PRP was added to some analyzed groups aiming to positively influence cell differentiation and induce an increased cellular viability (74% in group E [cells + HA] and 79% in group A [cells + HA + PRP with thrombin + HP]). In a similar study, Kim et al. found 72% viability of mesenchy-
mal human stem cells with high molecular weight HA (170,000 Da) in the same period, without the addition of specific growth factors. However, when bone morphogenetic protein-2 was added to the HA gel, cellular viability increased to 81%.

PRP is a well-known source of growth factors that play an important role in cell proliferation and differentiation processes, such as platelet-derived growth factor, transforming growth factor-β, and insulin-like growth factor-1. Also, human thrombin was added in group A (cells + HA + PRP with thrombin + HP) and group F (cells + PRP with thrombin) for platelet activation, and a small improvement in cellular viability was observed within these groups. However, these results were not statistically significant when compared to the nonthrombin groups.

In similar studies, Krasna et al. and Arpornmaeklong et al. tested the effects of the application of PRP in vitro on the proliferation of human fibroblastic cells. In both studies, the highest PRP concentrations (approximately 20%) exhibited better responses by the MTT assay, indicating that PRP cellular stimulation is dose-dependent. These findings directly influenced the determination of the final volume of PRP applied to the OFCOL II cells in the present study.

The advantages of PRP applied to cell cultures have been observed in different investigations. In the present study, group F (cells + PRP with thrombin) and group G (cells + PRP) showed higher cell viability compared with other groups with HA. When compared with control group, however, PRP alone was not able to act as an influential agent to improve cellular proliferation.

Regarding the presence of HP, a positive result in cell proliferation was verified, obtaining 99% viability in group D (cells + HP). Group C (cells + HA + HP) presented one of the lowest cellular viabilities, suggesting that HP might not have interfered or contributed with the inhibitory effect verified in HA groups.

The present findings were obtained from an in vitro evaluation, which undoubtedly presents some limitations. On the topic of tissue engineering, in vitro studies do not allow the exact reproduction of a living tissue environment, which involves among other things application of forces by the musculoskeletal system, which is an important player on the physiological induction of bone formation. Kim et al. reported marked differences when comparing results from HA applications both in vitro and in vivo, stating that the in vivo findings were significantly better in terms of bone formation outcome.

Even considering that the present study indicated an acceptable average of cell viability of 72% in the presence of HA, additional in vivo studies may be needed to confirm its indication in bone tissue engineering. Changes in the HA molecular weight might be tested to achieve even more expressive cell viability percentages. Hence, other aspects to be investigated might include the addition of other esters to the HA formula and/or fracturing of the HA molecule to possibly improve its structural characteristics while maintaining its viscoelasticity and biological functions.

REFERENCES

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