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Abstract: 350 words

Background: Hyaluronic acid(HA) has been reported to have a positive effect on periodontal wound healing following nonsurgical and surgical therapy. However to date, few basic *in vitro* studies have been reported investigating the potential of HA on human periodontal ligament(PDL) cell regeneration. Therefore, the aim of this study was to investigate the effect of HA on PDL cell compatibility, proliferation and differentiation in vitro.

Methods: Either non-cross-linked(HA_ncl) or cross-linked(HA_cl) HA was investigated. Human PDL cells were seeded in 7 conditions as follows 1) Control tissue culture plastic(TCP) 2) dilution of HA_ncl(1:100), 3) dilution of HA_ncl(1:10), 4) HA_ncl directly coated onto TCP, 5) dilution of HA_cl(1:100), 6) dilution of HA_cl(1:10) and 7) HA_cl directly coated onto TCP. Samples were then investigated for cell viability using a live/dead assay, an inflammatory reaction using real-time PCR and ELISA for MMP2, IL-1 and cell proliferation via an MTS assay. Furthermore, the osteogenic potential of PDL cells was assessed by alkaline phosphatase(ALP) activity, collagen1(COL1) and osteocalcin(OCN) immunostaining, alizarin red staining, and real-time PCR for genes encoding Runx2, COL1, ALP and OCN.

Results: Both HA_ncl and HA_cl showed high PDL cell viabilitity irrespective of conditions demonstrating its excellent biocompatibility. Furthermore, no significant difference in both mRNA and protein levels of pro-inflammatory cytokines including MMP2 and IL-1 expression was observed. Both diluted HA_ncl and HA_cl significantly increased cell numbers compared to control TCP samples at 3 and 5 days. HA_ncl and HA_cl in standard cell growth media significantly decreased ALP staining, COL1 immunostaining and down-regulated early osteogenic differentiation including Runx2, COL1 and OCN mRNA levels when compared to control samples. However when osteogenic differentiation medium(ODM) was added, interestingly the expression of early osteogenic markers increased by demonstrating higher levels of COL1 and ALP expression; especially in HA 1:10 diluted condition. Late stage osteogenic markers remained inhibited.

Conclusions: Both non-cross-linked and cross-linked HA were biocompatible, increased proliferation and early osteogenic differentiation. HA was however consistently associated with a significant decrease in late osteogenic differentiation on human PDL cells. Future in vitro and animal research is necessary to further characterize the optimal clinical delivery of HA for periodontal regeneration.

Key words: Hyaluronic acid, hyaluronan, periodontal regeneration, soft tissue regeneration, connective tissue regeneration

Introduction

Hyaluronic acid (HA; also termed hyaluronan or hyaluronate) is an anionic, nonsulfated glycosaminoglycan and considered an optimal biomaterial for tissue engineering, given its broad expression in connective tissue as well as the significant role it plays during organogenesis, cell migration and development in general [1-4]. Non-cross-linked HA (HA_ncl) is biodegradable, biocompatible, bioresorbable and also well known to improve tissue lubrication in cartilage, guides cell growth and differentiation, and speeds the healing and repair of chronic wounds [5]. Cross-linked HA (HA_cl) has also been utilized for tissue engineering as a scaffold to further improve the overall mechanical performance of the scaffolding material and rigidity supporting the growth of various cells [6-9]. HA has been widely used for patients with knee osteoarthritis due to its ability to provide cartilage tissue integrity [10, 11]. Furthermore, in the oral maxillo-facial area, HA injections have been used as a treatment option to manage symptoms of temporomandibular joint disorders [12, 13]. More recently, HA has also been utilized for applications for aesthetic purposes in the oral facial regions primarily to reduce or eliminate facial creases, interdental papilla loss and various other abnormalities [8, 14, 15].

Due to the growing use of HA in dentistry, HA has also been hypothesized to have influences on periodontal regeneration [16]. The management of periodontal defects is mainly a result of the fact that the tissues which comprise the periodontium, including the periodontal ligament, cementum and alveolar bone, represent three unique tissues [6]. HA is an essential component of the periodontal ligament matrix and has been shown to play various important roles in cell adhesion, migration and differentiation mediated by various HA binding proteins and cell-surface receptors such as CD44 [17]. CD44 is expressed in periodontal tissue and HA-CD44 interaction has been associated

with their proliferation and mineralization activities [18]. Furthermore, other advantages of HA include its anti-inflammatory activity promoting better soft and hard tissue healing response, which may be of significant interest during periodontal regeneration [19]. Based on these assumptions, exogenous HA has already been tested in patients with chronic periodontitis in several clinical studies reporting significant improvements in reducing bleeding on probing scores and probing depths [2, 20-23]. However, to date the in vitro effect of HA on periodontal ligament activity has not been clearly investigated.

Therefore, the aim of the present study was to investigate the effects of HA_ncl and HA_cl on PDL cells by stimulating cells with 3 different conditions of either diluted samples with HA (co-existing at 2 concentrations of 1:10 and 1:100 dilutions) with HA or directly pre-coating HA on HA surfaces. PDL cells cultured with either HA_ncl or HA_cl were assessed for cell viability at 24 hours, inflammatory cytokines expression at 1 day, cell proliferation at 1, 3 and 5 days and osteogenic differentiation markers expression at 7 days and 14 days.

Materials and Methods

Regents and cell culture

HA was kindly provided by Regedent (Zürich, Switzerland) utilizing 2 compositions of HA including non-cross-linked native HA (hyaDENT, BioScience GmbH, Dümmer, Germany) as well as a cross-linked HA (hyaDENT BG, BioScience GmbH). HyaDENT (HA_ncl) contains a formulation of 14.0 mg/mL of sodium hyaluronate (synthesized by bacterial fermentation in *Streptococcus* [24], non-cross-linked), and hyaDENT BG (HA_cl) contains 2.0 mg/mL of sodium hyaluronate and 16.0mg/mL of sodium hyaluronate cross-linked with butanediol diglycidyl ether (BDDE). Both HA compositions were cultured under three in vitro conditions including (1) dilution of HA (1:100), (2) dilution of HA (1:10), and (3) 100 µl of HA coated directly to tissue culture plastic (TCP) based on our previous report [25]. In short, HA were diluted in standard cell culture growth medium consisting of DMEM (Gibco), 10% fetal Bovine serum (FBS; Gibco) and 1% antibiotics (Gibco). The 100 µl of HA were directly pre-coated in per 24-culture well and then the amount of HA were adjusted the same between 1:10 dilution and coating conditions per well in the end post cell seeding.

The primary human PDL cells were obtained from the middle third portion of the each teeth extracted from three healthy patients with no signs of periodontal disease extracted for orthodontic reasons as previously described [26, 27]. PDL cells were detached from TCP using 0.25% EDTA-Trypsin (Gibco, Life technologies, Carlsbad, CA, USA) prior to reaching confluency. Cells used for experimental seeding were from passages 4-6. Cells were cultured in a humidified atmosphere at 37°C in cell growth medium. For in vitro experiments, cells were seeded with HA in contained within cell culture media at a density of 10,000 cells in 24 well culture plates for cell proliferation experiments and 50,000 cells per well in 24 well dishes for real-time PCR, ELISA, ALP

assay, immunostaining and alizarin red experiments. For experiments lasting longer than 5 days, medium was replaced twice weekly.

Cell viability

Primary human PDL cells were seeded in at a density of 12,500 cells / cm^2 with either coated on control (TCP), non-crossed-linked HA at a dilution of 1:100, 1:10 or pre-coated HA as well as cross-linked HA at a dilution of 1:100, 1:10 or pre-coated with HA_cl on chamber slides (Sigma, St. Louis, MO, USA). At 1 day post cell seeding, cells were evaluated using a live-dead staining assay according to the manufacturer's protocol (Enzo Life Sciences AG; Lausen, Switzerland) as previously described [28]. Fluorescent images were quantified with a fluorescent microscope (OLYMPUS BX51, Tokyo, Japan).

Proliferation assay

PDL cells were seeded in 24-well plates at a density of 10,000 cells per well in a 24 well culture plate with the various concentrations of HA including dilutions of 1) 1:100, 2) 1:10 and 3) pre-coated culture wells. Cells were quantified using fluorescent MTS assay (Promega, Madison, WI, USA) at 1, 3 and 5 days for cell proliferation as previously described [29]. At desired time points, cells were washed with phosphate buffered solution (PBS) and quantified using a ELx808 Absorbance Reader (BIO-TEK, Winooski, VT, USA).

Real-time PCR analysis

PDL cells were first cultured for 1 day with HA in order to investigate inflammatory marker expressions including matrix metalloproteinase-2 (MMP2) and

interleukin-1 (IL-1). Moreover in order to investigate the effects of HA on osteogenic differentiation, the cells were stimulated for 7 days within each concentration of HA with and without osteogenic differentiation medium (ODM), which consisted of DMEM supplemented with 10% FBS, 1% antibiotics, 50 μg/mL ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma) to promote osteogenic differentiation as previously described [30]. The RNA expressions of runt-related transcription factor 2 (Runx2), collagen1a2 (COL1a2), alkaline phosphatase (ALP), osteocalcin (OCN) in either condition were measured. Total RNA was harvested using High Pure RNA Isolation Kit (Roche, Basel, Switzerland). Primer and probe sequences were fabricated with primer sequences according to Table 1. Real-time RT-PCR was performed using Roche Master mix and quantified on an Applied Biosystems 7500 Real-Time PCR machine. A Nanodrop 2000c (Thermo, Wilmington, DE, USA) was used to quantify total RNA levels. The ΔΔCt method was used to calculate gene expression levels normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Protein quantification with ELISA

The supernatant culture media was collected at 1 and 3 days post cell seeding. MMP2 (DY902, range = 0.625 – 20.00 ng/mL) and IL-1β/IL-F2 (DY201, range = 3.91 - 250 pg/mL) were quantified using an ELISA assays (R&D Systems, Minneapolis, MN, USA) according to manufacturer's protocol as previously described [31, 32]. Briefly, 100 μL of assay diluents and 100 μL of sample were incubated for 2 hours at room temperature in antibody-precoated 96-well plates. Wells were washed 3 times with washing buffer, incubated for 2 hours with peroxidase-conjugated antibody solution, washed again, followed by addition of 100 μL of substrate solution for 20 minutes and 50 μL of stopping solution for 20 minutes. Absorbance was measured at 450 nm and 570

nm on an ELx808 Absorbance Reader and subtract at 570 nm from the readings at 450 nm.

ALP activity assay

PDL cells were stimulated within each concentration of HA with and without ODM. At 7 days, cells were quantified for alkaline phosphatase expression utilizing a cell imaging system. Alkaline phosphatase activity was monitored using Leukocyte alkaline phosphatase kit (procedure No. 86, Sigma) as previously described [33]. PDL cells were fixed by immersion in a citrate-acetone-formaldehyde fixative solution for 5 minutes. Alkaline dye mixture were prepared by mixing 1 mL Sodium Nitrite Solution and 1 mL of fast red violet alkaline solution dissolved in 45 mL of distilled water and 1 mL of Naphtol AS-Bl alkaline solution. Surfaces were then placed in alkaline dye mixture solution for 15 minutes protected from light followed by rinsing in deionized water. All images were captured on a Wild Heerbrugg M400 ZOOM Makroskop (WILD HEERBRUGG, Heerbrugg, Switzerland) at the same magnification and light intensity and imported into Image J software (NIH, Bethesda, MD, USA). Thresholding was used to generate percent stained values for each field of view.

Immunofluorescent staining

At 14 days post PDL cell seeding, the cells were fixed with 4% formaldehyde for 10 minutes, followed by permealized within PBS containing 0.2% Triton X-100 and blocked in PBS containing 1% bovine serum albumin (BSA, Sigma) for 1 hour. Subsequently, cells incubated for over night at 4°C either with polyclonal rabbit to collagen type I antibody (sc-28657, Santa Cruz, CA, USA) or polyclonal rabbit to osteocalcin antibody (sc-30044, Santa Cruz) at dilution of 1:75 in PBS containing 1%

BSA. After washing with PBS, cells were incubated for 1 hour at 37°C with TR-conjugated-goat-anti-rabbit antibodies (sc-2780, Santa Cruz) (1:100) diluted in PBS containing 1% BSA. Prior to viewing, samples were mounted with Vectashield containing DAPI nuclear staining (Vector, Burlingame, CA, USA). Images were captured from each surface on an OLYMPUS BX51 fluorescence microscope. The optical density (OD) of the fluorescent staining was quantified from 3 independent experiments using Image J software.

Mineralization assay

Alizarin red staining was performed to determine the presence of extracellular matrix mineralization. PDL cells were stimulated for 14 days within each concentration of HA in ODM. After 14 days, cells were fixed in 96% ethanol for 15 minutes and stained with 0.2% alizarin red solution (Alizarin Red S, Sigma) in water (pH 6.4) at room temperature for 1 hour as previously described [30]. All images were captured and the percentage of staining was evaluated in the same manner as the ALP assay.

Statistical analysis

All experiments were performed in triplicate with three independent experiments for each condition. Mean and standard error (SE) were analyzed for statistical significance using one-way analysis of variance with Turkey test (*, p values < 0.05 was considered significant) by GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Cell viability and proliferation in response to HA

To investigate the biocompatibility of HA towards human PDL cells, a live/dead assay was utilized. It was determined that both HA_ncl or HA_cl maintained complete cell viability in the presence of HA at various concentrations (Fig. 1). Moreover, the mRNA expression of inflammatory cytokine genes encoding MMP2 and IL-1 of PDL cells treated with either HA_ncl or HA_cl demonstrated little differences at 1 day post seeding when compared to control samples (Fig. 2A, C). Inflammatory cytokine release of MMP and IL-1 from PDL cells treated with either HA_ncl or HA_cl was investigated at 1 day and 3 days post cell seeding (Fig. 2B, D). There was no significant difference of MMP2 and IL-1 release among both HA_ncl and HA_cl treated goups as well as mRNA expression (Fig. 2 B, D). Thereafter, PDL cell proliferation was investigated in response to HA_ncl and HA_cl at 1, 3 and 5 days post seeding (Fig. 3). It was found that while cell numbers were indifferent at 1 day post seeding (Fig. 3A), a significant increase was observed at 3 and 5 days in either diluted conditions for both HA_ncl and HA_cl (Fig. 3B, C).

Cell differentiation in response to HA

Thereafter, human PDL cells were investigated for their ability to differentiate when cultured with HA_ncl and HA_cl (Fig. 4-7). The mRNA expression of osteogenic markers was compared by real-time PCR for genes encoding Runx2, COL1a2, ALP and OCN at 7 days (Fig. 4). All HA_ncl and HA_cl treatments significantly down-regulated Runx2, COL1a2, ALP and OCN mRNA levels when compared with control samples (Fig. 4A, C, E, G), in normal growth medium. On the other hand, when ODM supplement was added to culture media, it was found that no significant difference was observed in Runx2 mRNA expression (Fig. 4B), whereas both HA_ncl and HA_cl in 1:10 diluted condition demonstrated a 3-fold significant increase in COL1a2 mRNA levels (Fig. 4D)

and a 20-fold increase in ALP levels. Nevertheless, OCN mRNA expression was significantly downregurated in either 1:10 diluted or coated conditions for both HA_ncl and HA_cl despite culture with ODM at 7 days post cell seeding (Fig. 4H).

Moreover it was generally found that cell culture with HA_ncl significantly decreased ALP staining, HA_cl treatment demonstrated no significant changes in ALP staining at 7 days likely as a result of the cross-linking HA (Fig. 5A). Interestingly, when ODM was added to cell culture media to promote osteogenic differentiation, HA_ncl at a dilution of 1:10 significantly increased up to 5-fold ALP staining when compared to all other treatment modalities (Fig. 5B, depicted by **). In addition, both HA_ncl and HA_cl coated samples demonstrated significant increase of ALP staining under ODM supplement when compared with control at 7 days (Fig. 5B). Thereafter, it was demonstrated that both HA_ncl and HA-cl significantly decreased COL1 immunofluorescent staining at 14 days post seeding (Fig. 6A, C) as well as mRNA expression via realtime PCR at 7 days (Fig. 4C).

Cells were further investigated by OCN immunostaining and alizarin red staining when induced by HA_ncl and HA_cl treatment (Fig. 6 B, D, 7). All HA_ncl and HA_cl treatment significantly decreased OCN protein expression and alizarin red staining when compared to control samples as well as OCN mRNA expression (Fig. 4G, H).

Discussion

HA has been employed as a biomaterial for medical devices such as dermal fillers, adhesion barriers, drug delivery carriers and scaffolds for tissue engineering due to its high biocompatibility [16, 34, 35]. With respect to periodontal tissue regeneration, HA has recently been investigated due to its ability to limit tissue inflammation, improve wound healing (soft tissue repair), and possibly speed alveolar bone regeneration [21, 23]. In the present study the effect of high molecular weight (HMW) HA_ncl and a HA_cl were evaluated on periodontal ligament cells. It was first found that both HA_ncl and HA_cl did not elucidate PDL cell apoptosis even at higher concentrations (Fig. 1). Both compositions of HA were shown extremely and equally biocompatible on PDL cells.

We then sought to investigate the inflammatory response of HA on PDL cells. It has previously been shown that low molecular weight (LMW, 100-500 kDa) HA, but not the native HMW HA molecules (~4,000 kDa), stimulated inflammatory cells and LMW-HA is prominent in the gingival tissue of patients with initial stage of periodontitis [36]. Nakatani et al. reported that the expression of MMP-1 in cultured human PDL cells was enhanced by the treatment with HAoligo through p38MAPK signaling pathway, suggesting that the degradation of periodontal tissue under pathologic conditions may involve MMP-1 induction by HAoligo [37]. On the other hand, the majority of products used in connection to periodontal therapy contain HMW HA [8]. The mechanism of anti-inflammatory effects of HMW HA has been widely investigated previously due to the pronounced impact and desire for treatment modalities in the field of osteoarthritis and periodontitis. HA has demonstrated to modulate inflammation in articular chondrocytes and synoviocytes, due to the specific inhibition of MMPs [19, 38] and down-regulation of TNF-α, IL-8, and inducible nitric oxide synthase [39]. In the present study, HMW HA and HA_cl did not affect the mRNA expressions of inflammatory cytokines including MMP-2

and IL-1 under any conditions at 1 days post seeding of healthy PDL cells (Fig. 2). The present study was assessed on healthy human PDL cells, whereas in the presence of inflammation, HMW HA within the tissues is reportedly broken down to LMW HA by reactive oxygen species or by bacterial hyaluronidase [8, 40-42]. Therefore, these combined results suggest that although HA demonstrated little influence on inflammation in the current study utilizing healthy PDL cells, a more pronounced effect could be observed in diseased tissues due to gingival tissue inflammatory in periodontitis.

Thereafter, the effects of HA demonstrated an increase in PDL cell proliferation under the present in vitro conditions (Fig. 3). During the granulation phase of periodontal tissue repair, HA has been shown to be a key protein highly expressed in various tissues responsible for promoting cell proliferation, migration and granulation tissue organization [19]. In non-mineralized tissues, HA is transiently elevated during the formation of tissue repair and helps with the re-establishment of the epithelium [40]. In our study, both 1:100 and 1:10 dilution of HA_ncl as well as HA_cl promoted cell proliferation at 3 and 5 days post PDL cell seeding (Fig. 3). It is demonstrated by Takeda et al. in the same manner that HMW HA enhanced cell adhesion and proliferation on human periodontal ligament cells [9]. Both exogenous co-exsisting HA compositions but not coated HA promoted PDL cell proliferation, which suggests a positive effect for periodontal tissue regeneration.

The effect of HMW HA on cell differentiation still provoked controversies, while most studies reported that cell differentiation was increased by low-MW HA [16, 43-45]. Huang et al. [43] reported osteogenic cell behavior of HMW HA in a concentration-dependent manner on rat mesenchymal stem cells. Moreover it was demonstrated that sulfated HMW HA could enhance the osteogenic differentiation of human mesenchymal

stem cells [46]. On the other hand, some studies showed that cell differentiation was not affected by HMW HA [16, 47-50]. Kaneko et al. demonstrated that HA inhibited BMP-induced osteoblastic differentiation through the CD44 receptor in osteoblasts [47]. Furthermore, the animal pure bone defect models demonstrated debatable osteogenic effects of HA. HA-gelatin hydrogels were loaded into biphasic calcium phosphate (BCP) ceramics in rabbit femurs promoted new bone formation and collagenous mineralization [48]. HA gel has also been shown to accelerate the healing process in tooth sockets of rats stimulating the expression of osteogenic proteins such as bone morphogenetic protein (BMP)-2 and osteopontin (OPN) in vivo [49]. On the other hand, Atilgan et al. reported that demineralized bone matrix (DBM) + tricalcium phosphate + HA combination showed more new bone formation without HA [50]. Therefore, the remains great interest to determine what may be causing the reported variability in the literature.

In the present study, the expression of osteogenic markers stimulated by HA_ncl and HA_cl were investigated with and without ODM. Both HA_ncl and HA_cl down-regulated Runx2, COL1a2 and OCN mRNA levels suggesting that HA treatment of PDL cells inhibits osteogenesis in regular growth medium (Fig. 4). Moreover, it was observed that HA significantly decreased ALP activity at 7 days, COL1 and OCN immunofluorescent staining at 14 days when compared with control sample in growth medium (Fig. 5A, 6). Interestingly however, we found that either HA_ncl or HA_cl in 1:10 diluted condition demonstrated 3-fold and 20-fold increase in COL1a2 and ALP mRNA levels respectively in ODM whereas these genes were down-regulated in routine cell culture medium (Fig. 4D, F). Moreover HA at a dilution of 1:10 increased a 5-fold significant increase in ALP staining compared to control samples however only in the presence of osteogenic medium (ODM) (Fig. 5B). Irrespective of ODM, HA consistently

down-regulated OCN mRNA expression as well as alizarin red staining thus demonstrating that it prevents full osteoblast maturation (Fig. 7). Takeda et al. demonstrated that HMW HA at concentration of 1, 25, 50 and 100 μg/mL did not increase cell differentiation on human periodontal ligament cells, suggesting that HMW HA affects cell events at early phases rather than those at late phases [9]. In the present study, the effect of HA_ncl and HA_cl at 1.6 mg/mL (1:10) and 160 μg/mL (1:100) were investigated. Both HA_ncl and HA_cl in 1:10 diluted condition demonstrated a 3-fold significant increase in COL1a2 mRNA levels (Fig. 4D) and a 20-fold increase in ALP levels in ODM, which suggested that higher concentration of HMW HA might induce early osteoblastic differentiation. For periodontal regeneration, HMW HA was investigated in a dog intrabony defect model and neither significant periodontal tissue nor bone tissue regeneration was observed [9]. Most recently Kim et al. reported that HA improved wound healing and bone formation of hemisection-performed extraction sockets with communication of the periodontal lesion in a canine model [51]. The effect of HMW-HA on periodontal regeneration was still unclear and controversial as well as the effect on bone regeneration however, its contradiction might be explained because of the varieties of HA molecular weight, modification methods, concentration, existence of inflammation and also cell types. It may be that HA requires an osteoconductive matrix during periodontal regeneration to improve the osteogenic phase of PDL regeneration however this hypothesis certainly requires further investigation.

Conclusion

Both HA_ncl and HA_cl were shown to be extremely biocompatible and at both concentrations were associated with a significant increase in PDL cell numbers. The early osteogenic differentiation markers such as Runx2, COL1 and ALP were

significantly down-regulated in standard cell growth media. Interestingly, with the addition of ODM, the expression of early osteogenic markers were shown to significantly increase in COL1 and ALP especially at higher concentration (1:10) utilizing both HA_ncl and HA_cl diluted condition. Nevertheless, the late stage osteogenic marker such as OCN as well as calcium formation was inhibited regardless of ODM addition. Future in vitro models including three-dimensional culture and animal research are necessary to further characterize the optimal uses as well as delivery systems of HA for improved clinical use.

Abbreviations

HA: Hyaluronic acid

HA_ncl: non-cross-linked hyaluronic acid

PDL: periodontal ligament

HA_cl : cross-linked HA

TCP: tissue culture plastic

ALP : alkaline phosphatase

OCN: osteocalcin

MMP2: matrix metalloproteinase-2

IL-1: interleukin-1

COL: collagen

OCN: osteocalcin

BDDE: butanediol diglycidyl ether

DMEM: Dulbecco's modified eagle medium

EDTA: ethylenediaminetetraacetic acid

FBS: fetal Bovine serum

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

PBS: phosphate buffered solution

RT-PCR: reverse transcription polymerase chain reaction

Runx2: runt-related transcription factor 2

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

BSA: bovine serum albumin

OD: optical density

SE: standard error

HMW: high molecular weight

LMW: low molecular weight

OPN: osteopontin

ODM: osteogenic differentiation medium

BCP: biphasic calcium phosphate

DBM: demineralized bone matrix

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Competing interests

Patrick Schmiddlin has received a research grant from Regedent, Switzerland. Both non-cross-linked and cross-linked HA utilized in the present study were also provided by Regedent. All authors declare that they have no competing interests and financial interests to disclose.

Authors' contributions

MFK, HDM, AM, AL, AS, PRS and RJM have made substantial contributions to conception and design, MFK, HDM, AM and PRS have done the acquisition of data, and MFK, HDM, AL, AS, and RJM have performed analysis and interpretation of data. MFK, RJM, AS and RJM have been involved in drafting the manuscript or revising it critically for important

intellectual content. MFK, HDM, AM, AL, AS, PRS and RJM have given final approval of the version to be published.

Availability of Data and Materials

The data supporting the findings from this study can be found entirely within the content of the present manuscript. Data sets are available upon request.

Ethics and Consent statement

Not applicable. The human PDL cells within the present manuscript did not require an ethical approval as the tissues were to be discarded and was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Canton of Bern, University of Bern, Switzerland.

Consent for publication

Not applicable

Figure Legends

Figure 1: Cell viability staining of primary human primary PDL cells exposed to control (TCP), non-cross-linked HA (HA_ncl) and cross-linked HA (HA_cl) surfaces. For cell viability, Live-Dead staining was done with viable cell appearing in green and dead cells in red. The results from these experiments demonstrated that both HA_ncl and HA_cl are highly biocompatible at dilutions of 1:100 and 1:10 as well as pre-coated onto cell culture plastic.

Figure 2: Real-time PCR of PDL cells seeded with HA_ncl and HA_cl for genes encoding (A) matrix metalloproteinase-2 (MMP2), (C) Interleukin-1 (IL-1), at 1 days post seeding. Protein release at 1 and 3 days of (B) MMP2, (D) IL-1, (** denotes significantly higher than all other modalities among HA treated groups, $p<0.05$).

Figure 3: Proliferation assay of PDL cells seeded with HA_ncl and HA_cl at (A) 1, (B) 3 and (C) 5 days post seeding. It was found that both HA and HA_cl at dilutions of 1:100 and 1:10 significantly increased cell numbers at 3 days and 5 days post seeding when compared to control samples († denotes significantly higher than control, $p<0.05$).

Figure 4: Real-time PCR of PDL cells seeded with HA_ncl and HA_cl treatment for genes encoding (A, B) Runx2, (C, D) Collagen 1 alpha 2 (COL1a2), (E, F) alkaline phosphatase (ALP) and (G, H) osteocalcin (OCN) at 7 days post seeding. Cells were treated (A, C, E, G) in regular growth medium or (B, D, F, H) with ODM. (* denotes significant difference, $p<0.05$; # denotes significantly lower than control, $p<0.05$; † denotes significantly higher than control, $p<0.05$; ** denotes significantly higher than all other treatment modalities, $p<0.05$).

Figure 5: Alkaline phosphatase staining of PDL cells treated by HA_ncl and HA_cl (A) in growth medium or (B) within ODM at 7 days post seeding. Both HA_ncl and HA_cl significantly decreased ALP staining without ODM while with ODM both HA_ncl and HA_cl significantly increased ALP staining when compared to control samples (* denotes significant difference, $p<0.05$; # denotes significantly lower than control, $p<0.05$; † denotes significantly higher than control, $p<0.05$; ** denotes significantly higher than all other treatment modalities, $p<0.05$).

Figure 6: Immunofluorescent COL1 staining and OCN staining at 14 days post cell seeding with HA_ncl and HA_cl. (A) The merged images of immunofluorescent detection of COL1 (red) and DAPI (blue). (B) The merged images of immunofluorescent detection of OCN (red) and DAPI (blue). (C, D) Quantified data of (C) COL1 and (D) OCN immunostaining at 14 days (# denotes significantly lower than control, $p<0.05$).

Figure 7: Alizarin red staining denoting mineralization at 14 days post seeding. (A) Alizarin red staining images and (B) quantified data of alizarin red staining from colour thresholding software for PDL cells treated with HA_ncl or HA_cl (** denotes significantly higher than all other treatment modalities, $p<0.05$). It was found that both HA_ncl and HA_cl treatment significantly decreased alizarin red staining.

Table 1: PCR primers for genes encoding MMP2, IL-1, Runx2, ALP, COL1a2, OCN and GAPDH

Gene	Primer Sequence
hMMP2 F	ccccaaaacggacaaaga
hMMP2 R	cttcagcacaaacaggttgc
hIL-1 F	ggttgagtttaagccaatcca
hIL-1 R	ggtgatgacctaggcttgatg
hRunx2 F	tcttagaacaaattctgcccttt
hRunx2 R	tgctttggtcttgaaatcaca
hCOL1a2 F	cccagccaagaactggtatagg
hCOL1a2 R	ggctgccagcattgatagtttc
hALP F	gacctcctcggaagacactc
hALP R	tgaagggcttcttgtctgtg
hOCN F	agcaaaggtgcagcctttgt
hOCN R	gcgcctgggtctcttcact
hGAPDH F	agccacatcgctcagacac
hGAPDH R	gcccaatacgaccaaatcc

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