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^{99m}Tc-Hydroxydiphosphonate quantification of extracellular matrix mineralization in 3D human mesenchymal stem cell cultures

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Objectives

Bone tissue engineering is one of the fastest growing branches in modern bioscience. New methods are being developed to achieve higher grades of mineral deposition by osteogenically inducted mesenchymal stem cells. In addition to well established monolayer cell culture models, 3D cell cultures for stem cell-based osteogenic differentiation have become increasingly attractive to promote *in vivo* bone formation. One of the main problems of scaffold-based osteogenic cell cultures is the difficulty in quantifying the amount of newly produced extracellular mineral deposition, as a marker for new bone formation, without destroying the scaffold. In recent studies, we were able to show that ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP), a gamma radiation-emitting radionuclide, can successfully be applied as a reliable quantitative marker for mineral deposition as this tracer binds with high affinity to newly produced hydroxyapatite (HA).

Methods

Within the present study, we evaluated whether this promising new method, using ^{99m}Tc-hydroxydiphosphonate (^{99m}Tc-HDP), can be used to quantify the amount of newly formed extracellular HA in a 3D cell culture model. Highly porous collagen type II scaffolds were seeded with 1 × 106 human mesenchymal stem cells (hMSCs; n = 6) and cultured for 21 days in osteogenic media (group A – osteogenic (OSM) group) and in parallel in standard media (group B – negative control (CNTRL) group). After incubation with ^{99m}Tc-HDP, the tracer uptake, reflected by the amount of emitted gamma counts, was measured.

Results

We saw a higher uptake (up to 15-fold) of the tracer in the OSM group A compared with the CNTRL group B. Statistical analysis of the results (Student`s *t*-test) revealed a significantly higher amount of emitted gamma counts in the OSM group (p = 0.048). Qualitative and semi-quantitative analysis by Alizarin Red staining confirmed the presence of extracellular HA deposition in the OSM group.

Conclusion

Our data indicate that ^{99m}Tc-HDP labelling is a promising tool to track and quantify non-destructive local HA deposition in 3D stem cell cultures.

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Keywords: Osteogenesis, Hydroxylapatite quantification, Scaffold-based tissue repair, Technetium labeling

Article focus

- Non-destructive quantification of hydroxyapatite (HA) synthesized *in vitro* by mesenchymal stem cells (MSCs) is a challenging problem in osteogenic tissue engineering.
- ^{99m}Tc-hydroxydiphosphonate (^{99m}Tc-HDP) is one of the most common tracers in nuclear medicine for bone imaging as it binds to newly formed HA *in vitro* and *in vivo*.

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- This study evaluated the usability of ^{99m}Tc-HDP for non-destructive HA quantification in a 3D scaffold model.

Key messages

- ^{99m}Tc-HDP binds with high significance to newly synthesized HA produced by osteogenically-induced MSCs *in vitro*, which represents a promising new method for non-destructive osteogenic quantification.
- The amount of tracer uptake can be measured using a common gamma camera, which allows a relative uptake quantification.
- The results suggest that the new ^{99m}Tc-HDP labelling method is more sensitive than the actual benchmark Alizarin red staining.

Strengths and limitations

- The individual osteogenic potential of each donor may influence the uptake of the tracer, nevertheless, the statistical analysis showed significant results.
- It is unclear if the method also works with HA scaffolds or if it is limited to collagen scaffolds.

Introduction

The treatment of critical-size bone defects is still one of the most challenging problems in clinical practice.[1] One of the main topics in today's bone tissue engineering is the development of osteoinductive and/or osteoconductive scaffolds, which are preloaded with progenitor cells such as mesenchymal stem cells (MSCs), capable of differentiating into osteoblast-like cells in order to stimulate regeneration of bone defects.[1-3] The general principle of scaffold-based tissue engineering is the application of cell-seeded and pre-cultured scaffolds, which are osteoconductive and/or inductive, in order to stimulate new bone formation *in vivo*.[4-6] Published data indicate that collagen-based scaffolds are highly suitable for seeding with MSCs and capable of undergoing further osteogenic differentiation.[7-13]

The proof of successful osteogenic differentiation *in vitro* is usually found when using Alizarin Red or von Kossa staining as these two well established qualitative methods react with the calcium, e.g. phosphorus from the extracellular hydroxyapatite (HA) deposits.[14-17] More elaborate methods to prove osteogenic differentiation of MSCs include the detection and quantification of bone-specific proteins[18] such as alkaline phosphatase (AP), a glycoprotein essential for the extracellular secretion of HA as it acts as a calcium transporter and interacts with ß-glycerophosphate to form a calcium complex.[19] To quantify the presence of AP, so as to track the mineralization process, it is necessary to destroy and lyse the mono- or 3D cell culture constructs.[20] Therefore, these cultures are no longer available for further experiments.

Recent studies have evaluated some novel, non-destructive approaches to quantify the osteogenic potential of human mesenchymal stem cells (hMSCs) using ultrasound and µ-CT.[21-23] Other published methods are scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy-dispersive x-ray microanalysis (EDX), and x-ray diffraction analysis (XRD). All of these methods are highly elaborate techniques to quantify cell-mediated osteogenesis,[24-28] but they have at least one or more disadvantages: 1) they are harmful and destructive for the cell culture and scaffolds, therefore eliminating the availability to undertake further experiments or analytical methods; 2) the equipment and the method are very costly; 3) most of these methods only allow a qualitative proof of osteogenesis; 4) if a quantitative proof of HA measured by these methods is possible, it is measured indirectly, which is a potential source of error for the exact assessment of the mineral content of the specimen.

Thus, another author has already stated that there is a strong need for new non-destructive methods to image and quantify cellular response to osteogenic stimuli within 3D scaffolds, e.g. cell-based osteogenesis.[22]

Since the late 1990s, well known radioactive tracers such as ^{45}Calcium, ^{3}Hydrogen, ^{99m}Technetium, and ^{18}Fluorine were used to investigate the osteogenic pathway.[26,29,30] In 2004, Wang et al[31] performed a novel method to quantify the amount of HA produced by monolayer MC3T3 cells cultured on polystyrene and titanium alloy (Ti-6Al-4V) disks. They used the ability of the well-established nuclear medicine tracer ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP; a polyphosphonate) to bind to newly formed HA *in vitro* and *in vivo*. It is well known that polyphosphonates accumulate in areas with high bone metabolism. Here, the phenomenon of chemosorption occurs, as the polyphosphonates establish a strong chemical bond with the inorganic HA of the bone matrix. The binding energy is characteristically around 800 kJ/mol.[32-37]

The ^{99m}Tc-MDP labelling method was validated using inductively coupled plasma to evaluate the calcium content of the cultures.[31]

In a recent study, we were able to show the usage of ^{99m}Tc-MDP and ^{99m}Tc-hydroxydiphosphonate (^{99m}Tc-HDP) for the quantification of extracellular matrix mineralization to assess the osteogenic potential of human and goat MSCs in monolayer culture.[38]

Within this paper, we present a novel, easy-to-use, time-saving, and non-destructive method to quantify the amount of extracellular HA deposition in a 3D-culture setting, employing osteogenic-induced hMSCs seeded on type II collagen scaffolds using ^{99m}Tc-HDP.

Materials and Methods

Human mesenchymal stem cell isolation. Bone marrow aspirates were obtained from the proximal femoral bone

cavity of six healthy human donors under general anaesthesia during an elective surgical procedure for total hip arthroplasty after informed consent and with the approval from the local ethics committee board (No. S-309/2007).

During preparation of the proximal femoral bone cavity, 10 ml to 15 ml of bone marrow was collected using a 20 ml syringe (Becton, Dickinson and Co., Franklin Lakes, New Jersey) containing 1000 IU of heparin (5000 IU/ml; B. Braun Melsungen AG, Melsungen, Germany). Individual samples were diluted 1:1 with phosphate-buffered saline (PBS; Invitrogen, Carlsbad, California) and then centrifuged, followed by resuspension in PBS for washing. This procedure was repeated twice. The mononuclear cell fraction was isolated by Ficoll gradient centrifugation (Ficoll–Paque PLUS; GE Healthcare, Chicago, Illinois). Mononuclear cells were plated in T-150 polystyrene tissue culture flasks (Falcon; Corning Life Sciences, Tewksbury, Massachusetts) at a density of $5 \times 10^5/cm^2$ and cultured in low-glucose Dulbecco's Modified Eagle Medium (DMEM; 1g/l D-glucose with 4 mM L-glutamine and with 1 mM sodium pyruvate; Invitrogen) containing 10% foetal calf serum (FCS; Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen) in a humidified 5% CO_2 atmosphere at 37°C. After 48 hours, non-adherent cells were removed by washing with PBS. Media were changed three times a week. At 80% to 90% of confluence, the cells were trypsinized (0.25% trypsin/0.1% ethylenediaminetetraacetic acid (EDTA); Invitrogen) and cryopreserved for further experiments by freezing 0.5 ml aliquots containing 5×10^5 to 1×10^6 cells in low-glucose DMEM (DMEM-LG; Invitrogen) with 20% FCS (Biochrom AG) and 10% dimethyl sulfoxide (DMSO; Sigma–Aldrich, Merck KGaA, Darmstadt, Germany) in liquid nitrogen.

Media preparation. Within this study two different types of media were used for 3D cell culture and differentiation of hMSCs.

Medium A: to obtain osteogenic differentiation, a well-established osteogenic medium (OSM; medium A) was used[39], having undergone minor modifications. The final osteogenic medium contained Minimum Essential Medium Eagle - Alpha Modification (Alpha MEM; Invitrogen) with 10% FCS and 1% penicillin and streptomycin, 100 nM dexamethasone (Sigma-Aldrich), 10 mM ß-glycerophosphate (Sigma-Aldrich), and 0.17 mM ascorbic acid (Sigma-Aldrich).

Medium B: for cell expansion (EXP) and for the negative control (CNTRL) group, Alpha-MEM was supplemented with 10% FCS and 1% penicillin and streptomycin but without osteogenic supplements.

Cell expansion and hMSC culture. One vial of each donor (n = 6; MSC donor 1 to 6) was thawed and subsequently seeded into T-150 flasks (Falcon) with 30 ml of the expansion medium (EXP, medium B) at a density of $3.3 \times 10^3/cm^2$. Cells were split when reaching 80% to 90% of confluence. Replating density was $3.3 \times 10^3/cm^2$ in T-150 cell culture flasks. Cells were cultured for ten days until passage 3 (P3) to obtain a sufficient number of cells with media change every second day at 37°C and 5% CO^2. Finally, at 90% of confluence, cells were trypsinized (Trypsin/EDTA; Biochrom AG) and prepared for scaffold seeding.

Preparation of the scaffolds. Collagen type II scaffolds were fabricated by freeze drying following an established protocol.[40] Porcine cartilage-derived type II collagen-glycosaminoglycan (GAG) material was provided by Geistlich Biomaterials (Geistlich Pharma AG, Wolhusen, Switzerland). The collagen sheets were blended and dissolved in 0.001 M hydrochloric acid to a final concentration of 1% (w/v). The suspension was centrifuged to remove air bubbles, and 25 ml of the slurry was poured into an aluminium pan (9 cm x 9 cm) and placed into the chamber of a freeze dryer (VirTis AdVantage 2.0 XL, SP Scientific, Warminster, Pennsylvania) at 20ºC to obtain highly porous, 2 mm thick scaffolds. The shelf temperature was lowered at a constant rate (approximately 1.4ºC per minute) to a final temperature of -10ºC and maintained for one hour to ensure complete freezing. Subsequent removal of the ice crystals by sublimation (vacuum of < 700 mTorr at 0ºC for 17 hours) formed the architecture of the scaffolds with a pore diameter of 350 µm. The scaffolds were then punched out with a biopsy punch (8 mm diameter; Stiefel Laboratories, GlaxoSmithKline, Research Triangle, North Carolina).[41,42] The final scaffolds measured 8 mm in diameter and 2 mm in height.

Sterilization and crosslinking of the scaffolds. To achieve optimal stability, the scaffolds were crosslinked. The dehydrothermal crosslinking process was performed in a vacuum oven (105°C at 50 mTorr) following a standard protocol.[41] For additional mechanical stabilization, further chemical crosslinking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) was performed.[43,44]

Seeding of the scaffolds. Two 12-well plates (Falcon) were prepared for scaffold culture.

Agarose powder (PeqLab Biotechnologie GmbH, VWR International, Erlangen, Germany) was dissolved in Aqua Dest (2% w/v) and then heated in an autoclave. Next, 1 ml of the suspension was added to each well and stored in a refrigerator at 4°C to allow the agarose to become solid. The purpose of the agarose coating was to prevent the cells or scaffolds from sticking to the plate surface.

The P3 cells were trypsinized, spun down, and resuspended to a concentration of 1×10^6 cells/20 µl cell solution. In total, 24 scaffolds were split into two groups (group A and group B). The scaffolds in group A were cultured in osteogenic media (medium A) while the scaffolds in group B acted as a negative CNTRL and were treated with CNTRL media (medium B).

The scaffolds were then soaked in the corresponding medium for ten minutes. The scaffolds were placed on filter paper for two minutes and then transferred to the agarose-coated wells.

Cells from each donor (n = 6) were seeded in duplicates on the scaffolds of each group. Therefore, from every donor, two scaffolds underwent osteogenic differentiation while two scaffolds served as a negative CNTRL. The double setup of the experiment was performed so as to have additional scaffolds available if needed for other investigations. Then, 10 µl cell suspension (containing 500 000 cells) was added with a pipette in the center of the scaffold, then the scaffold was turned to the other side and again 10 µl cell suspension was applied. In total, each scaffold received 20 µl cell suspension containing 1 $\times$ 10^6cells.

Next, 2 ml corresponding medium was added to each well and cultured in an incubator at 37°C and 5% CO_2 for 21 days with medium change every second day.

Preparation of the scaffolds and ^{99m}Tc –HDP labelling. One scaffold from each donor and group (total of 12 scaffolds) was placed in a 15 ml conical bottom tube (Falcon) and washed three times with PBS (Invitrogen). 240 MBq ^{99m}Tc–HDP (Technescan-HDP; Mallinckrodt Pharmaceuticals, Staines-upon-Thames, United Kingdom) was dissolved in 12 ml sodium chloride (NaCl) 0.9% (B. Braun Melsungen AG). Next, 1 ml of the solution, containing 20 MBq ^{99m}Tc–HDP activity, was added to 2.5 ml conical bottom tubes (Eppendorf International, Hamburg, Germany). To ensure that the same amount of activity was applied to each scaffold, each tube was measured in advance in a dosimeter (Aktivimeter ISOMED 2010; MED Nuklear-Medizintechnik Dresden GmbH, Dresden, Germany). Each scaffold was transferred into a single tube and incubated for two hours at room temperature. The scaffolds were then washed five times with PBS and subsequently transferred into 1 ml conical bottom tubes (Eppendorf) containing 1 ml fresh PBS.

The tubes were immediately placed on the posterior detector of a gamma camera (Siemens E-CAM+, Siemens AG, Munich, Germany). A single detector acquisition was performed, using a 256 $\times$ 256 detector grid. The gamma counts over the whole detector area were measured over 180 seconds. Finally, the scaffolds were removed from the detector and safely stored to allow the radioactivity decay to return to background levels.

To measure the activity emitted by each scaffold, the acquisition software Xeleris 3 (GE Healthcare) was used to define regions of interest (ROIs) around each scaffold within the detection area. Each ROI had the exact same pixel size. The amount of gamma counts for each ROI was calculated by the software, reflecting the amount of bound tracer.

Embedding/histology. The scaffolds were fixed in 10% phosphate-buffered formalin (Sigma) for one hour, followed by paraffin embedding using standard protocols.[45]

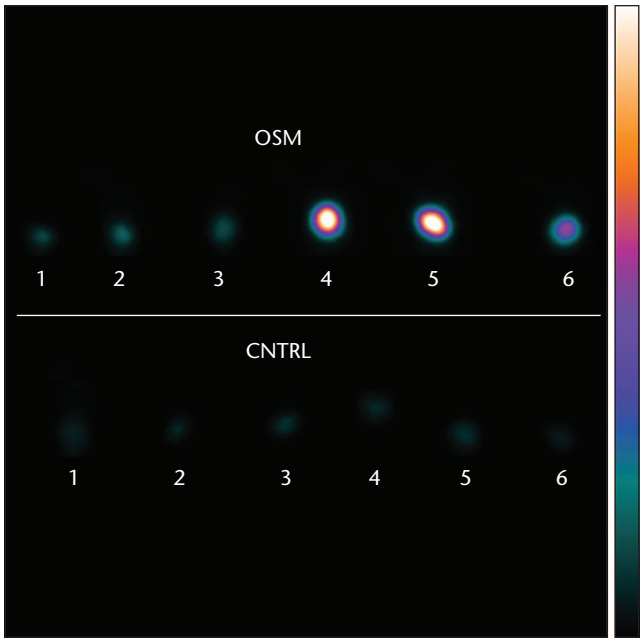

Fig. 1

Gamma camera image of the ^{99m}Tc-hydroxydiphosphonate (^{99m}Tc-HDP)-labelled scaffolds. In the upper line, the osteogenic group (OSM) emits much more gamma radiation from the scaffolds then from the control group (CNTRL) in the lower line, indicated by the intensity. The scaffolds are numbered with the mesenchymal stem cell donor number. On the right-hand side is the free scalable radiation reference intensity bar (maximum white/yellow to minimum green/black).

The central sections of the paraffin blocks were cut transversally (Jung Histo Slide 2000R; Leica Biosystems Nussloch GmbH, Nußloch, Germany), into sections 6 µm thick. These were mounted on glass slides, deparaffinized, and stained as described below using standard histological techniques.

Staining and quantification. For general scaffold architecture assessment and cell distribution, a standard haematoxylin and eosin (H&E) staining (Waldeck GmbH & Co. KG, Münster, Germany) was performed on the sections, followed by Masson's trichrome staining for collagen fibres (Merck KGaA).

To detect newly formed HA deposits within the scaffolds, Alizarin Red staining was performed. For the preparation of the dye, 2 g Alizarin Red S powder (Sigma-Aldrich) was dissolved in 100 ml Aqua Dest. Next, the pH was adjusted to 7.2 by adding ammonium hydroxide (Sigma-Aldrich). The slides were placed in the solution for 120 seconds and the excess dye was washed off. Using a microscope (Axioplan 2 Imaging Microscope; Carl Zeiss AG, Oberkochen, Germany) with a 5$\times$ and 10$\times$ magnification, sections of all 12 scaffolds were examined and classified by a ranking system. Each scaffold was graded according to the amount of Alizarin Red stain: '0' = 0% stained area; + = 1% to 25% stained area; ++ = 26% to 50% stained area; +++ = 51% to 75% stained area; and ++++ = 76% to 100% stained area.

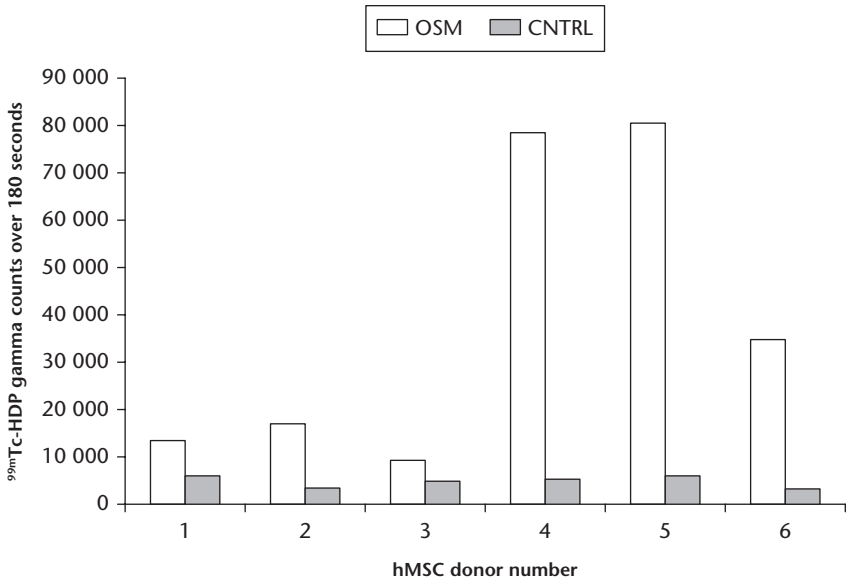

Fig. 2

99mTc-hydroxydiphosphonate (99mTc-HDP) uptake over 180 seconds in collagen type II scaffolds. This figure shows the individual gamma counts per 180 seconds for each donor (1 to 6). OSM, osteogenic group; CNTRL, control group; hMSC, human mesenchymal stem cell.

Statistical method. To determine a significant difference regarding the emitted gamma counts between the OSM group and the CNTRL group, a Student's *t*-test was performed. Prior to the test, the results were assessed for normal distribution using the Kolmogorov–Smirnov test.

For statistical analysis, SPSS Statistics version 20 (IBM, Armonk, New York) was used. Statistical significance was set to $p < 0.05$.

Results

99mTc–HDP labelling - gamma counts. There was substantial variability among human donors with respect to the tracer uptake. The values of the gamma counts showed a tracer uptake two to 15 times higher in the OSM group compared with the CNTRL group. The highest uptake within the six scaffolds treated with osteogenic media was 80.337 counts/180 seconds while the lowest activity was still 9.463 counts/180 seconds. The mean activity was calculated with 38.906 counts/180 seconds (SD) (32.520 counts/180 seconds; Figs 1 to 3).

Within the OSM group, donors 4, 5, and 6 showed a much higher degree of 99mTc-HDP uptake (34.747; 78.414; 80.337; counts per 180 seconds) then donors 1, 2, and 3 (9.464; 13.539; 16.935; counts per 180 seconds). Nevertheless, even the donor with the lowest uptake in the OSM group still had almost 50% more uptake then the scaffold with the highest uptake in the CNTRL group (Figs 1 to 3).

In contrast, within the CNTRL group, the highest activity measured for a scaffold was as low as 6.149 per 180 seconds, and the lowest activity was 333 counts per 180 seconds. The mean value for the CNTRL group was 4.325 counts per 180 seconds (SD 2.169 counts per 180 seconds; Figs 1 to 3).

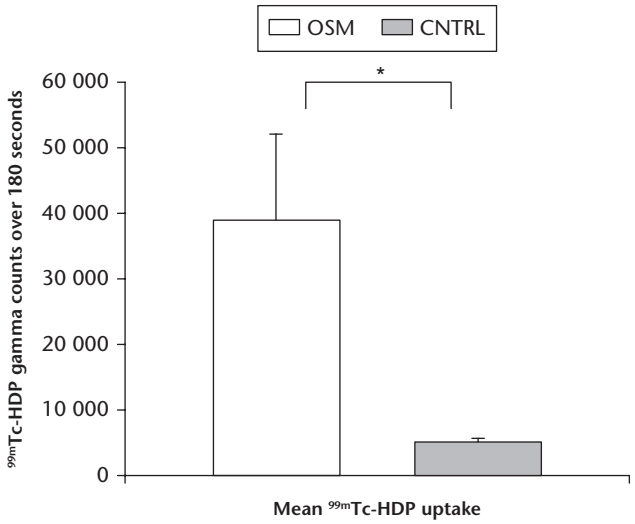

Fig. 3

Mean 99mTc-hydroxydiphosphonate (99mTc-HDP) uptake over 180 seconds in collagen type II scaffolds. This figure shows the mean gamma counts per 180 seconds for the osteogenic group (OSM) and control group (CNTRL). *Significant difference regarding the mean gamma counts between the groups, $p = 0.048$. SEM, scanning electron microscopy.

Macroscopically, the osteogenically inducted scaffold had a much more condensed and solid appearance, while the negative CNTRL scaffold was very loose. In particular, the CNTRL group scaffold (#6) with the uptake of only 333 counts per 180 seconds had a distinct loose structure. These findings correspond to the histological architecture found during microscopy of the stained scaffolds.

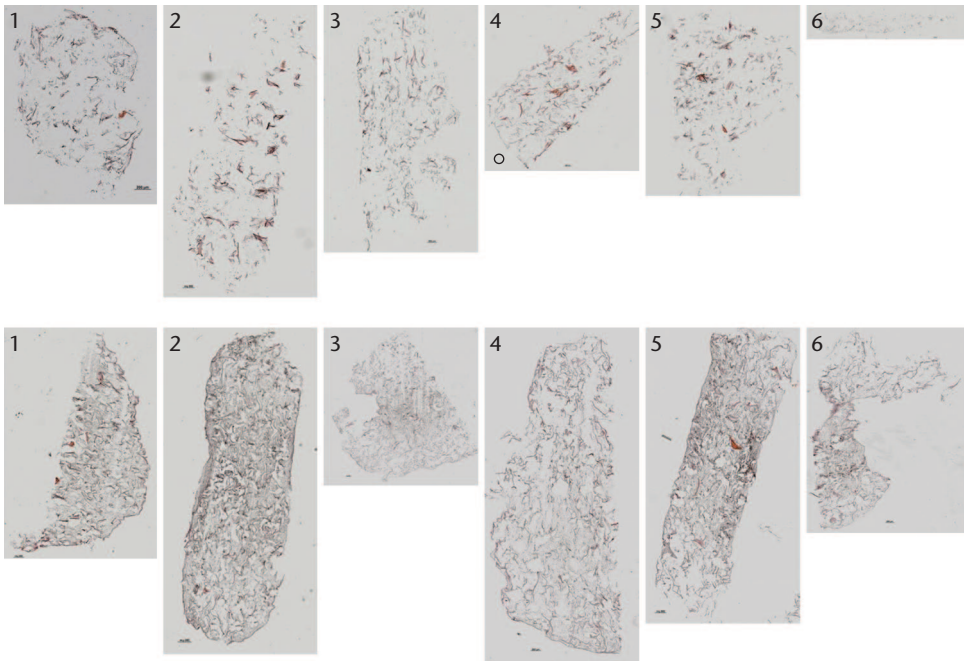

Fig. 4

6 µm histology section, 5 × magnification, haematoxylin and eosin (H&E) staining, donors 1 to 6, control group (upper line) and osteogenic group (lower line).

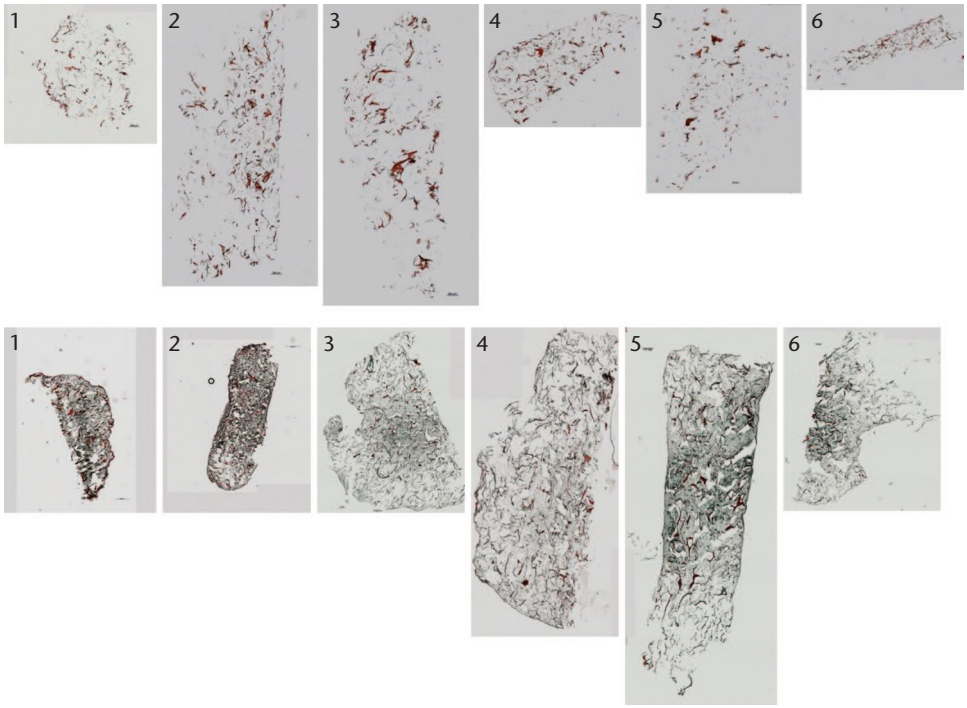

Fig. 5

6 µm histology section, 5 × magnification, Masson's trichrome staining, donors 1 to 6, control group (upper line) and osteogenic group (lower line).

The Kolmogorov–Smirnov test confirmed normal distribution of the data. Student's *t*-test revealed a significant difference in the amount of emitted gamma counts between the OSM group and the CNTRL group (two-sided significance, $p = 0.048$; Fig. 3).

Quantitative and qualitative histology. The H&E and Masson's trichrome microscopic examination of the osteogenically treated scaffolds showed a much denser collagen structure compared with the negative CNTRLs. Nuclei were well dispersed throughout the scaffold.

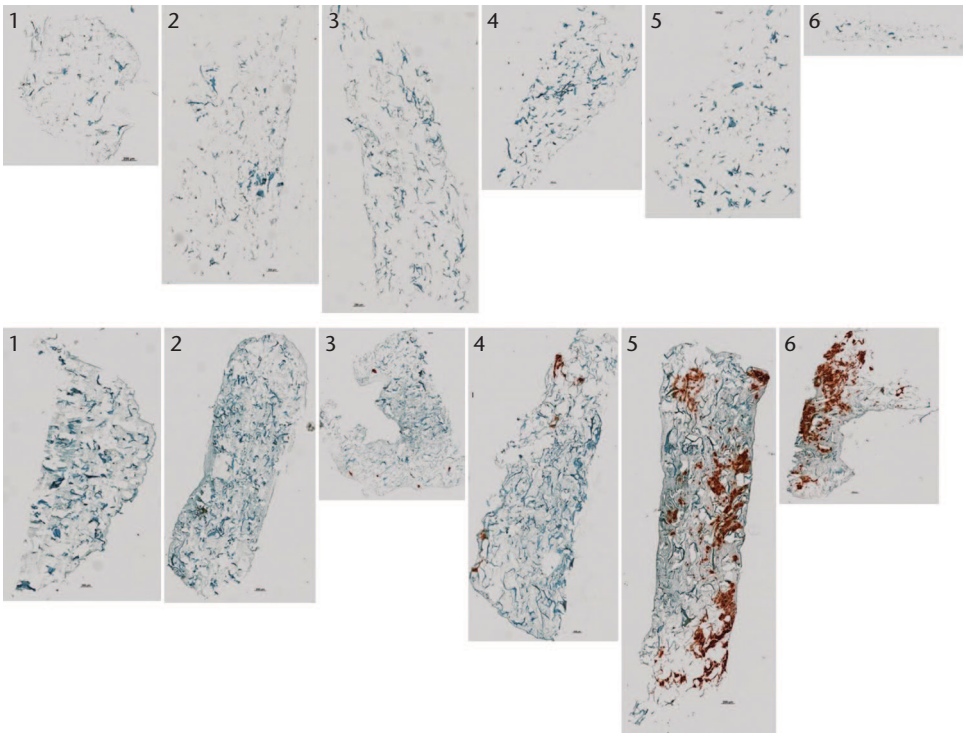

Fig. 6

6 µm histology section, 5 × magnification, Alizarin red staining, donors 1 to 6, control group (upper line) and osteogenic group (lower line).

The architecture of the CNTRL group scaffolds was very loose while the collagen fibres were not as compact as in the OSM group scaffolds (Figs 4 and 5).

Histological evaluation of the Alizarin Red-stained sections revealed a positive staining in the OSM group, indicating extracellular calcium deposition in three of the six donors (donors 4, 5, and 6). While donors 5 and 6 showed a strong positive stain in almost 50% of the scaffold area, donor 4 had only a few spots confirming the presence of HA, e.g. calcium deposition. Donors 1, 2 and, 3 showed no or minimal positive Alizarin Red stain (Fig. 6). None of the scaffolds of the CNTRL group showed any positive Alizarin Red staining for extracellular calcium deposition (Fig. 6).

Table I shows the amount of positive stained area for each scaffold compared with the measured gamma counts.

Table I. Showing the mesenchymal stem cell donor number and the emitted gamma counts (Tc) over 180 seconds for each scaffold. The ARS column indicates the amount of Alizarin Red stained area over the whole scaffold. o, o% stained area; +, 1% to 25% stained area; ++, 26% to 50% stained area; +++, 51% to 75% stained area; ++++, 76% to 100% stained area

n	OSM Tc	OSM ARS	CNTRL Tc	CNTRL ARS
1	13.539	0	5.821	0
2	16.935	0	3.441	0
3	9.463	0 (+)	4.970	0
4	34.747	+	5.235	0
5	80.337	++	6.149	0
6	78.414	++	333	0

ARS, Alizarin Red stained OSM, osteogenic group; CNTRL, control group

Discussion

Within the presented work we were able to establish a new method for the direct quantification of HA deposition by osteogenically induced hMSCs in a collagen II-based 3D cell culture model. This study was conducted as there is a strong need for new and efficient non-destructive methods to track and quantify osteogenic differentiation *in vitro* as 3D bone tissue engineering is a growing field in modern regenerative medicine.[22]

As there is continuous development to optimize methods for osteogenic differentiation, there is a great need for the prompt evaluation of these new protocols. One of the great advantages of the presented method is that it is fast, cost-efficient, and non-destructive. The scaffolds remain intact for further experiments. Additionally, the HA content is detected directly by ^{99m}Tc–HDP labelling. We found a strong variability in the uptake of ^{99m}Tc-HDP in different donors in the OSM group, which may represent a strong interindividual variability in hMSCs undergoing osteogenic differentiation. This is a well described phenomenon during osteogenic differentiation.[46-49] Nevertheless, all scaffolds in the OSM group showed a significantly higher uptake of the nuclear tracer when compared with untreated CNTRLs, reflected by the amount of emitted gamma counts. We were able to show a significant difference between the emitted gamma counts, reflecting a much higher amount of mineral

deposition in the OSM group compared with the negative CNTRL group (Student's *t*-test: two-sided significance, $p = 0.048$).

However, the histological mineral staining results were by far not as impressive as the results from the technetium labelling. A possible explanation might be that the radioactive labelling method is much more sensitive than the Alizarin Red and von Kossa staining methods. Therefore, it is quite likely that the radioactive tracer binds small amounts of existing mineral deposits that cannot even be visualized by the histological staining. Nevertheless, we observed a cohesion within the OSM group between the gamma counts and the positive Alizarin Red-stained area. These results correspond with the results from our previous work, where we could show that there is a robust correlation between the uptake of the tracer, reflected by the gamma counts, and the amount of Alizarin Red stain within monolayer cell cultures.[38]

The H&E and Masson's trichrome staining showed the dense architecture of the osteogenic scaffolds while the cells were distributed evenly through the whole scaffold. The scaffolds of the negative CNTRL group showed fewer distributed cells with much less extracellular matrix, resulting in a very soft and loose architecture.

As the tracer was applied after the cell cultures were terminated, the radioactivity was very unlikely to have an effect on the cell proliferation or on the mineral deposition. Also, the applied activity of 20 MBq is extremely low, especially in comparison with the amount of activity that is used in human nuclear medicine, where patients are exposed to about 500 MBq to 800 MBq activity per examination. Therefore, it is very unlikely that the laboratory personnel were exposed to a harmful amount of radiation while performing the ^{99m}Tc–HDP labelling assay for the quantification of mineral deposits in 3D-MSC cultures.

In the near future, further experiments are needed to refine this novel method. First of all, there is a need to evaluate whether the incubation time, as well as the applied dosage of radioactivity, could be reduced. Following this, further experiments will be performed to test the novel method against the benchmark Alizarin Red staining in terms of sensitivity, as well as to perform a toxicity assay to proof the harmlessness of the tracer. To have the gamma counts standardized to a certain amount of HA, a defined amount of HA could be added to each collagen slurry followed by a native ^{99m}Tc-HDP labelling.

In summary, our data show that ^{99m}Tc-HDP labelling is capable of quantifying the amount of extracellular HA deposition in 3D-MSC cultures on a time-saving and non-destructive basis.

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- T. L. Grossner: Planned the study, Cultured the cells, Provided radioactive labelling, Analyzed the results.
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