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ORIGINAL ARTICLE

Effect of hyaluronic acid on paracrine signaling of osteoblasts from mesenchymal stromal cells: potential impact on bone regeneration

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Abstract

Objectives This study evaluated hyaluronic acids (HA) with different molecular weights as potential matrices for tissue-engineered bone grafting and their possible influence on the paracrine mechanisms of adipose-derived mesenchymal stromal cells.

Material and methods Murine adipose mesenchymal stromal cells (mASCs) on the fourth passage were seeded in 96-well plates, osteoinduced for 27 days and exposed for 3 days to low (HA-LW) and high/low molecular weight (HA-HLW) at previously defined concentrations. Cytokines IGF-1, VEGF, FGF-2, and BMP-2 were evaluated by quantification in the supernatant.

Results Greater expression of growth factors was observed in groups with HA-HLW compared to HA-LW. Results indicated that differentiated cells secreted fewer cytokines, namely VEGF, FGF, and BMP-2 than undifferentiated mASCs ($p < 0.05$). IGF-1 showed its greatest expression in the mASC HA-LW group ($p < 0.05$).

Conclusions The application of HA-HLW as cell matrix in tissue engineering did not compromise mASC paracrine effect. Also, the association of HA-HLW matrix and mASCs resulted in greater expression of osteogenic growth factors. Longer periods of cell differentiation seemed to negatively affect their capacity for local paracrine stimulation.

Clinical relevance The use of HA-HLW as matrix for undifferentiated ASCs can be positive for bone regeneration, favoring its application as cell matrix in bone grafting procedures.

Keywords Cytokine · Bone regeneration · Mesenchymal stem cell · Paracrine

Introduction

The regeneration of bone defects in orthopedics and dentistry remains a critical challenge. Bone tissue engineering, using a combination of cell culture techniques, signaling molecules, and specific matrices, is emerging as a promising alternative to improve or replace autogenous, allogeneic, and synthetic biomaterial-based bone grafting [1]. Mesenchymal stromal cells (MSCs) have demonstrated great potential for application in tissue-engineered products due to their multipotency, ease of access, and potential for in vitro isolation and subsequent culture [2]. In addition, they secrete a wide range of bioactive molecules, namely cytokines or growth factors, that mainly through paracrine mechanisms act as immunoregulatory agents on peripheral cells organizing regenerative microenvironments in tissue injuries [3]. These mechanisms may also influence angiogenesis, stimulate the remodeling of extracellular matrix, restrict local inflammation, reduce cell apoptosis and fibrosis, and participate in local tissue immune response [4, 5]. This way, MSCs directly or through paracrine mechanisms openly affect tissue regeneration, mainly by modulating the tissue healing process [6, 7].

In addition to the presence of signaling factors, cells do need an appropriate support matrix for adherence, proliferation, and protein synthesis [8]. Particularly, hyaluronic acid (HA), a sequence of repeated disaccharide units containing glucuronic acid and *N*-acetylglucosamine, has been described as potentially applicable as a cell matrix for tissue engineering [9, 10]. Being present in the connective tissue, it plays an important role in several biological phenomena, such as cell

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signaling, adhesion, proliferation, and differentiation [2, 11]. Synthetic HA also presents suitable physicochemical properties for in vivo application, being biodegradable, biocompatible, and presenting consistent viscosity and elasticity [12]. Nevertheless, HA's primary characteristic to be considered aiming its application in living tissues would be the molecular weight, as variations on this property have been said to elicit pro- or anti-inflammatory tissue reactions, along with distinct angiogenic effects [13]. Intending to maximize physical properties and local tissue responses, HA hybrid formulas combining both high (> 1000 kD) and low (50 to 1000 kD) molecular weight acids have been proposed, presenting promising features for application in living tissues [14]. Thus, the present study aims to evaluate the possible influence of the use of HA as extracellular matrix and/or scaffold for tissue-engineered products presenting distinct molecular weights on the paracrine mechanisms of adipose-derived MSCs.

Materials and methods

Animals

The present study protocol was approved by the Animal Research Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (CEUA_1471005851428). Three 60-day-old male Lewis rats were used as mesenchymal stromal cell donors obtained from their epididymal adipose tissue. Animals were kept in conventionally controlled sanitary standards with 12-h light/dark photoperiod and controlled room temperature (21 to 24 °C).

Euthanasia and characterization of adipose tissue-derived mesenchymal stromal cells

Stromal cell lines derived from murine adipose tissue (mASCs) were characterized according to previously established criteria [12]. For cell harvesting, animals were euthanized by inhalation of an anesthetic (isoflurane) at 5%. Adipose tissue was collected from the epididymal region and digested in type I collagenase solution (250 U/mL) for approximately 30 min at 37 °C. Digested tissue was centrifuged (400*g*) and the isolated cells seeded in 6-well plates. On > 80% confluence, cells were trypsinized (0.05% trypsin–0.2% EDTA) and subsequently passaged by splitting them into two flasks.

For osteogenic and adipogenic differentiation, cell cultures were subjected to differentiation processes in adipocytes and osteoblasts in the fourth passage. To induce adipogenic differentiation, cells were cultured for 4 weeks in 12-well plates, in complete medium, namely Dulbecco's modified Eagle's medium/low glucose (DMEM) with 10% fetal bovine serum (GIBCO, Grand Island, NY, USA), supplemented with 10^{-8} M dexamethasone, 2.5 μg/mL of bovine pancreas insulin, 100 mM indomethacin, and 5 mM rosiglitazone. The adipocyte phenotype was evaluated by the Oil Red O staining, evidencing the presence of fat vacuoles. For osteogenic differentiation, cells were maintained in a 12-well plate with complete medium with 10^{-5} M dexamethasone, 100 μg ascorbic acid, and 10 mM β-glycerophosphate for the same 4 weeks and later formed the osteoinduced cells groups (OSTEO). Osteoblastic activity was evaluated by Alizarin Red S staining evidencing the calcium-rich extracellular matrix.

Flow cytometry analysis was then performed as follows: cells were then trypsinized, centrifuged, and incubated for 30 min at 4 °C, with specific murine antibodies for CD11b, CD34, CD45, CD73, CD90, and CD105, conjugated with phycoerythrin (PE) or fluorescein isothiocyanate (FITC) (BD Pharmingen, San Diego, CA). Data from at least 10,000 events were collected using the FACSCalibur cytometer (Becton Dickinson, San Diego, CA), equipped with a 488-nm laser and the CELLQuest program (Becton Dickinson). The obtained data were analyzed using the WinMDI 2.8 program.

Analysis of mASC cell viability (MTT test)

The cytotoxicity of low molecular weight hyaluronic acid (HA-LW) (Hyaloss®–HYAFF™, Meta, Italy) was previously evaluated in vitro in mASC cultures by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2yl-tetrazolic bromide) test [15]. Results showed negligible levels of cytotoxicity after 72 h at an HA-LW concentration of 75% [15]. The same applied methodology was used here to evaluate the cytotoxicity of the high/low molecular weight hyaluronic acid (HA-HLW) (Profhilo®, IBSA, Italy). For this, 256 mg of HA-HLW were diluted in 4 mL (64 mg/mL, 100%) DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA) and 10 μg/mL gentamicin (GIBCO, Grand Island, NY, USA) at 37 °C. From the maximum concentration (100%), dilutions of 75%, 50%, 25%, and 15% were subsequently made and assembled in groups, along with a positive control group (DMEM only) and a negative control group (1% sodium hypochlorite). The experiment was carried out in triplicates and repeated 3 times under the same conditions.

The mASCs in the fourth passage were seeded in a 96-well plate with complete medium at a density of 4×10^4 cells/well. After 24 h of incubation, the complete culture medium was removed and HA-HLW was added with volume and dilutions as presented in Table 1.

After application of HA-HLW in triplicate, the culture medium was removed and 10% solution of MTT (5 mg/mL) in PBS was added to each well. Then, cultures were incubated at 37 °C protected from light until the presence of violet

Table 1 Concentrations (%) of high/low molecular weight hyaluronic acid (HA-HLW) (mg/mL) cells and Dulbecco's modified eagle medium/low glucose (DMEM) applied on each well for cell viability analysis

Percent	Cells	HA-HLW (mg/mL)	DMEM + HA-HLW (μL/well)	DMEM (μL/well)	Total volume (μL/well)
100	4×10^4	64	100	100	200
75	4×10^4	48	75	125	200
50	4×10^4	32	50	150	200
25	4×10^4	16	25	175	200
15	4×10^4	14.1	15	185	200
+ control	4×10^4	–	–	200	200
− control	4×10^4	–	–	200	200

formazan crystals was observed. For solubilization of formazan crystals, 100 μL of dimethylsulfoxide (DMSO) was added to each well and the absorbance of the samples at 570 nm was determined by an ELISA reader (Bio-Rad Microplate Reader Benchmark, Inc., USA) after 24, 48, and 72 h. The group of cells that showed the highest cell viability determined the concentration of HA-HLW to be used in the experiment.

Dosage of growth factors

The cytokines IGF-1, VEGF, FGF-2, and BMP-2 were quantified using the enzyme-linked immunoabsorbent assay (ELISA), according to the manufacturer's instructions (Quantikine® ELISA Kits, USA). For that, the cells in the fourth passage were seeded in 96-well plates in triplicate, grown for 28 days in a humidified incubator at 37 °C and 5% CO_2 and divided into 6 groups (Table 2). The cells were exposed to HAs at concentrations defined by the MTT assay and the growth factors present in the supernatant were quantified.

Statistical analysis

Results are shown as means ± standard error of the mean (SEM). The significance of the differences between two groups of values was calculated using paired Student's *t* test. Significance of the differences among groups was analyzed by ANOVA followed by Tukey's test. In all statistical tests, a significance level of 5% was adopted ($p < 0.05$).

Results

Morphological analysis and ASC adipogenic and osteogenic differentiation assay

As expected, ASCs exhibited elongated and fusiform fibroblast morphology, with central nucleus and extensions. Phenotypically stable, they showed high adherence to plastic and replicative rates. When subjected to each specific chemical induction, they were able to differentiate into osteogenic and adipogenic strains (Fig. 1).

Analysis of ASC cell viability (MTT test) in HA-HLW

The absorbance results of each group with a concentration variation of 15, 25, 50, 75, and 100%, at 24, 48, and 72 h in percentage of cell viability, are shown in Fig. 2. The absorbance verified in the control group (DMEM) was considered 100% of cell viability. HA-HLW was considered non-cytotoxic for mASCs within the safety range of 75% concentration. From this established HA concentration of 75%,

Table 2 Distribution of in vitro experimental groups

Group 1: mASCs	Mesenchymal stromal cells derived from adipose tissue cultured in complete medium (P4).
Group 2: mASCs + HA-HLW	Mesenchymal stromal cells derived from adipose tissue cultured in complete medium for 28 days. After this, high and molecular weight hyaluronic acid (HA-HLW) was added and kept in culture for another 3 days.
Group 3: mASCs + HA-LW	Mesenchymal stromal cells derived from adipose tissue cultured in complete medium for 28 days. After this, low molecular weight hyaluronic acid (HA-LW) was added and kept in culture for another 3 days.
Group 4: OSTEO	Mesenchymal stromal cells derived from adipose tissue differentiated into osteoblasts by osteogenic medium for 28 days.
Group 5: OSTEO + HA-HLW	Mesenchymal stromal cells derived from adipose tissue differentiated into osteoblasts by osteogenic medium for 28 days. After this, high and low molecular weight hyaluronic acid (HA-HLW) was added and kept in culture for another 3 days.
Group 6: OSTEO + HA-LW	Mesenchymal stromal cells derived from adipose tissue differentiated into osteoblasts by osteogenic medium for 28 days. After this, low molecular weight hyaluronic acid (HA-LW) was added and kept in culture for another 3 days.

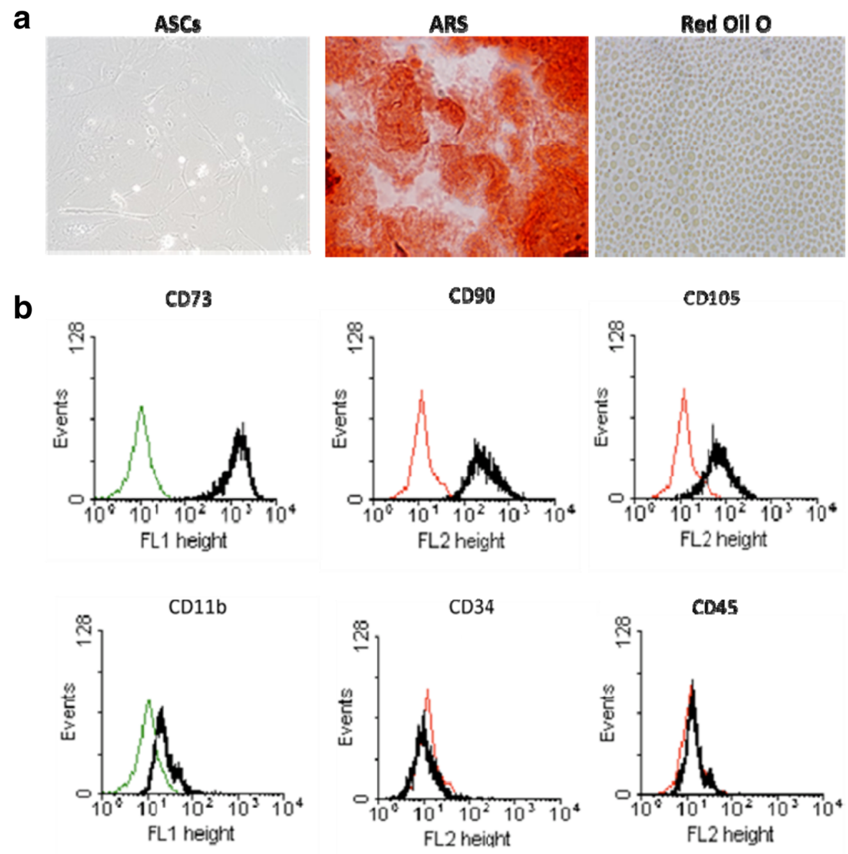

Fig. 1 **a** Undifferentiated ASCs (P4), differentiated into osteoblasts stained with Alizarin Red S (ARS) and differentiated into adipocytes with fat vacuoles stained with Red Oil O, at 40× magnification using phase-contrast microscopy. **b** Immunophenotyping of ASCs by flow cytometry. Peaks are the expression of the selected molecules (black trace), compared to the negative isotype control (green or red trace)

Table 3 shows the total volume of medium, cells, HA-HLW, and HA-LW applied for all mASCs and OSTEO groups.

Quantification of growth factors

Quantification of vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF), and bone morphogenetic protein 2 (BMP-2) present in the culture supernatant are depicted in Fig. 3.

Vascular endothelial growth factor

Figure 3a compares the expression of VEGF (pg/mL) between mASCs and OSTEO groups. Notoriously, the amount of VEGF in the mASC supernatant (2.474 ± 0.054) was significantly higher than in the OSTEO groups (0.083 ± 0.024) ($p < 0.05$). The presence of VEGF was significantly higher in mASCs and mASCs + HA-HLW (2.544 ± 0.050) groups ($p < 0.05$). Also, the addition of HA-LW to mASCs significantly reduced VEGF expression (1.135 ± 0.080) ($p < 0.05$). When

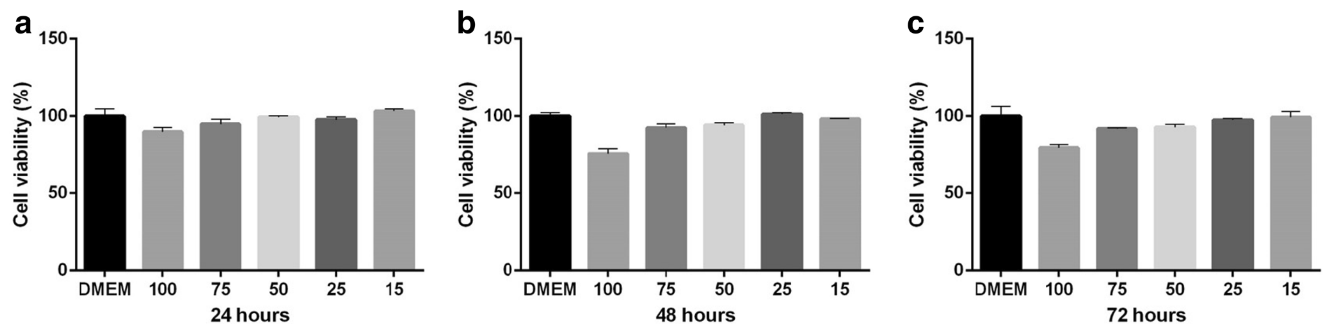

Fig. 2 Adipose-derived stem cells viability (%) exposed to different concentrations of high and low molecular weight hyaluronic acid (HA-HLW) for 24 h (**a**), 48 h (**b**), and 72 h (**c**), verified through the MTT assay. Bars indicate means and standard deviations, and different letters indicate statistical differences ($p < 0.05$)

Table 3 Experimental group distribution and their respective volumes

Group	Cells	CM (μL/well)	OM (μL/well)	HA-HLW (μL/well)	HA-LW (μL/well)	Total volume/well
mASCs	4×10^4	200	–	–	–	200
mASCs + HA-HLW	4×10^4	125	–	75	–	200
mASCs + HA-LW	4×10^4	125	–	–	75	200
OSTEO	4×10^4	–	200	–	–	200
OSTEO + HA-HLW	4×10^4	–	125	75	–	200
OSTEO + HA-LW	4×10^4	–	125	–	75	200

CM complete medium: Dulbecco's modified Eagle's medium/low glucose (DMEM) with 10% fetal bovine serum (FBS), *OM* osteogeneic medium, *HA-HLW*, high/low molecular weight hyaluronic acid, *HA-LW* low molecular weight hyaluronic acid

baseline values of mASCs were converted into percentage considering them 100%, no significant difference in VEGF expression was verified in the addition of HA-HLW. However, in the OSTEO groups, there was a significant increase in VEGF in the presence of HA-HLW (0.422 ± 0.029) and HA-LW (0.166 ± 0.018) compared to its baseline condition ($p < 0.05$).

Insulin-like growth factor-1

Secretion of IGF-1 is shown in Fig. 3b. A significant increase in IGF-1 secretion (pg/mL) was present in mASCs + HA-LW (0.092 ± 0.010) group when compared to other groups ($p < 0.05$). Also, when considering the basal condition of the cells, the expression of IGF-1 in the OSTEO groups (0.065 ± 0.002) was greater than mASCs groups (0.054 ± 0.001) ($p < 0.05$). The addition of both HAs did not significantly affect the basal expression of IGF-1 in the OSTEO groups ($p > 0.05$).

Fibroblast growth factor receptor

Considering FGF secretion in the supernatant (pg/mL), it was only detected in the mASCs (0.054 ± 0.001 pg/mL) and mASCs + HA-HLW groups, with a significantly higher expression verified in the mASCs + HA-HLW (0.096 ± 0.010) (Fig. 3c) ($p < 0.05$).

Bone morphogenetic protein 2

Secretion of BMP-2 presented significant expression (pg/mL) in the mASCs (0.060 ± 0.008) and mASCs + HA-HLW

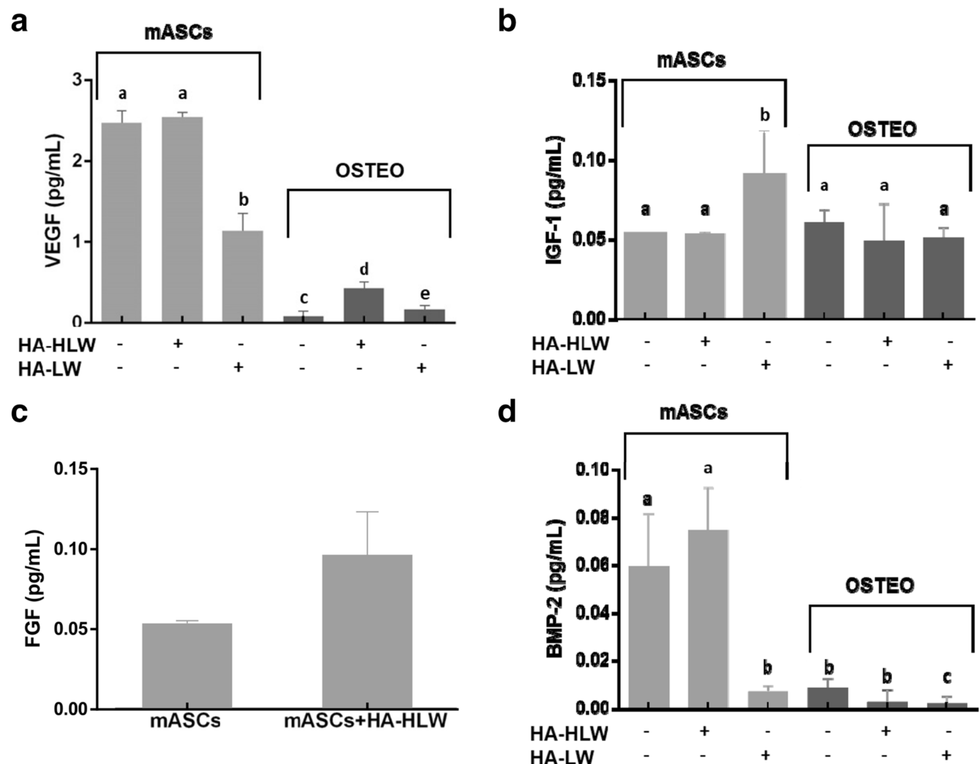

Fig. 3 Expression of vascular endothelial growth factor (VEGF) (**a**), insulin-like growth factor-1 (IGF-1) (**b**), fibroblast growth factor (FGF) (**c**), and bone morphogenetic protein-2 (BMP-2) (**d**) in the experimental groups. Values are expressed as mean ± standard error. Different letters indicate statistical differences ($p < 0.05$). Positive (+) and negative (-) signs indicate the presence and absence of hyaluronic acids in each group, respectively

(0.074 ± 0.016) groups ($p < 0.05$). The lowest secretion of BMP-2 occurred in the OSTEO + HA-LW group (0.004 ± 0.001), statistically differing from the other groups ($p < 0.05$). The association of HA-LW significantly reduced BMP-2 secretion in the mASC group (0.007 ± 0.001) when compared to their baseline condition ($p < 0.05$), while the OSTEO group presented greater reduction of BMP-2 expression when associated with HA-LW compared to OSTEO baseline (0.008 ± 0.001) and HA-HLW groups (0.005 ± 0.001) ($p < 0.05$) (Fig. 3d).

Discussion

Present in perivascular regions of all adult tissues, including bone marrow, periosteum, muscle tissue, and parenchymal organs [12, 16], MSCs have the capacity of self-renewal and differentiation into a wide range of adipogenic, chondrogenic, osteogenic, myogenic, angiogenic, neurogenic, and cardiomyogenic cells [17]. Among the possible sources of mesenchymal stromal cells, those derived from adipose tissue stand out, as they might be easily obtained. Adipose tissue harvesting from the epididymal region of Lewis rats may be considered a highly invasive procedure characterized by the full removal of present fat tissue from each animal. We performed adipose cell harvesting from euthanized animals, as keeping the animals alive during and/or after harvesting procedures would technically impose significant adversities in terms of animal suffering, equipment, and personnel. We considered a more appropriate adipose cell harvesting from euthanized animals, as recommended by the applied Vivisection Protocols for Animal Research. Also, it has been said that ASC success rate of isolation could reach 100%, and the yield of viable stem cells in adipose tissue is generally 40 times higher than in bone marrow [18].

MSCs express a large number of bioactive molecules such as specific cell adhesion molecules, extracellular matrix proteins, cytokines, and receptors for growth factors, allowing sound interactions with other cells [19]. These molecules act locally modulating tissue inflammatory response, angiogenesis, and mitosis of the cells involved in the tissue reparative process [20]. Such characteristics involved with cell plasticity, cytokine, and growth factor secretion [21] and immunoregulatory function [22, 23] directly influence local cell behavior, mainly by activation of cell signaling pathways and contributions to the angiogenesis and tissue regeneration processes [24–26].

Associated with MSCs, one scaffold of particular interest considered applicable in tissue engineering is HA, a hydrogel polymer of natural origin, belonging to the family of glycosaminoglycans (GAGs) and present in the extracellular matrix of living tissues [15]. However, there is still no consensus on the ideal presentation and concentration of HA for application as cell scaffold in tissue engineering, since it might elicit distinct effects on cultured and local cells based on its physicochemical properties, especially molecular weight [27]. In fact, HA stimulates a local environment that loosens cellular anchorage to the extracellular matrix, facilitating their migration and division [28]. Right after a tissue injury, there is a marked increase in local high molecular weight HA (HA-HW) that binds to fibrinogen, which is essential for the initial formation of blood clots and future tissue repair, in addition to the opening of spaces for polymorphonuclear leukocyte migration to the wound site for removal of dead tissue, debris, and bacteria. Later, in the inflammatory stage of tissue regeneration, an accumulation of HA-LW generated from the degradation of HA-HW may be verified, being responsible for stimulation of cytokine production and angiogenesis [29].

Previous investigations have reported a better chemical stability and a prolonged effect in vivo of the HA-HLW, even subjected to the effect of local hyaluronidases [30]. Also, when evaluating the effect of HA-HLW on the proliferation and differentiation of ASCs in vitro, an increase in the proliferation rate and differentiation improvement of adipocytes from ASCs compared to HA-LW has been reported [31].

In terms of growth factor expression, results of the present investigation showed that VEGF secretion, which positively regulates FGF expression, and BMP-2 by ASCs presented more expressive values when exposed to HA-HLW. Traditionally, the angiogenic cytokine VEGF (mitogenic and vasoactive) was considered to be specific for endothelial cell growth. However, in addition to ASCs, studies have confirmed the presence of VEGF receptors in several other cells, including osteoblasts [32]. When comparing undifferentiated to osteodifferentiated cell groups, results of the present study revealed a better expression of VEGF, BMP-2, FGF, and IGF-1 in ASC groups. Osteodifferentiated cell groups expressed significantly lower values of VEGF and BMP-2, suggesting that cells at earlier stages of differentiation might be more influenced by the paracrine effect in terms of their cytokine secretion capacity. Furthermore, the low levels of BMP-2 verified in the OSTEO groups might be associated with the advanced differentiation stage of induced osteoblasts. It has been said that BMP-2 has its maximum expression in the first days of bone healing [33], suggesting the importance of its early presence in the cascade of cellular events involved in bone repair and confirming its greater paracrine secretion in lower stages of cell differentiation [34]. Therefore, considering cell culture-based tissue engineering applications, it might be hypothesized that the expression levels of important growth factors such as BMPs during bone healing might be directly influenced by local cell differentiation process and their actual maturation stage.

Considering FGF expression on undifferentiated and osteodifferentiated cell groups, results verified here pointed to a significantly positive effect of HLW-HA exposure on mASCs compared to unexposed mASCs and OSTEO groups, even presenting undetectable levels of FGF expression for the

OSTEO groups. This finding corroborated the concept of stimulation of FGF expression by the marked presence of VEGF in undifferentiated groups, which also presented a pronounced expression in the mASCs + HLW-HA group. The combination of FGF and VEGF has been considered important for the process of differentiation of human ASCs in osteogenic and vasculogenic strains at early stages of bone healing [35].

Results reported here indicated that differentiated ASCs had their growth factors production negatively affected in comparison with undifferentiated ASCs. Corroborating these findings, similar cellular responses were observed when a combination of ASCs and HA-LW was evaluated in bone tissue engineering in vivo [15], where results suggested that the combination of HA-LW and uninduced ASCs favored bone formation in critical defects.

Conclusion

Results indicate a tendency for a promising cytokine expression response for undifferentiated cells (mASCs) compared to cells in later stages of differentiation. Also, longer periods of cell culture aiming differentiation seemed to lead to a progressive reduction in their capacity for local paracrine stimulation.

The association of mASCs and hyaluronic acids of different molecular weights do result in different levels of growth factor secretion. Our findings suggest that the use of HA-HLW generated no impairment in the paracrine effect of mASCs, even favoring the expression of some growth factors. Within the limitations of an in vitro study, the present results might also indicate that the combination of HA-HLW matrix with mASCs might represent a promising alternative and point to the viability of extended investigations, as greater secretion of these osteogenic cytokines was verified in the tested group, even though in vivo studies involving bone regeneration are needed to confirm this hypothesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The present study protocol followed national and institutional guidelines for the care and use of animals and was approved by the Animal Research Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul, Brazil (CEUA_1471005851428).

Informed consent The present study does not require formal informed consent.

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