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Bone Substitutes Enhance Osteogenic Differentiation of Mesenchymal Stem Cells in Three-dimensional Scaffolds

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Abstract. *Aim: The present study aimed to find bone substitutes to enhance osteogenic differentiation of mesenchymal stem cells in three-dimensional scaffolds in the absence of dexamethasone. Materials and Methods: Seven commercial bone substitutes were added to a three-dimensional fibrin-matrix containing rat mesenchymal stem cells in a biocompatible poly-L-lactic-acid mesh. Cell viability, cytotoxicity and alkaline phosphatase activity were followed for three weeks. Expression of bone markers was examined by qualitative evaluation of corresponding transcripts. Results: Six out of the seven bone derivatives exhibited an osteogenic-enhancing effect. Conclusion: The osteogenic-enhancing effect of the evaluated bone substitutes suggests their potential clinical application for preparation of autologous bone replacement material in three-dimensional carriers.*

Osteogenic differentiation of mesenchymal stem cells (MSCs) *in vitro* is well-established by complementing the culturing medium with dexamethasone, β-glycerolphosphate and ascorbic acid phosphate (1-5). However, upon application of the cells to a patient, this osteogenic condition cannot be maintained for further osteogenic differentiation. In contrast, bone substitutes are solid or semi-solid and therefore remain at the application site. Should a bone substitute have an osteogenic differentiation-enhancing effect, it can be included in preparation of the carriers with cells *in vitro* for the implantation (6). Bone substitutes can be safely used in tissue engineering because they are readily-applied in routine clinical practice (7-9).

In this study, we examined commercially available bone substitutes for their potential osteogenic effect on rat MSCs in three-dimensional fibrin matrices held by poly-L-lactic-acid (PLLA) mesh scaffolds. The carriers with various bone substitutes were cultured under standard culturing condition. Cell viability, cytotoxicity and alkaline phosphatase activity (APA) of the cells were monitored for three weeks and the expression of bone markers was examined qualitatively by an evaluation of their transcripts.

Materials and Methods

Bone substitutes. The commercially-available bone substitutes Cerasorb® M, Ostim®, Fortoss Vital®, TriCos®, NanoBone®, BioOss® and OsteoBiol® mp3® were used. For further details see Table I.

3-D Cell culture. The PLLA mesh scaffolds were disinfected in 70% ethanol for 24 h, and subsequently dried at 37˚C for 48 h. MSCs were prepared from bone marrow of Sprague-Dawley-rats as described elsewhere (10). A total of 500,000 cells were mixed with fibrinogen (Tissuecol®; Baxter, Unterschleiβheim, Germany) without or with one of the seven bone substitutes. After adding thrombin, the mixture was filled into a sterile PLLA tube (2 mm × 5 mm diameter) scaffold, which was placed in one well of a 96-plate. These constructs were left at 37˚C for 2 h for complete coagulation. Cells and the bone substitutes were evenly distributed in the three-dimensional fibrin gels supported by the PLLA mesh scaffold. Each polymerized carrier was then placed into one well of a 24-well-plate and covered with 1 ml standard culture medium (Dulbecco's Modified Eagle's Medium 4.5 g/l glucose, 10% Fetal Bovine Serum; BioWest, Renningen, Germany). As a positive control, MSCs in carriers without any bone substitute were cultured under osteogenic differentiation conditions with dexamethasone (1 μM; Sigma-

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Table I. *Compositions and features of the seven bone substitutes evaluated in the study.*

Bone substitute	Supplier	Composition	Structure	Type	Particle size
Cerasorb® M	Curasan, Kleinostheim, Germany	100% β-TCP	Granulate	Ceramic	1-2 nm
Ostim®	Heraeus Kulzer, Wehrheim, Germany	100% Hydroxyapatite	Paste	Ceramic	100 nm
Fortoss Vital®	Biocomposites, Keele, Staffordshire, England	β-TCP hydroxysulfate-matrix	Paste	Ceramic	Unclear
TriCos®	Baxter Healthcare, Wallisellen, Suisse	60% Hydroxyapatite 40% β-TCP	Granulate	Ceramic	1-2 mm
NanoBone®	ARTOSS, Rostock, Germany	76% Hydroxyapatite 24% SiO2	Granulate	Ceramic	600-2,000 μm
BioOss®	Geistlich Biomaterials, Baden-Baden, Germany	Mineralised part of bovine bone	Granulate	Xenogenic	250-1,000 μm
OsteoBiol® mp3®	Tecnoss Dental, Torino, Italy	Mix of pre-hydrated collagenated cortico-cancellous porcine bone (90% granulates, 10% collagen gel)	Paste	Xenogenic	600-1,000 μm

Aldrich, Hamburg, Germany), α-glycerolphosphate (0.5 M; Sigma-Aldrich) and ascorbic acid phosphate (20 mM; Sigma-Aldrich) in the medium. Each combination was examined in four to eight replicates.

Assays and expression study. Conditioned medium (100 μl) was taken from each well containing the scaffold with cells for measuring cell proliferation, cytotoxicity and APA using CellTiter-Blue® (Promega, Mannheim, Germany), CytoTox-One® (Promega) and a *p*-nitrophenol (Sigma-Aldrich) based assay, respectively. Cell numbers of each sample were determined using a calibration curve based on the values of a series of samples with known cell numbers. To exclude APA from serum, carriers with cells were kept in serum-free medium for 45 min prior to the assay.

On days 1, 7, 14 and 21, RNA was extracted from MSCs from the 3-D carriers using an RNease mini kit® (Qiagen, Hilden, Germany) and used for preparing cDNA using a REDTaq® PCR Reaction Mix (Sigma-Aldrich). RNA extracted from a 3-D carrier with no cells seeded was used as a negative control. Polymerase chain reaction (PCR) was carried out using these cDNAs to evaluate expression of bone-related genes coding for Runt-related transcription factor 2 (RUNX2), collagen type 1, osteopontin and osteocalcin. The polymerase chain reaction (PCR) products were visualized on an agarose gel. Information for PCR conditions and primers are available upon request.

Statistical analysis. Each of the parameters viability, cytotoxicity and alkaline phosphatase activity was measured in 4 replicates, resulting in a dataset consisting of 4 values. Mean and standard deviation were calculated for each data set and used to show the time course of these parameters.

Results

Under standard culture conditions in carriers with and without bone substitute, cell viability dropped at the first measurement to below that of the seeded 500,000 cells, but recovered quickly to yield more than 500,000 cells (Figure 1). Afterwards, cell viability fluctuated or remained around 500,000. Only with OsteoBiol did cell viability decrease gradually to that corresponding to approximately 200,000 cells. As expected, no increased cytotoxicity was observed in cells cultured in carriers with bone substitutes compared to that in cells in carriers without bone substitutes (Figure 1).

APA dropped from day 0 to day 3 under all cultural conditions. For the negative control of cells in carriers without any bone substitute in standard cultural medium, APA remained low and decreased slightly further from day 14. For the positive control of cells in carriers cultured under osteogenic conditions, APA increased from day 3 to day 10, decreased slightly from day 10 to day 14 and increased again from day 14. The same pattern was observed for APA in cells in carriers containing Cerasorb M, Ostim, BioOss and OsteoBiol. A similar pattern with a stable phase between day 3 and day 14 and increasing phase from day 14 was observed for cells in carriers with Cerasob M, TricCos, NanoBone and Fortoss Vital (Figure 2A). In summary, three patterns were observed: (i) decrease–increase–slight decrease–final increase; (ii) decrease–stable–slight further decrease; and (iii) decrease–stable–increase, as illustrated in Figure 2B.

Expression of most of the bone markers, RUNX2, collagen type I, osteopontin and osteocalcin, in cells cultured under standard conditions was enhanced by most of the bone substitutes tested (Figure 3).

Discussion

We found that all seven bone substitutes tested in this study elevated APA, an indicator of osteogenic differentiation, in cells cultured in standard medium to a level compatible with that of cells cultured under osteogenic differentiation conditions. Furthermore, most of the substitutes also enhanced expression of bone markers collagen type 1, osteoponin and osteocalcin.

Interestingly, a pattern of decrease–increase–decrease–increase of the APA was found under osteogenic

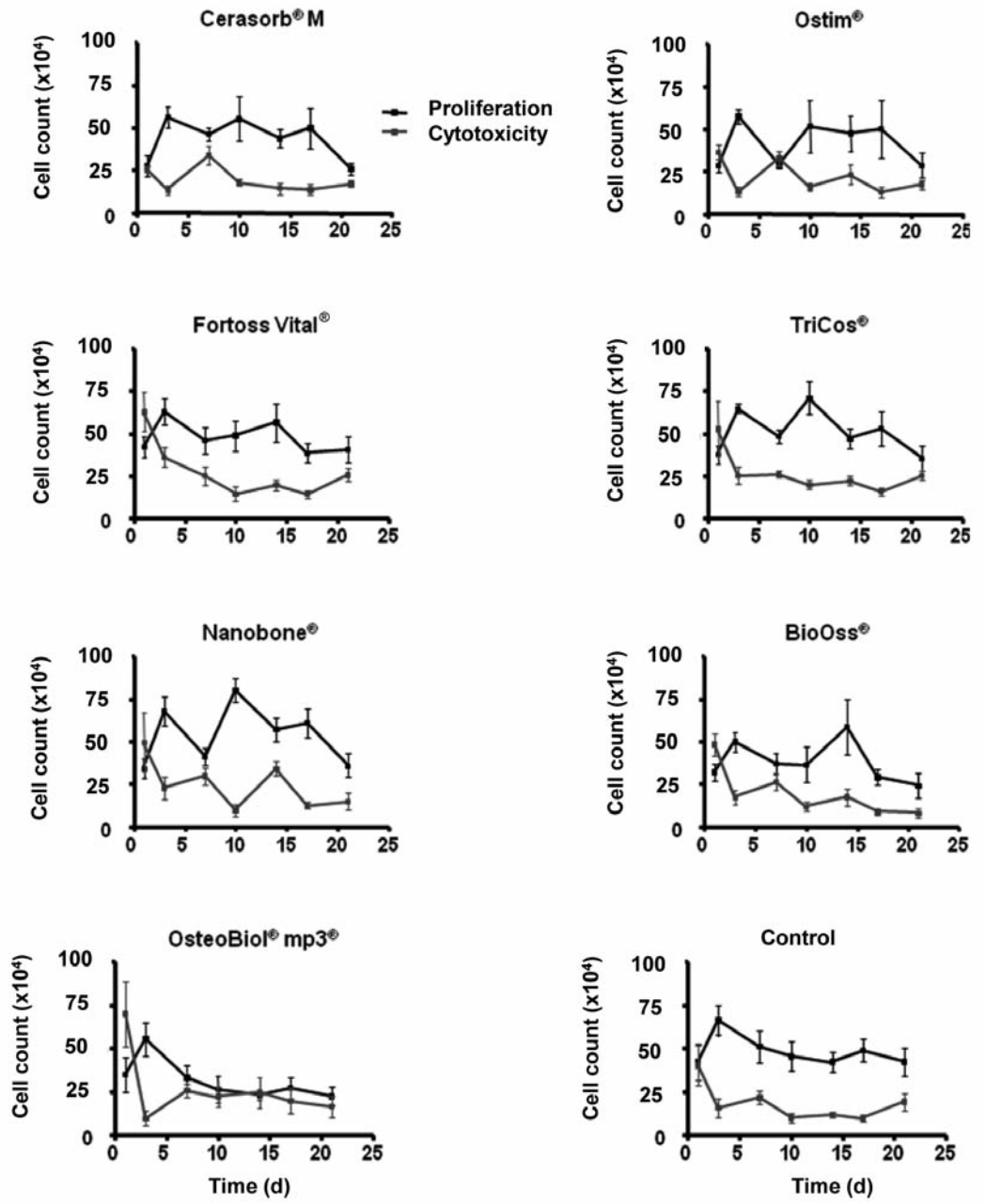

Figure 1. *Viability and cytotoxicity of mesenchymal stem cells in 3D carriers with poly-L-lactic-acid. A total of 500,000 cells were seeded in fibrin carriers with and without bone substitutes as indicated.*

differentiating conditions as well as under standard conditions with several bone substitutes. This pattern differs from that of APA in monolayer cells which increased to day 7 and decreased afterwards (11). The initial decrease, which was found under all cultural conditions, can be explained by the initial adaptation of the cells to the three-dimensional fibrin matrix. The first increase followed by a decrease corresponds to that of cells in monolayer culture. The second increase may reflect distinctive behavior of the cells in three-dimensional matrix surrounded by the PLLA mesh scaffold. Future studies should examine the corresponding time course of calcium sedimentation. Since 21 days are the standard period for osteogenic differentiation *in vitro* (12), our experiments were also designed only for 21 days. Therefore, the second increase of APA was only partially followed. This remains another issue to be clarified.

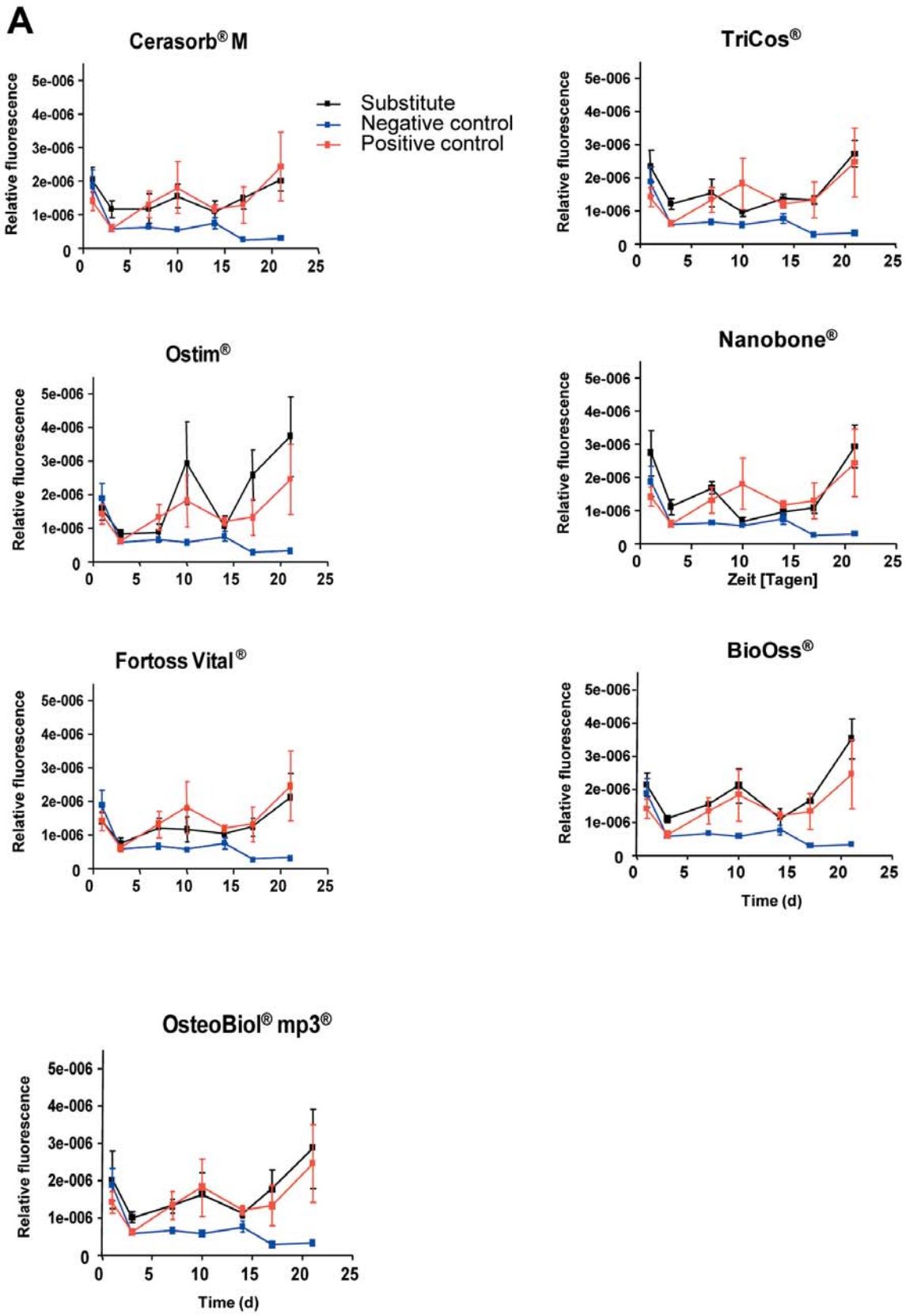

Figure 2. *continued*

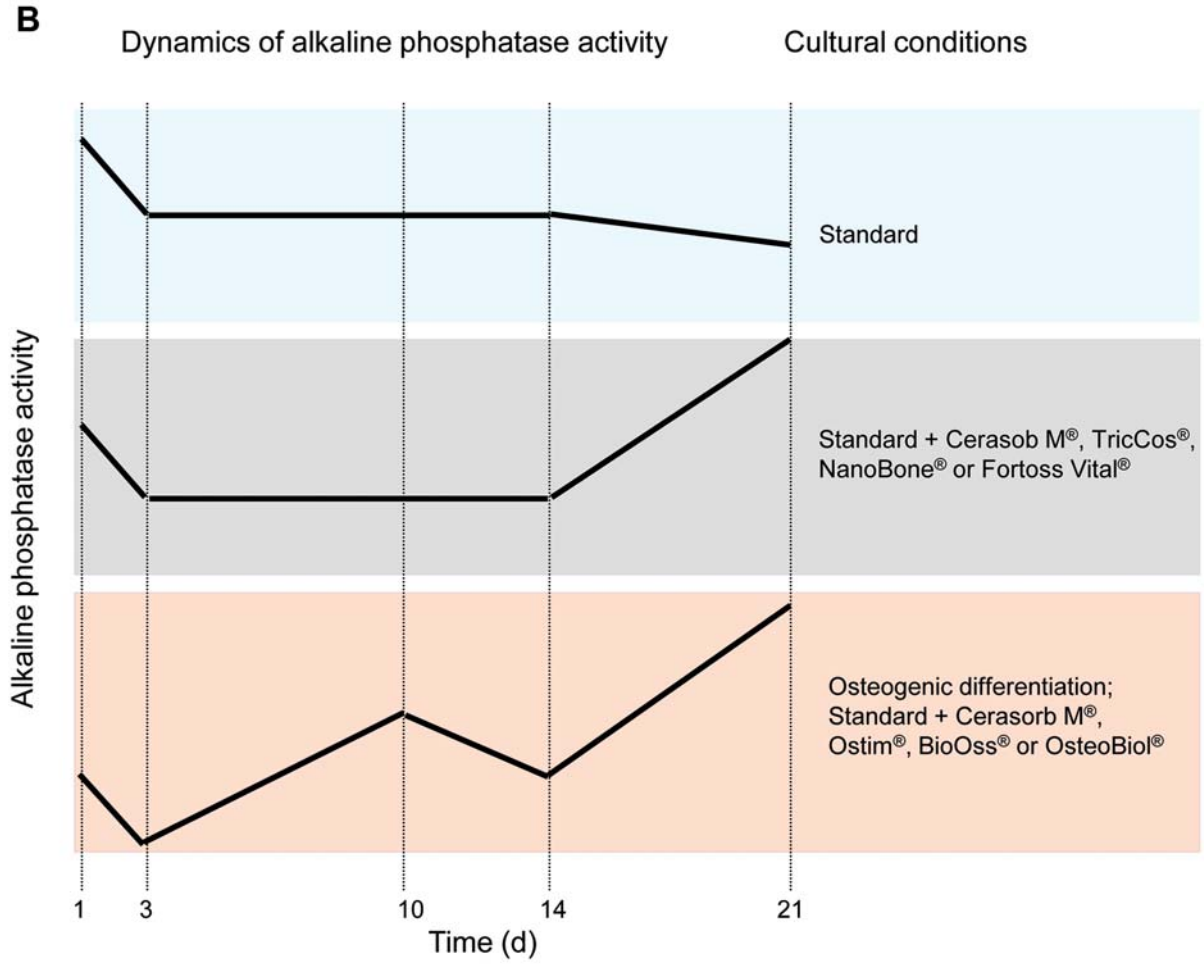

Figure 2. *A: Alkaline phosphatase activity of mesenchymal stem cells (MSC) in 3D carriers with poly-L-lactic-acid (PLLA). A totaI of 500,000 cells were seeded in fibrin carriers with and without bone substitutes as indicated. B: General patterns of alkaline phosphatase activity of MSCs in 3D carriers with PLLA.*

This *in vitro* study shows the enhancing effect of bone substitute materials on differentiation behavior of MSCs in a three-dimensional matrix. This is consistent with findings of *in vivo* studies examining different techniques of bone augmentation in the upper or lower jaw (13, 14). In contrast to the osteogenic differentiating behavior of cells in two-dimensional cultures, the three-dimensional set-up of this study seems to imitate the *in vivo* situation more adequately. This study adds to the knowledge of stem cell differentiation in three-dimensional carriers and their functional behavior in combination with bone substitutes.

Conclusion

In summary, our results suggest that some bone substitutes such as Ostim® and BioOss® have osteogenic-enhancing effect and can be used to prepare bone substitute in three-dimensional carriers.

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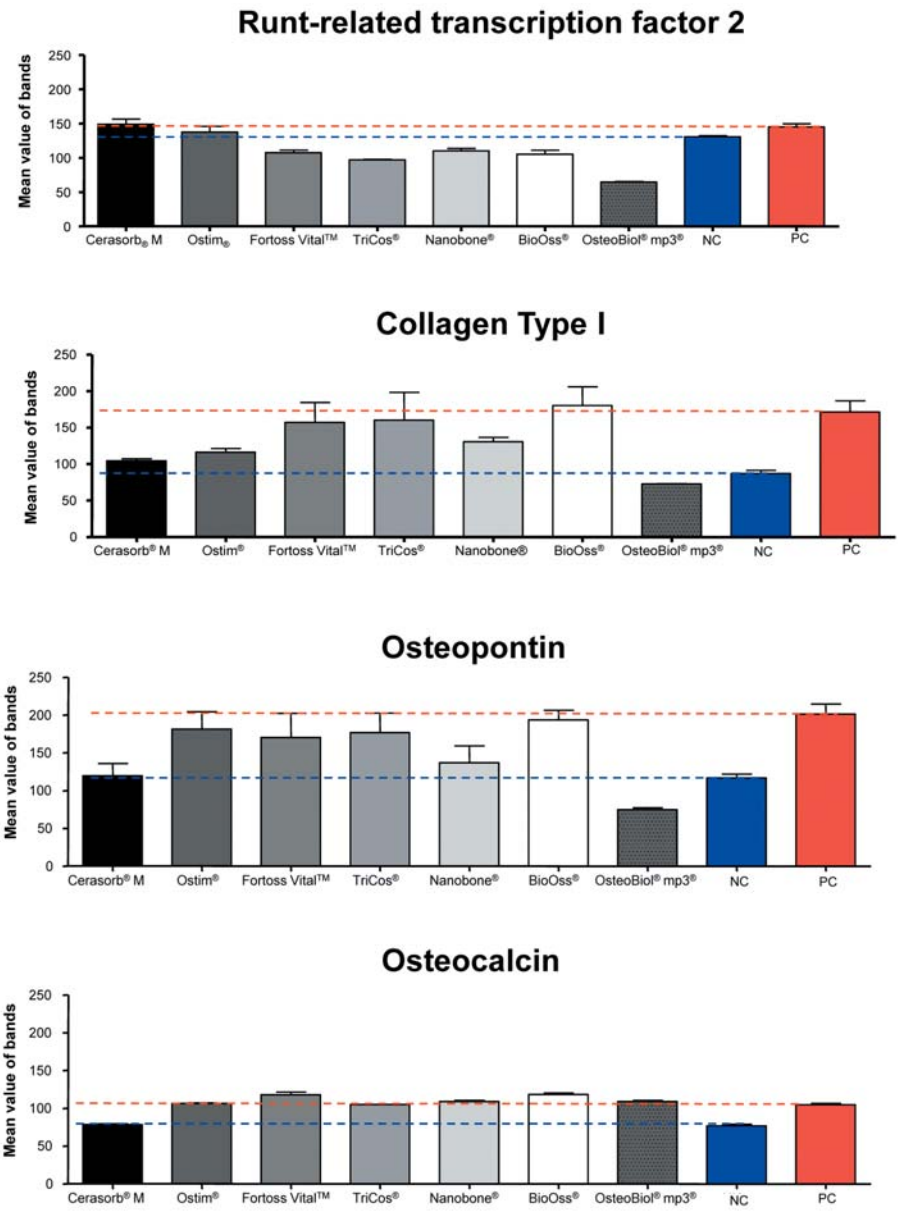

Figure 3. *Gene expression analysis of runt-related transcription factor 2 (RUNX2), collagen type I, osteopontin and osteocalcin in mesenchymal stem cells in 3D carriers with poly-L-lactic-acid. Maximum values of a 14-day period are presented. NC: Negative control; PC: positive control.*

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