COLLAGENATED HETEROLOGOUS CORTICO-CANCELLEUS BONE MIX STIMULATED DENTAL PULP DERIVED STEM CELLS

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Collagenated heretologous cortico-cancelleus bone mix (CHCCBM) is largely employed in maxillary and dental surgery for regeneration procedures, and is similar to human bone from chemical and physical point of view and promotes osteogenesis. In order to get more inside how this biomaterial induces osteoblast gene expression to promote bone formation, the mRNA levels of bone related genes were compared in human osteoblasts and dental pulp stem cells, using real time RT-PCR. The obtained results demonstrated that CHCCBM enhance stem cells differentiation and deposition of matrix by the activation of osteoblast related genes SP7, FOSL1 and SPP1.

Collagenated heretologous cortico-cancelleus bone mix (CHCCBM) is a bovine bone derived employed in maxillary and oral surgery. Some reports have demonstrated its clinical efficacy to restore alveolar ridge in pre-prosthetic surgery (1-3).

To investigate the molecular mechanism by which CHCCBM promotes osteoblast differentiation and proliferation, the quantitative expression of the mRNA of specific bone related genes, was examined in derived dental pulp stem cells treated CHCCBM. Dental pulp stem cells (DPSCs) represents an ideal source of stem cells because approachable niches containing a high number of stem cells compared to equal volumes of bone marrow (4-6). In this study the expression levels of specific genes were examined by means of real time RT-PCR in DPSCs after treatment with CHCCBM.

Gene expression in DPSCs was then compared with the gene expression in treated Human Osteoblasts (HOb) to evaluate the potential effect of this material in osteoblasts differentiation.

MATERIALS AND METHODS

Stem cells isolation from dental pulp, flow cytometric analyses and primary human osteoblasts cell culture were performed as previously described (4-6).

Cell treatment

DPSCs and HOb were seeded with CHCCBM (Gen-Os, Tecnoss srl, Giaveno, Torino, Italy) at the concentration of 10 mg/ml. Another set of wells containing untreated cells were used as control. The cells were maintained in a humidified atmosphere of 5% CO2 at 37°C. Cells were harvested at two time points, 15 and 30 days, for RNA extraction. RNA processing, RT-PCR and statistical analyses were conducted as previously described (4-6).

Key words: bone, dental pulp, stem cells, gene expression, osteoblast differentiation

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RESULTS

Cell cultures were phenotipically characterized by flow cytometric analyses as previously reported (4-6). To study if CHCCBM stimulates osteoblasts differentiation and proliferation in DPSCs, several osteoblast genes and mesenchymal stem cells marker, were analyzed by quantitative real-time PCR after 14 days of treatment with CHCCBM. Treated DPSCs resulted in up-regulation of SP7, FOSL1 and SPP1 genes and down-regulation of ENG, RUNX2, COL3A1, COL1A1 and ALPL during the first 14 days of treatment.

Analyzing the results obtained in HOb, we observed that after 14 days of treatment SP7, ENG, FOSL1, COL1A1, COL3A1 and SPP1 were up-regulated. The expression of RUNX2 and ALPL was down-regulated. "Two tailed ANOVA" comparison between DPSCs and HOb after the treatment showed that all genes were significantly differentially expressed after 14 days of treatment. (Table I).

DISCUSSION

CHCCBM (Gen-Os, Tecnoss srl, Giaveno, Torino, Italy) is widely used in several bone regeneration procedures in oral surgery due to its characteristics of resorption rate and capability of promotes osteogenesis. It is of paramount importance since several situation determine bone loss. Among them are socket preservation after tooth extraction, peri-implants bone maintenance (7-16), ridge reconstruction in syndromic patients (17-23) and oral rehabilitation after oncological treatments (24-35)

In order to get more inside how CHCCBM acts on DPSCs, changes in expression of bone related marker genes (RUNX2, SPP1, SP7, COLIA1, COL3A1, ALPL and FOSL1) and mesenchymal stem cells marker (ENG) were investigated by real-time RT–PCR. DPSCs were isolated by enzymatic digestion and phenotipically characterized by flow cytometric analyses. Dental pulp derived cell were homogenously $CD105^{+}$, $CD90^{+}$, $CD34^{-}$, $CD45^{-}$, $CD14^{-}$, which is a typical mesenchymal stem cells surface antigen profile.

The osteoinductive properties of CHCCBM were demonstrated by the up-regulation of SP7 during all the entire treatment both in treated DPSCs than in treated HOb. SP7 is a transcriptional factor involved in bone formation and osteoblast differentiation downstream of RUNX2 pathway. CHCCBM also modulate the expression of collagenic extracellular matrix genes, collagen type 1α1 (COL1A1) and collagen type 3α1 (COL3A1).

After 14 days, in treated DPSCs was observed the

Table I. *Differentially expressed genes between DPSCs and HOb after 14 days of treatment.*

Genes	**DPSCs**	**HOb**	**Differentially expressed genes**
	Log10 RQ	**Log10 RQ**	**p<0,005**
SP7	1,22	0,52	**0,001**
ENG	-0,42	0,03	**0,009**
FOSL1	0,25	0,26	**0,001**
RUNX2	-0,42	-0,03	**0,002**
COL3A1	-1,46	0,02	**0,001**
COL1A1	-0,71	0,13	**0,001**
ALPL	-0,54	-0,23	**0,001**
SPP1	0,93	1,64	**0,001**

down-regulation of COL1A1 and COL3A1. Instead, in HOb, the two collagens type were up-regulated. Osteopontine (SPP1) was up-regulated during all the treatment both in DPSCs than in HOb. The up-regulation of this gene suggests the differentiation effect of treatment.

ENG, also named CD105, a surface marker used to define a bone marrow stromal cell population capable of multilineage differentiation was down-regulated in DPSCs and up-regulated in HOb during the entire treatment. Another gene involved in osteoblast differentiation and modulated by CHCCBM was FOSL1. Both in DPSCs than in HOb this gene was up-regulated. FOSL1 is a component of the dimeric transcription factor AP-1 involved in the transcription of bone related genes. This study demonstrates that CHCCBM is capable of supporting osteoblast differentiation and extracellular matrix deposition and mineralization in mesenchymal stem cells by the activation of osteoblast related genes SP7, SPP1 and FOSL1.

Within the limits of this *in vitro* study related to the period of observation (2 weeks), it possible to infer that CHCCBM is a bone substitute that positively active gene related to bone formation.

REFERENCES

1. Lopez MA, Manzulli N, Casale M, Ormianer Z, Carinci F. The use of resorbable heterologous cortical lamina as a new sinus lift floor: a technical note. J Biol Regul Homeost Agents 2016; 30(2 Suppl 1):75-9.
2. Giuliani A, Iezzi G, Mazzoni S, Piattelli A, Perrotti V, Barone A. Regenerative properties of collagenated porcine bone grafts in human maxilla: demonstrative study of the kinetics by synchrotron radiation microtomography and light microscopy. Clin Oral Investig 2018; 22(1):505-13.
3. Marconcini S, Giammarinaro E, Derchi G, Alfonsi F, Covani U, Barone A. Clinical outcomes of implants placed in ridge-preserved versus nonpreserved sites: A 4-year randomized clinical trial. Clin Implant Dent Relat Res 2018; 20(6):906-14.
4. Palmieri A, Pezzetti F, Graziano A, Riccardo D, Zollino I, Brunelli G, Martinelli M, Arlotti M, Carinci F. Comparison between osteoblasts derived from human dental pulp stem cells and osteosarcoma cell lines. Cell Biol Int 2008; 32(7):733-8.
5. Laino G, Carinci F, Graziano A, d'Aquino R, Lanza V, De Rosa A, Gombos F, Caruso F, Guida L, Rullo R, Menditti D, Papaccio G. In vitro bone production using stem cells derived from human dental pulp. J Craniofac Surg 2006; 17(3):511-5.
6. Laino G, d'Aquino R, Graziano A, Lanza V, Carinci F, Naro F, Pirozzi G, Papaccio G. A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J Bone Miner Res 2005; 20(8):1394-402.
7. Baj A, Bolzoni A, Russillo A, Lauritano D, Palmieri A, Cura F, Silvestre FJ, Giannì AB. Cone-morse implant connection system significantly reduces bacterial leakage between implant and abutment: an in vitro study. J Biol Regul Homeost Agents 2017; 31(2 Suppl 1):203-8.
8. Baj A, Beltramini GA, Bolzoni A, Cura F, Palmieri A, Scarano A, Ottria L, Giannì AB. Bacterial colonization of the implant-abutment interface of conical connection with an internal octagon: an in vitro study using real-time PCR. J Biol Regul Homeost Agents 2017; 31(2 Suppl 1):163-8.
9. Candotto V, Lauritano D, Carinci F, Bignozzi CA, Pazzi D, Cura F, Severino M, Scarano A. Silver-Based Chemical Device as an Adjunct of Domestic Oral Hygiene: A Study on Periodontal Patients. Materials (Basel) 2018; 11(8). pii: E1483.
10. 1Lauritano D, Carinci F, Palmieri A, Cura F, Caruso S, Candotto V. Reuterinos(®) as adjuvant for peri-implant treatment: A pilot study. Int J Immunopathol Pharmacol 2019; 33:2058738419827745.
11. Carinci F, Lauritano D, Bignozzi CA, Pazzi D, Candotto V, Santos de Oliveira P, Scarano A. A New Strategy Against Peri-Implantitis: Antibacterial Internal Coating. Int J Mol Sci 2019; 20(16): ii: E3897.
12. Lauritano D, Boccalari E, Di Stasio D, Della Vella F, Carinci F, Lucchese A, Petruzzi M. Prevalence of Oral Lesions and Correlation with Intestinal Symptoms of Inflammatory Bowel Disease: A Systematic Review. Diagnostics (Basel) 2019; 9(3):pii: E77.
13. Lauritano D, Moreo G, Della Vella F, Di Stasio D, Carinci F, Lucchese A, Petruzzi M. Oral Health Status and Need for Oral Care in an Aging Population: A

Systematic Review. Int J Environ Res Public Health 2019; 16(22)pii: E4558.
14. Lauritano D, Moreo G, Carinci F, Borgia R, Lucchese A, Contaldo M, Della Vella F, Bernardelli P, Moreo G, Petruzzi M. Aging and Oral Care: An Observational Study of Characteristics and Prevalence of Oral Diseases in an Italian Cohort. Int J Environ Res Public Health 2019; 16(19)pii: E3763.
15. Lauritano D, Lucchese A, Di Stasio D, Della Vella F, Cura F, Palmieri A, Carinci F. Molecular Aspects of Drug-Induced Gingival Overgrowth: An In Vitro Study on Amlodipine and Gingival Fibroblasts. Int J Mol Sci 2019; 20(8)pii: E2047.
16. Lauritano D, Palmieri A, Lucchese A, Di Stasio D, Moreo G, Carinci F. Role of Cyclosporine in Gingival Hyperplasia: An In Vitro Study on Gingival Fibroblasts. Int J Mol Sci 2020;16:21(2)pii:E595.
17. Eller-Vainicher C, Rossi DS, Guglielmi G, Beltramini GA, Cairoli E, Russillo A, Mantovani G, Spada A, Chiodini I. Prompt clinical and biochemical response to denosumab in a young adult patient with craniofacial fibrous dysplasia. Clin Cases Miner Bone Metab 2016; 13(3):253-6.
18. Segna E, Pucciarelli V, Beltramini GA, Sforza C, Silvestre FJ, Giannì AB, Baj A. Parry Romberg Syndrome and linear facial scleroderma: management in pediatric population. J Biol Regul Homeost Agents. 2017 Apr-Jun;31(2 Suppl 1):131-138.
19. Lauritano D, Palmieri A, Candotto V, Carinci F. Regenerative Dentistry and Stem Cells: A Multilineage Differentiation as a Safe and Useful Alternative Way of Harvesting and Selection Adipose Derived Mesenchymal Stem Cells. Curr Drug Targets 2018; 19(16):1991-7.
20. Cullati F, Rusconi FME, Mapelli A, Zago M, Beltramini GA, Giannì AB, Sforza C. Three-dimensional longitudinal evaluation of facial mimicry in orthognathic class III surgery. Int J Oral Maxillofac Surg 2019; 48(3):355-63.
21. Zavattero, E., Romano, M., Gerbino, G., Rossi, D.S., Giannì, A.B., Ramieri, G., Baj, A. Evaluation of the accuracy of virtual planning in orthognathic surgery: A morphometric study . Journal of Craniofacial Surgery 2019; 30(4):1214-20.
22. Lauritano D, Attuati S, Besana M, Rodilosso G, Quinzi V, Marzo G, Carinci F. Oral and craniofacial manifestations of Ellis-Van Creveld syndrome: a systematic review. Eur J Paediatr Dent 2019; 20(4):306-10.
23. Palmieri A, Scapoli L, Carrozzo M, Cura F, Morselli PG, Pannuto L, Nouri N, Carinci F, Lauritano D, Martinelli M. ROCK1 is associated with non-syndromic cleft palate. J Oral Pathol Med 2020; 49(2):164-8.
24. Baj A, Bellocchio G, Laganà F, Beltramini GA, Testori T, Giannì AB. Vascularized fibula free flap for implant rehabilitation in the case of extreme atrophy. Minerva Stomatol 2010; 59(4):223-8, 228-31.
25. Baj A, Lovecchio N, Bolzoni A, Mapelli A, Giannì AB, Sforza C. Stair ascent and descent in assessing donor-site morbidity following osteocutaneous free fibula transfer: a preliminary study. J Oral Maxillofac Surg 2015; 73(1):184-93.
26. Bolzoni A, Mapelli A, Baj A, Sidequersky FV, Giannì AB, Sforza C. Evaluation of three-dimensional mandibular movements after reconstruction with free fibula flap. Acta Otorhinolaryngol Ital 2015; 35(6):371-8.
27. Segna E, Bolzoni AR, Baserga C, Baj A. Free flap loss caused by heparin-induced thrombocytopenia and thrombosis (HITT): a case report and literature review. Acta Otorhinolaryngol Ital 2016; 36(6):527-33.
28. Bardazzi A, Beltramini GA, Autelitano L, Bazzacchi R, Rabbiosi D, Pedrazzoli M, Tewfik K, Rezzonico A, Biglioli F. Use of Buccinator Myomucosal Flap in Tongue Reconstruction. J Craniofac Surg 2017; 28(4):1084-7.
29. Baserga C, Massarelli O, Bolzoni AR, Rossi DS, Beltramini GA, Baj A, Giannì AB. Fibula free flap pedicle ossification: Experience of two centres and a review of the literature. J Craniomaxillofac Surg 2018; 46(9):1674-8.
30. Segna E, Bolzoni AR, Giannì AB, Baj A, Beltramini GA. Impact of reconstructive microsurgery on patients with cancer of the head and neck: a prospective study of quality of life, particularly in older patients. Br J Oral Maxillofac Surg 2018; 56(9):830-4.
31. Di Giuli R, Zago M, Beltramini GA, Pallotta ML, Bolzoni A, Baj A, Giannì AB, Sforza C. Donor-Site Morbidity After Osteocutaneous Free Fibula

Transfer: Longitudinal Analysis of Gait Performance. J Oral Maxillofac Surg 2019; 77(3):648-57.

32. Bolzon, AR, Segna E, Beltramini GA, Sweed AH, Giannì AB, Baj A. Computer-Aided Design and Computer-Aided Manufacturing Versus Conventional Free Fibula Flap Reconstruction in Benign Mandibular Lesions: An Italian Cost Analysis Journal of Oral and Maxillofacial Surgery 2019 pii:S0278-2391(19)30260-5.
33. Lauritano D, Oberti L, Gabrione F, Lucchese A, Petruzzi M, Carinci F, Lo Muzio L. Liquid biopsy in head and neck squamous cell carcinoma: Prognostic significance of circulating tumor cells and circulating tumor DNA. A systematic review. Oral Oncol 2019; 97:7-17.
34. Contaldo M, Lauritano D, Carinci F, Romano A, Di Stasio D, Lajolo C, Della Vella F, Serpico R, Lucchese A. Intraoral confocal microscopy of suspicious oral lesions: a prospective case series. Int J Dermatol 2019; doi: 10.1111/ijd.14574.
35. Lauritano D, Lucchese A, Gabrione F, Di Stasio D, Silvestre Rangil J, Carinci F. The Effectiveness of Laser-Assisted Surgical Excision of Leukoplakias and Hyperkeratosis of Oral Mucosa: A Case Series in A Group of Patients. Int J Environ Res Public Health 2019; 13:16(2):pii: E210.