ASCORBIC ACID ENHANCES BONE PARAMETER EXPRESSION IN HUMAN GINGIVAL MESENCHYMAL STEM CELLS

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Ascorbic acid (AS), also known as vitamin C or ascorbate, is an essential dietary nutrient which plays a vital role in biological processes through various different mechanisms, in particular for the biosynthesis of collagen. The aim of the study was to establish the possibility of enhancing the osteogenic differentiation potential by manipulating the cellular micro-environment, through AS supplementation in human gingival mesenchymal stem cells (hGMSCs) at different concentrations, such as 60 and 90 µg/mL, for three weeks. Human GMSCs are considered a stem cell population, easily obtainable and displaying a remarkable immunotherapeutic potential and regenerative repair expression. Osteogenic differentiation level induced from AS was assayed by histochemical characterization, using light microscopy through Alizarin red S staining. The transcript levels of Collagen 1A1 (COL1A1), runt-related transcription factor 2 (RUNX2), bone morphogenetic protein 2/4 (BMP2/4), osteopontin (OPN) and osteonectin (SPARC) were determined by quantitative RT-PCR. Protein expression of COL1A1, RUNX2, BMP2/4, OPN, SPARC were studied trough Western blotting and confocal laser scanning microscopy (CLSM). Our results demonstrate that AS supports osteogenic differentiation in stem cells from gingiva niche as shown by osteogenic marker upregulation and by *de novo* production of calcium phosphate deposits as revealed by Alizarin red S staining. In summary, the results of the current study provide evidence that hGMSCs undergo osteogenic differentiation with AS treatment, for that reason AS could be a promising candidate for the prevention and healing of bone-related diseases.

Bone is a specialized, highly dynamic connective tissue, in which pre-osteoblasts, osteoblasts, osteocytes, osteoclasts, and blood vessels play a key role in the maintenance of its homeostasis. In the past two decades, several studies have been carried out to understand the processes that regulate physiological bone turnover during lifetime (1, 2). In particular, during aging, bone diseases, such as osteopaenia and osteoporosis, cause a structural deterioration of tissue that can affect bone functional properties associated with an increased risk of fractures and representing a major global health problem that affects people all over the world (3). For these reasons, several drugs have been developed but they do not offer an effective solution, because they do not completely reduce the incidence of fractures and decline of functionality (4).

Nowadays, there is a growing interest in the development of alternative natural molecules that can contribute to the replacement and repair of injured

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bone tissue. Ascorbic acid (AS) is an important antioxidant and cofactor which is involved in the development, function and maintenance of several cell types in the body such as adipocytes, myoblast, chondroblasts, odontoblasts and osteoblasts (5). Current evidence suggests that AS is involved in collagen matrix synthesis. Several studies have shown that the addition of AS to cultured osteoblast-like cells stimulates the initial deposition of a collagenous extracellular matrix followed by the induction of specific genes associated with the osteoblast phenotype, such as alkaline phosphatase and osteocalcin (6).

AS, during the differentiation process of mouse calvaria-derived MC3T3-EI cells, promotes the induction of collagen matrix formation (5). Mesenchymal stem cells (MSCs), which were first identified in the bone marrow, can also now be obtained from various tissues, such as fat, synovial membrane, muscle, skin, trabecular bone, articular cartilage, and oral cavity (7-9). MSCs are able to differentiate into osteoblasts, chondroblasts, myoblasts, adipoblasts, myocardium and skin (10, 11). Human gingival MSCs (hGMSCs) are a novel type of pluripotent MSCs that exhibit self-renewal, multipotent differentiation potential and immunomodulatory capacities (12, 13). hGMSCs have attracted great attention due to their easy isolation, high proliferative capacity, homogeneity, stable phenotype and, notably, the fact that they maintain a normal karyotype and telomerase activity during prolonged culture (14). Therefore, hGMSCs are considered to be an optimal candidate cell source for tissue engineering and cell-based therapies. In the present study, we evaluated the effect of AS treatment at different concentrations (60 and 90 µg/mL) on hGMSCs, in terms of proliferation and osteogenic differentiation. Mainly, the effects were evaluated of AS treatment on osteogenic marker modulation such as Collagen 1A1 (COL1A1), Runt-related transcription factor 2 (RUNX2), bone morphogenetic protein 2 (BMP2), osteopontin (OPN) and osteonectin (SPARC), to evaluate the potential role of AS in the development of novel therapeutic strategies that could support bone tissue homeostasis.

MATERIALS AND METHODS

The present study was approved by the Ethics Committee of the Medical School, "G. d'Annunzio" University, Chieti, Italy (number 266/April 17, 2014). Informative consent before enrolment in the study was signed from all subjects.

Cell culture

To obtain hGMSCs, the gingival tissue was collected by a procedure previously described (10). Healthy patients scheduled to remove the third molar for orthodontic purpose were enrolled in the study. Tissue explants were cultured in Petri dish with Mesenchymal stem cells growth medium chemically-defined (MSCGM-CD) (Lonza, Basel, Switzerland) (15). The medium was replaced with a fresh one every two days. After two weeks of culture, cells spontaneously migrated from tissue explants. All experiments were performed with cells at 2nd passage.

Experimental design

Cells at 2nd passage were divided into four experimental groups: CTRL: hGMSCs cultured with basal medium (MSCGM-CD); AS 60 µg/mL: hGMSCs treated with AS at a concentration of 60 µg/mL; AS 90 µg/mL: hGMSCs treated with AS at a concentration of 90 µg/mL; and DIFF OSTEO: hGMSCs cultured with osteogenic differentiation medium as reported hereafter. For osteogenic differentiation, cells were cultured in osteogenic differentiation medium (Lonza) for three weeks. The osteogenic differentiation and the effects of AS were then evaluated.

Cell proliferation and viability assay

Cell proliferation was evaluated through MTT assay, as previously reported (16). $2x10^3$ cells per well were seeded into 96-well plates in medium volume of 200 µL to test all experimental groups at different endpoints, 24, 48 and 72 h. 20 µL of MTT (Promega, Milan, Italy) solution were added to each well. Absorbance at 490 nm was measured with a reference wavelength of 630 nm (17).

Cell viability was assessed by trypan blue exclusion test. For this purpose all samples were incubated with trypan blue solution at the same endpoint used for MTT testing (24, 48 and 72 h) and subsequently analysed with Burker's chamber at inverted light microscopy, as previously described (18).

Osteogenic differentiation evaluation

Evaluation of calcium deposition and ECM mineralization was obtained by Alizarin Red S (ARS) staining assay performed after 6 weeks. Cells were washed with PBS, fixed in 10% (v/v) formaldehyde (Sigma-Aldrich, Milan, Italy) for 30 min and washed twice with abundant dH_2O prior to addition of 0.5% Alizarin red S in H_2O, pH 4.0, for 1 h at room temperature. After cell incubation under gentle shaking, cells were washed with dH_2O four times for 5 min. For staining quantification, 800 µL 10% (v/v) acetic acid was added to each well. Cells incubated for 30 min were scraped from the plate, transferred into a 1.5 mL vial and vortexed for 30 s. The obtained suspension, overlaid with 500 µL mineral oil (Sigma-Aldrich), was heated to 85°C for 10 min, then transferred to ice for 5 min, carefully avoiding opening the tubes until fully cooled, and centrifuged at 20,000× g for 15 min (19). In addition, 500 µL of the supernatant were placed into a new 1.5 mL vial and 200 µL of 10% (v/v) ammonium hydroxide was added (pH 4.1–pH 4.5). Furthermore, 150 µL of the supernatant obtained from cultures were read in triplicate at 405 nm by a spectrophotometer (Synergy HT, BioTek, Bad Friedrichshall, Germany).

Confocal laser scanning microscope (CLSM) analysis

For immunofluorescence detections, cells grown on 8-well chamber slides were fixed using 4% paraformaldehyde diluted in 0.1M sodium phosphate buffer (PBS, Lonza). After the fixation step, cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min, followed by blocking with 5% skimmed milk in PBS for 30 min (20). Primary antibodies used for immunofluorescence were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA). COL1A1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), RUNX2 (1:100, Santa Cruz Biotechnology), BMP2/4 (1:200, Santa Cruz Biotechnology), OPN (1:200, Santa Cruz Biotechnology) and SPARC (1:250, Santa Cruz Biotechnology) were used as primary antibodies. Then cells were incubated by Alexa Fluor 568 red fluorescence conjugated goat anti-rabbit as secondary antibodies (1:200, Molecular Probes, Invitrogen, Eugene, OR, USA). Alexa Fluor 488 phalloidin green fluorescence conjugate (1:400, Molecular Probes) was used to mark the cytoskeleton actin. After immunofluorescence labelling cells were washed and incubated with TOPRO (1:200, Molecular Probes) for 1 h at 37°C (15). Samples were observed under Zeiss LSM800 confocal system (Zeiss, Jena, Germany). All the experiments were performed in triplicate.

Western blot analysis

Proteins (30 µg) derived from all experimental groups were processed as previously reported (21). All antibodies used for Western blot were purchased from Santa Cruz Biotechnology. After protein separation, saturated sheets were incubated overnight at 4°C with COL1A1 (1:200, Santa Cruz Biotechnology), RUNX2 (1:1000, Santa Cruz Biotechnology), BMP2/4 (1:750, Santa Cruz Biotechnology), OPN (1:750, Santa Cruz Biotechnology), SPARC (1:1000, Santa Cruz Biotechnology) and β-Actin (1:1000, Santa Cruz Biotechnology) (22). Then samples were washed and incubated in secondary antibody diluted 1:1000 in 1x TBS, 5% milk, 0.05% Tween-20. Protein specific bands were visualized by means of the ECL method (23).

Gene expression

Total RNA was isolated from all experimental groups used in the present study through the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA, USA). ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used for qPCR of studied markers (COL1A1 Hs00164004_m1; RUNX2 Hs00231692_m1; BMP2/4 Hs00154192_m1; OPN Hs00959010_m1; SPARC Hs00234160_m1; ThermoFischer, Milan, Italy). Beta-2 microglobulin (B2M Hs00187842_m1, Hs99999907_m1; ThermoFischer) was used for template normalization (24). Comparative $2^{-\Delta\Delta Ct}$ relative quantification method was used to analyze the mRNA expression.

Statistical analysis

Graph Pad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used to perform the statistical evaluation. Student's *t*-test was used to analyze the differences between the groups. The obtained results are reported as means ± SEM. A P-value <0.05 was considered statistically significant.

RESULTS

Ascorbic acid effects on cell proliferation and viability

To evaluate cell proliferation and viability, MTT and Trypan blue assay were performed on all considered samples. Cells treated with AS 60 µg/mL and AS 90 µg/mL showed no significant differences when compared to the CTRL and to the DIFF OSTEO samples (Fig. 1).

Ascorbic acid effects on the osteogenic differentiation

To evaluate the osteogenic differentiation process Alizarin red S staining was performed on all samples after three weeks of culture. Calcium deposits, stained in red were visible in AS 90 µg/mL and in DIFF OSTEO samples when compared to the CTRL and to the AS 60 µg/mL, indicating that a high concentration of AS showed some effect on osteogenic commitment (Fig. 2A-D). Bar graphs showed the quantitative results of the *in-vitro* staining (Fig. 2E).

Ascorbic acid treatment modulates the osteogenic markers in hGMSCs

Immunofluorescence staining was performed to evaluate the expression of markers related to the osteogenic differentiation. All considered markers, COL1A1, RUNX2, BMP2/4, OPN and SPARC, showed an increase expression in differentiated cells (DIFF OSTEO) and in cells treated with AS 90 µg/mL, when compared to the untreated cells (CTRL) and with cells treated with a lower concentration of AS (AS 60 µg/mL) (Fig. 3).

Ascorbic acid treatment modulates genes and proteins related to the osteogenic commitment

COL1A1, RUNX2, BMP2/4, OPN and SPARC are osteogenic markers related to the differentiation process. To evaluate their expression RT-PCR analyses were performed. The mRNA levels of COL1A1, RUNX2, BMP2/4, OPN and SPARC increased in AS 90 µg/mL and DIFF OSTEO (Fig. 4A). In particular, the obtained data confirmed the expression of related proteins evidenced by immunofluorescence (Fig. 4B).

DISCUSSION

Skeletal regeneration is often initiated by a traumatic episode that involves damage to the bone, which often includes the periosteum, bone marrow spaces, and surrounding soft tissues (25). Trauma, such as fracture or surgical cutting and drilling, causes a physiological disruption of the mineralized tissue matrix, death of many types of cells, and interruption of the local blood supply (26). Nowadays, stem cells that combine a capacity for self-renewal with the ability to initiate multiple differentiation cascades, resulting in the generation of cells with specialized functions are attracting great interest, in particular for bone regeneration and homeostasis.

Deficiencies in AS can lead to conditions such as scurvy, which, among other ailments, causes bleeding gums, bone pain and impaired wound healing. This work examined the functional importance of vitamin C related to the development and maintenance of bone tissues. Analysis of several epidemiological and *in-vivo* studies regarding the effect of vitamin C showed a positive effect on bone health. Overall, vitamin C exerts a positive effect on trabecular bone formation by influencing expression of bone matrix genes in osteoblasts. Recent studies on the molecular pathway for vitamin C actions that include direct effects of vitamin C on transcriptional regulation of target genes by influencing the activity of transcription factors and by epigenetic modification of key genes involved in skeletal development and maintenance have been discussed. AS can be considered an essential dietary nutrient required for various biological functions, including the biosynthesis of collagen (27). AS used in basal concentrations showed an antioxidant role as a water□soluble factor. At pharmacologic or increased doses, AS is also known to act as a prooxidant *in vitro*. Antioxidant AS reduces oxidizing substances, such as hydrogen peroxide (28). AS used at the appropriate dose could potentially function as a preventative therapy for heterotopic ossification through the same mechanism used by radiation therapy, without the harmful risks or side effects (29). In the current study, we evaluated the biological response of hGMSCs to AS at 60 and 90 µg/mL after three weeks of culture.

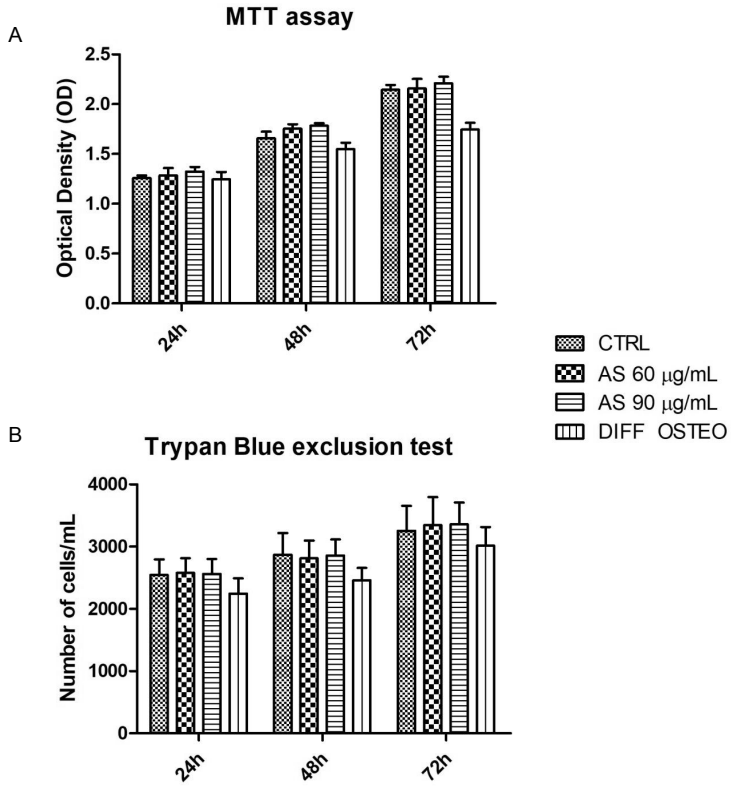

Fig. 1. *Cell proliferation and viability.* ***A)*** *MTT assay.* ***B)*** *Trypan blue exclusion test. Treatment with AS 60µg/mL and AS 90µg/mL showed no statistical differences in terms of cell proliferation and viability after 24, 48 and 72 h of culture.*

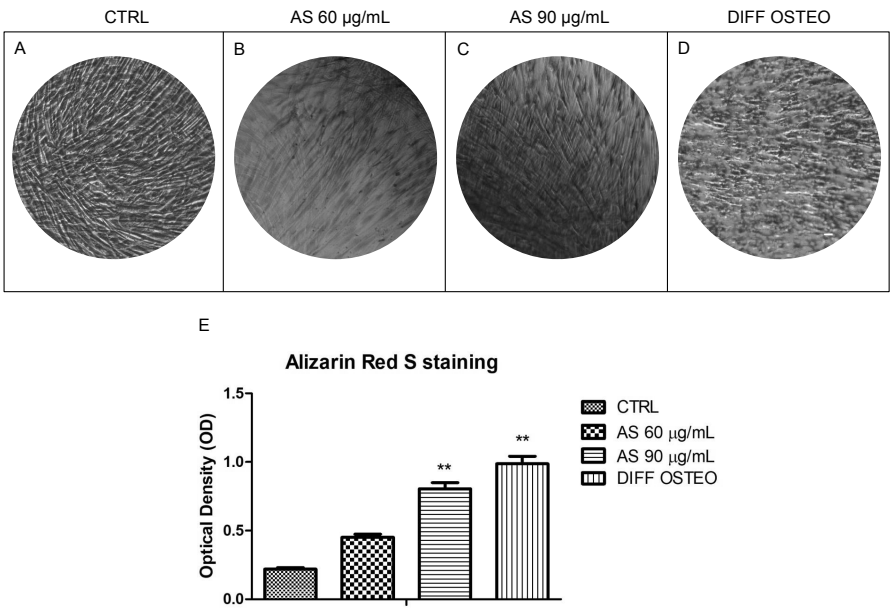

Fig. 2. *Osteogenic differentiation. Alizarin red S staining was used to evaluate the calcium depositions in (****A****) CTRL, (****B****) AS 60 µg/mL, (****C****) AS 90 µg/mL and (****D****) DIFF OSTEO by means of inverted light microscopy.* ***E)*** *Quantitative analysis of Alizarin red S staining was performed by measuring the absorbance of Alizarin red S at 562 nm. The results are expressed as mean – standard deviation (SD) (n = 3). **, $p < 0.01$ was recognized to be significant. Scale bar: 20 µm. Mag: 10X.*

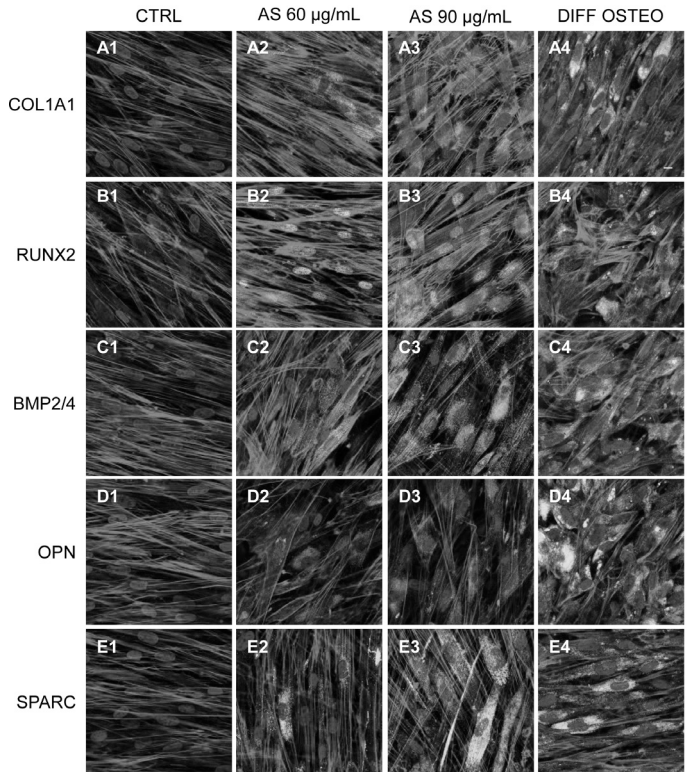

Fig. 3. *Immunofluorescence detection. Expression of COL1A1 in (A1) CTRL, (A2) AS 60µg/mL (A3) AS 90µg/mL and (A4) DIFF OSTEO. Expression of RUNX2 in (B1) CTRL, (B2) AS 60µg/mL (B3) AS 90µg/mL and (B4) DIFF OSTEO. Expression of BMP2/4 in (C1) CTRL, (C2) AS 60µg/mL (C3) AS 90µg/mL and (C4) DIFF OSTEO. Expression of OPN in (D1) CTRL, (D2) AS 60 µg/mL (D3) AS 90µg/mL and (D4) DIFF OSTEO. Expression of SPARC in (E1) CTRL, (E2) AS 60µg/mL (E3) AS 90µg/mL and (E4) DIFF OSTEO. Scale bar: 10 µm. Mag: 63X.*

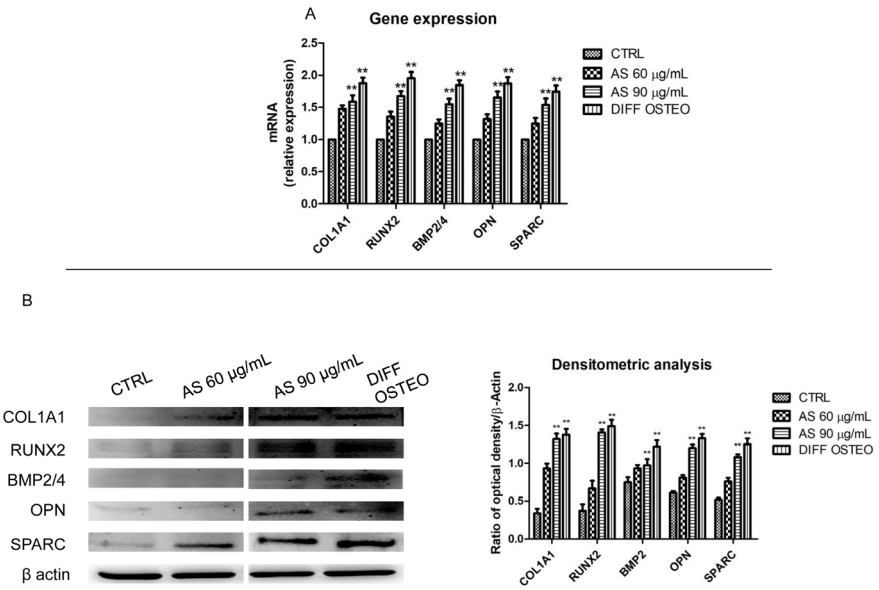

Fig. 4. *Gene expression. **A**) RT-PCR showed the expression of mRNA levels in CTRL, AS 60µg/mL, AS 90µg/mL and DIFF OSTEO of specific osteogenic related markers. **B**) Western blot analysis of COL1A1, RUNX2, BMP2/4, OPN and SPARC and their densitometric analysis using the expression ratios of β actin used as housekeeping protein.*

Alizarin red S staining indicated that the extent of mineralization was higher in the hGMSC cultures in presence of osteogenic medium compared to cultures supplemented with AS. The mRNA levels of several classic osteogenic genes, such as COL1A1, RUNX2, BMP2/4, OPN and SPARC, were analyzed in presence of AS. RUNX2 is a transcriptional factor essential for the activation of osteoblast-associated genes, and therefore is an important early indicator of osteoblast differentiation and bone formation (30). RUNX2 directly activates the transcription of genes such as Collagen type I, Osteopontin (OPN) or Osteonectin (SPARC).

In our experiments it was possible to detect that after three weeks of cultures the expression profile of RUNX2 was mainly upregulated in presence of differentiated medium in respect to samples treated with 60 and 90 μg/mL of AS. These data demonstrate that AS works as an osteogenic inductor in hGMSCs, but other cofactors are necessary for a complete differentiative pathway. The expression of SPARC, a non-structural glycoprotein secreted by osteoblasts, shows the identical trend of RUNX2 after three week in osteogenic differentiated culture conditions compared to AS-treated samples. The mRNA level of osteogenic-related markers is also confirmed by Western blot analysis. This protein binds calcium in bone, has an affinity for collagen and is also involved in cell-matrix interactions (31).

However, given that SPARC is more strongly expressed during the early stages of osteogenesis (i.e. during the proliferative and matrix deposition periods (32), our results may suggest that in the presence of the AS, osteogenic differentiation process was accelerated relative to the control groups. Upregulation of non-collagenous proteins, such as OPN and SPARC, with an important role in osteogenesis, confirmed the osteogenesis-promoting effect of the AS on hGMSCs. OPN codes for one of the most predominant non-collagenous proteins in bone extracellular matrix produced by osteoblasts, and it also promotes cell adhesion to the bone surface (33). OPN regulates cell–matrix interactions and signaling through binding to integrin- and CD44-receptors (34). Integrins are a family of heterodimeric transmembrane glycoproteins mediating cell-cell matrix and cell-cell adhesion, migration, proliferation and survival (35). Likewise, receptors of OPN integrins and CD44 also expressed in host stromal cells and in hGMSCs (36). OPN, which plays an important role in the regulation of vessel regeneration, is produced by mature osteoblasts during the bone formation process and is recognized as a major marker of osteogenic differentiation (37). During bone resorption, OPN is reported to be involved in the attachment of osteoclasts, and during bone mineralization plays a key role in osteogenesis and in the regulation of the crystal size (38).

Collagen 1A1, known as an early marker of osteoprogenitor cells, is considered fundamental for extracellular matrix synthesis (27), and in our study, COL1A1 was upregulated in DIFF OSTEO and AS 90 μg/mL.

Based on these results, validated through multiparametric analyses, we can conclude that AS is an antioxidant natural molecule which promotes osteogenesis, resulting in faster bone tissue formation in gingival-derived stem cells and can assume a strategic role in physiological and healthy bone remodelling.

REFERENCES

1. Diomede F, Gugliandolo A, Cardelli P, et al. Three-dimensional printed PLA scaffold and human gingival stem cell-derived extracellular vesicles: a new tool for bone defect repair. Stem Cell Res Ther 2018; 9:104.
2. Diomede F, D'Aurora M, Gugliandolo A, et al. A novel role in skeletal segment regeneration of extracellular vesicles released from periodontal-ligament stem cells. Int J Nanomed 2018; 13:3805-25.
3. Diomede F, D'Aurora M, Gugliandolo A, et al. Biofunctionalized scaffold in bone tissue repair. Int J Mol Sci 2018; 19. doi: 10.3390/ijms19041022.
4. Khosla S, Hopbauer LC. Osteoporosis treatment: recent developments and ongoing challenges. Lancet Diabetes Endo 2017; 5:898-907.
5. Franceschi RT, Iyer BS, Cui YQ. Effects of ascorbic-acid on collagen matrix formation and osteoblast differentiation in murine Mc3t3-E1 cells. J Bone

Miner Res 1994; 9:843-54.
6. Franceschi RT, Young J. Regulation of alkaline-phosphatase by 1,25-Dihydroxyvitamin-D3 and ascorbic-acid in bone-derived cells. J Bone Miner Res 1990; 5:1157-67.
7. Gugliandolo A, Diomede F, Cardelli P, et al. Transcriptomic analysis of gingival mesenchymal stem cells cultured on 3D bioprinted scaffold: A promising strategy for neuroregeneration. J Biomed Mater Research A 2018; 106:126-37.
8. Trubiani O, Toniato E, Di Iorio D, et al. Morphological analysis and interleukin release in human gingival fibroblasts seeded on different denture base acrylic resins. Int J Immunopathol Pharmacol 2012; 25:637-43.
9. Di Nisio C, De Colli M, di Giacomo V, et al. A dual role for beta1 integrin in an in vitro Streptococcus mitis/human gingival fibroblasts co-culture model in response to TEGDMA. Int Endod J 2015; 48:839-49.
10. Diomede F, Gugliandolo A, Scionti D, et al. Biotherapeutic effect of gingival stem cells conditioned medium in bone tissue restoration. Int J Mol Sci 2018; 19. doi: 10.3390/ijms19020329.
11. De Colli M, Zara S, di Giacomo V, et al. Nitric oxide-mediated cytotoxic effect induced by zoledronic acid treatment on human gingival fibroblasts. Clin Oral Investig 2015; 19:1269-77.
12. Mitrano TI, Grob MS, Carrion F, et al. Culture and characterization of mesenchymal stem cells from human gingival tissue. J Periodontol 2010; 81:917-25.
13. Zhang Q, Shi S, Liu Y, et al. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. J Immunol 2009; 183:7787-98.
14. Diomede F, Zini N, Pizzicannella J, et al. 5-Aza Exposure improves reprogramming process through embryoid body formation in human gingival stem cells. Front Genet 2018; 9:419.
15. Pizzicannella J, Diomede F, Merciaro I, et al. Endothelial committed oral stem cells as modelling in the relationship between periodontal and cardiovascular disease. J Cell Physiol 2018; 233:6734-47.
16. Diomede F, Zingariello M, Cavalcanti M, et al. MyD88/ERK/NFkB pathways and pro-inflammatory cytokines release in periodontal ligament stem cells stimulated by Porphyromonas gingivalis. Eur J Histochem 2017; 61:2791.
17. Diomede F, Merciaro I, Martinotti S, et al. miR-2861 is involved in osteogenic commitment of human periodontal ligament stem cells grown onto 3D scaffold. J Biol Regul Homeost Agents 2016; 30:1009-18.
18. Cavalcanti MF, Maria DA, de Isla N, et al. Evaluation of the proliferative effects induced by low-level laser therapy in bone marrow stem cell culture. Photomed Las Surg 2015; 33:6101-6.
19. Ballerini P, Diomede F, Petragnani N, et al. Conditioned medium from relapsing-remitting multiple sclerosis patients reduces the expression and release of inflammatory cytokines induced by LPS-gingivalis in THP-1 and MO3.13 cell lines. Cytokine 2017; 96:261-72.
20. Pizzicannella J, Gugliandolo A, Orsini T, et al. Engineered extracellular vesicles from human periodontal-ligament stem cells increase VEGF/VEGFR2 expression during bone regeneration. Front Physiol 2019; 10:512.
21. Giacoppo S, Thangavelu SR, Diomede F, et al. Anti-inflammatory effects of hypoxia-preconditioned human periodontal ligament cell secretome in an experimental model of multiple sclerosis: a key role of IL-37. FASEB J 2017; 31:5592-608.
22. Trubiani O, Ballerini P, Murmura G, et al. Toll-like receptor 4 expression, interleukin-6,-8 and Ccl-20 release, and Nf-Kb translocation in human periodontal ligament mesenchymal stem cells stimulated with Lps-P-gingivalis. Eur J Inflamm 2012; 10:81-89.
23. Mammana S, Gugliandolo A, Cavalli E, et al. Human gingival mesenchymal stem cells (GMSCs) pre-treated with vesicular Moringin nanostructures as a new therapeutic approach in a mouse model of spinal cord injury. J Tissue Eng Regen Med 2019; 13:1109-21.
24. Pizzicannella J, Cavalcanti M, Trubiani O, Diomede F. MicroRNA 210 mediates VEGF upregulation in human periodontal ligament stem cells cultured on 3D hydroxyapatite ceramic scaffold. Int J Mol Sci 2018; 19.

25. Plotkin LI, Bruzzaniti A. Molecular signaling in bone cells: Regulation of cell differentiation and survival. Adv Protein Chem Struct Biol 2019; 116:237-81.
26. Scioli MG, Bielli A, Gentile P, Cervelli V, Orlandi A. Combined treatment with platelet-rich plasma and insulin favours chondrogenic and osteogenic differentiation of human adipose-derived stem cells in three-dimensional collagen scaffolds. J Tissue Eng Regen Med 2017; 11:2398-410.
27. Ferrazzo PC, Niccoli S, Khaper N, Rathbone CR, Lees SJ. Ascorbic acid diminishes bone morphogenetic protein 2-induced osteogenic differentiation of muscle precursor cells. Muscle Nerve 2019; 59:501-8.
28. Casciari JJ, Riordan NH, Schmidt TL, Meng XL, Jackson JA, Riordan HD. Cytotoxicity of ascorbate, lipoic acid, and other antioxidants in hollow fibre in vitro tumours. Br J Cancer 2001; 84:1544-50.
29. Lee A, Amin NP. Radiation therapy for heterotopic ossification prophylaxis. StatPearls. Treasure Island (FL) 2019.
30. Huang W, Yang S, Shao J, Li YP. Signaling and transcriptional regulation in osteoblast commitment and differentiation. Front Biosci 2007; 12:3068-92.
31. Llacua LA, Faas MM, de Vos P. Extracellular matrix molecules and their potential contribution to the function of transplanted pancreatic islets. Diabetologia 2018; 61:1261-72.
32. Matta C, Szucs-Somogyi C, Kon E, et al. Osteogenic differentiation of human bone marrow-derived mesenchymal stem cells is enhanced by an aragonite scaffold. Differentiation 2019; 107:24-34.
33. Florencio-Silva R, Sasso GRD, Sasso-Cerri E, Simoes MJ, Cerri PS. Biology of bone tissue: structure, function, and factors that influence bone cells. Biomed Res Int 2015; 2015:421746.
34. Zhang X, Xu W. Neutrophils diminish T-cell immunity to foster gastric cancer progression: the role of GM-CSF/PD-L1/PD-1 signalling pathway. Gut 2017; 66:1878-80.
35. Zhao H, Chen Q, Alam A, et al. The role of osteopontin in the progression of solid organ tumour. Cell Death Dis 2018; 9:356.
36. Diomede F, Rajan TS, Gatta V, et al. Stemness Maintenance Properties in Human Oral Stem Cells after Long-Term Passage. Stem Cells Int 2017; 2017:5651287.
37. Ganss B, Kim RH, Sodek J. Bone sialoprotein. Crit Rev Oral Biol Med 1999; 10:79-98.
38. McKee MD, Nanci A. Osteopontin: an interfacial extracellular matrix protein in mineralized tissues. Connect Tissue Res 1996; 35:197-205.