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Short communication

The effect of perfusion culture on proliferation and differentiation of human mesenchymal stem cells on biocorrodible bone replacement material

J. Farack[a,*], C. Wolf-Brandstetter[a], S. Glorius[b], B. Nies[b], G. Standke[c], P. Quadbeck[d], H. Worch[a], D. Scharnweber[a]

[a] Technische Universität Dresden, Max Bergmann Center of Biomaterials, Budapester Str. 27, D-01069 Dresden, Germany
[b] InnoTERE GmbH, Tatzberg 47–49, D-01307 Dresden, Germany
[c] Fraunhofer Institute for Ceramic Technologies and Systems (IKTS), Winterbergstr. 28, D-01277 Dresden, Germany
[d] Fraunhofer Institute for Manufacturing Technology and Advanced Materials (IFAM), Winterbergstr. 28, D-01277 Dresden, Germany

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ABSTRACT

Biocorrodible iron foams were coated with different calcium phosphate phases (CPP) to obtain a bioactive surface and controlled degradation. Further adhesion, proliferation and differentiation of SaOs-2 and human mesenchymal stem cells were investigated under both static and dynamic culture conditions. Hydroxyapatite (HA; $[Ca_{10}(PO_4)_6OH_2]$) coated foams released 500 μg/g iron per day for Dulbecco's modified eagle medium (DMEM) and 250 μg/g iron per day for McCoys, the unmodified reference 1000 μg/g iron per day for DMEM and 500 μg/g iron per day for McCoys, while no corrosion could be detected on brushite ($CaHPO_4$) coated foams. Using a perfusion culture system with conditions closer to the *in vivo* situation, cells proliferated and differentiated on iron foams coated with either brushite or HA while in static cell culture cells could proliferate only on Fe-brushite. We conclude that the degradation behaviour of biocorrodible iron foams can be varied by different calcium phosphate coatings, offering opportunities for design of novel bone implants. Further studies will focus on the influence of different modifications of iron foams on the expression of oxidative stress enzymes. Additional information about *in vivo* reactions and remodelling behaviour are expected from testing in implantation studies.

1. Introduction

Conventional implant materials used in orthopaedic surgery are mostly based on non-degradable, bioinert metal alloys such as stainless steel, cobalt based alloys, and the more biocompatible titanium alloys. However, for numerous applications especially as bone replacement materials the aspect of stress shielding, caused by an inappropriate stiffness of the implant, remains a still unsolved issue especially for not biodegradable implant materials retaining a constant strength over time. Furthermore, the interface area between implant and surrounding tissue is limited by the surface of the solid non-porous implant.

Metal foams have become a novel promising material for medical applications as bone implant. A key benefit is the open porous structure which increases the interacting surface between tissue and implant and enables a better integration of the implant. Nevertheless, non-degradable metal foams made of titanium or tantalum [1–3] will remain permanently in the body. Therefore biocorrodible, degradable metals, in particular magnesium (Mg) and iron (Fe), became increasingly attractive for researchers in recent years.

Mg is an osteoconductive material and stimulates bone generation [4,5]. A significant drawback however is the low corrosion resistance. Thus available Mg materials generally degrade too fast for orthopaedic application [6–8].

Iron is an essential co-factor for a plenty of enzymes involved in oxygen binding, DNA synthesis and redox processes. However, iron homeostasis is tightly controlled in order to prevent iron overload [9,10]. High iron concentrations pose a risk to cells and tissue [9,10]. Major reason for this is its ability to catalyze the generation of hydroxyl radicals from superoxide and hydrogen peroxide, summarized as reactive oxygen species [11]. However, several observations *in vivo* argue against a significant cytotoxic effect of iron and its corrosion products [12]. In contrast to Mg the corrosion of elementary iron *in vivo* is slow and has been shown to exhibit no local or systemic toxicity [13].

Vascularisation of bone implants and their entire integration into the surrounding tissue are key functions for the success of a bone graft material. Cellular metal foams meet these high demands due to their open porosity (structural biocompatibility) which enables bony ingrowth [14,15]. This is expected to result

* Corresponding author. Tel.: +49 351 463 39382.
E-mail address: jana.farack@tu-dresden.de (J. Farack).

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in a more stably anchored implant and thus facilitate the healing process.

In addition, surface coatings with bioactive calcium phosphate phases (CPP) like hydroxyapatite, the major mineral component of bone tissue [16], is an appropriate means to increase the bioactivity [17,18].

In the present study we developed CPP-bioactivated iron foams which are biocorrodible and offer new opportunities for design of novel bone implants. The complete implant degradation over time is aspired which would make revision surgery obsolete.

2. Experimental

2.1. Specimen

Cylindrical iron foams (Ø 10 mm, height 5 mm) with an open porosity of 85% and a pore size of 45 pores per inch (Fig. 1a) were manufactured by powder metallurgy processes [19] at the Fraunhofer institutes IKTS and IFAM in Dresden, Germany. Briefly, shaped parts of polyurethane-foam were coated with carbonyl iron powder suspension with 3.8% Fe_3P, dried, pyrolyzed and finally the remaining metal foam was sintered. Subsequently, InnoTERE (Dresden, Germany) processed iron foam coating with either brushite or hydroxyapatite (HA). Therefore, thoroughly with acetone and ethanol cleaned iron foams were coated with a brushite layer ($CaHPO_4{\cdot}2H_2O$, InnoTERE's patent pending, Fig. 1b). To obtain a hydroxyapatite coating (Fig. 1c) the brushite-coated iron foams were heat treated at 95–100 °C in 0.1 M NaOH for 24 h as described elsewhere [20].

To characterise the corrosion behaviour and for cell culture experiments the sterilised samples (gamma-radiation, 25 kGy) were preincubated in cell culture media (type of media and concentration of fetal serum albumin depending on cell type used in the later cell culture experiment) for 4 days with media exchange every day.

2.2. Corrosion behaviour

As corrosion relevant parameters iron release, oxygen saturation, and hydrogen peroxide concentration in the cell culture media were quantified as described in the following.

2.2.1. Iron release

For quantification of iron in the supernatant a photometric assay (DiaSys Diagnostic Systems, Holzheim, Germany) was used according to the manufacturer's protocol. Briefly, iron bound to transferrin is released in an acidic medium as ferric iron and subsequently reduced to ferrous iron in the presence of ascorbic acid. Ferrous iron forms a blue complex with Ferene [21]. The absorbance of ferrous ferene complex was measured using a UV VIS spectrometer (Spectrafluor Plus, Tecan, Crailsheim, Germany) at 590 nm and is directly proportional to the iron concentration.

2.2.2. Hydrogen peroxide

The amount of hydrogen peroxide in the medium was quantified with an Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine) assay (Invitrogen, Darmstadt, Germany) using horseradish peroxidase to produce the red-fluorescent oxidation product resorufin. According to the manufacturer's instructions 50 μL reactions mix, containing both Amplex® Red and horseradish peroxidase, were added to 50 μL preincubated cell culture medium. After incubation of 30 min at room temperature in the dark the fluorescence was measured using a 485 nm excitation filter and a 595 nm emission filter.

2.2.3. Oxygen saturation

To monitor the oxygen saturation over the incubation time an oxygen electrode system from SBU (Waldheim, Germany) was utilised.

2.3. Cell culture experiments

For the biochemical and cytobiological characterisation, *in vitro* cell culture experiments with human mesenchymal stem cells (hMSC) and osteosarcoma cells (SaOs-2) were performed.

2.3.1. Adhesion and proliferation of SaOs-2

The human osteoblast-like cell line SaOs-2 was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ; Germany). This cell line has been studied extensively and has been shown to possess the phenotypic features of normal osteoblasts [22]. SaOs-2 Cells were grown in McCoys 5a medium supplemented with 15% fetal calf serum, 1% penicillin-streptomycin and 2 mM L-glutamine. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

For adhesion and proliferation tests the cells were seeded by drop-seeding directly on samples which had been preincubated in cell culture media (McCoys with 15% fetal calf serum 1% penicillin and streptomycin; 2 mM L-glutamine) as described below. After 1 h of adhesion the seeded samples were incubated either statically in cell culture well plates with media exchange twice a week, or dynamically in perfusion containers from Minucells® (Bad Abbach, Germany,) with a flow rate of 1 mL/h (Fig. 1d). After 1 h, 3 days and 14 days the cells on the different bioactivated iron foams were rinsed in 1 mL PBS at 37 °C and stored at −80 °C.

2.3.2. Proliferation and differentiation of hMSCs

Human mesenchymal stem cells were obtained from the University Hospital Carl Gustav Carus Dresden of the Technische Universität Dresden (Germany). Cells were grown in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum, 1% penicillin-streptomycin and 2 mM L-glutamine in a humified atmosphere with 5% CO_2 at 37 °C and used for cell culture experiments in passage 4.

Cell seeding and transfer to either static or dynamic culture was performed as described above for the SaOs-2 cells. For osteogenic differentiation tests the cells on the samples were incubated either in basic medium (DMEM with 10% fetal calf serum; 1% penicillin and streptomycin; 2 mM L-glutamine) or in differentiation medium (basic medium supplemented with 10 mM β-glycerophosphate, 10 nM dexamethasone and 200 μM ascorbic acid) starting at day 4 after cell seeding. 7 days, 14 days, 21 days and 28 days after osteogenic induction, the cells on the different bioactivated iron foams were rinsed in 1 mL PBS at 37 °C and stored at −80 °C.

2.4. Biochemical analysis

Cellular lactate dehydrogenase was used both as a quantitative marker for cell number to study the cellular adherence and proliferation and for calculation alkaline phosphate levels *in vitro*.

The stored cells on different bioactivated iron foams were thawed for 30 min on ice and subsequently incubated in 0,5 mL lysis buffer (PBS with 1% Triton X-100) for 50 min on ice.

2.4.1. Lactate dehydrogenase (LDH)

For analysis of LDH a commercial kit (LDH Cytotoxicity Detection Kit, TaKaRa Bio Inc. Shiga, Japan) was used according to the

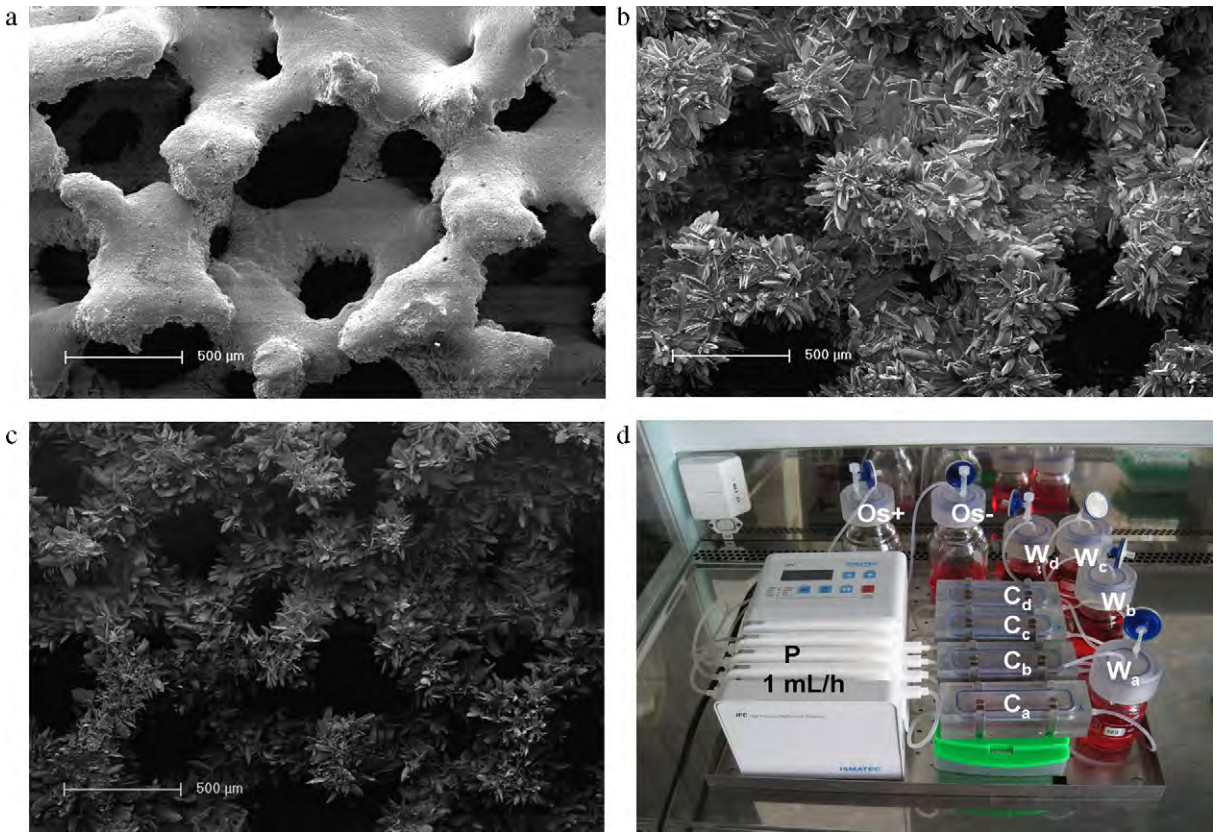

Fig. 1. (a) SEM-picture of unmodified iron foam (scale bar 500 µm); (b) SEM-picture of brushite-coated iron foam (scale bar 500 µm); (c) SEM-picture of HA-coated iron foam (scale bar 500 µm) and (d) perfusion culture: fresh basic medium (*Os−*); fresh differentiation medium (*Os+*); pump (*P*) perfusion container (*C1–C4*), waste bottle (*W1–W4*).

manufacturer's protocol. The absorbance of the formazan product was measured using a UV VIS spectrometer (Spectrafluor Plus, Tecan, Crailsheim, Germany) at 492 nm. Formazan concentrations are directly proportional to the concentration of LDH in the sample [23]. Lysis buffer and iron foams without cells were used as blanks.

2.4.1.1. Alkaline phosphatase (ALP). The ALP activity as a marker for osteogenic differentiation was spectroscopically determined at 405 nm using p-nitrophenylphosphate (Sigma–Aldrich Chemie GmbH, Germany) as substrate. 125 µL of 1 mg/mL substrate solution (p-nitrophenylphosphate in 0.1 M diethanolamine, 0.1% Triton X-100, and 1 mM $MgCl_2$, pH 9.8) was added to 25 µL lysate. After incubation for 30 min at 37 °C, the reaction was stopped with 63 µL 1 N NaOH and centrifuged at 14,000 × *g* for 30 min. Absorption was measured using a UV VIS spectrometer (Spectrafluor Plus, Tecan, Crailsheim, Germany) at 405 nm. Lysis buffer and iron foams without cells were used as blanks. The ALP activity was normalized to the LDH activity (per cells).

3. Results and discussion

3.1. Corrosion behaviour of bioactivated iron foams

It is known that during degradation of metallic iron, Fe(II) ions are released from the surface following Eq. (1)

$$Fe \rightarrow Fe^{2+} + 2e^{-} \quad (1)$$

The incubation of unmodified and modified iron foams in cell culture medium resulted in different iron release rates over time (Fig. 2a). Unmodified iron foams show by far the highest initial corrosion (≈1 mg/g iron per day) which decreases within 4 days to half of the initial value due to formation of a protein containing iron oxide layer. HA-coated foams (Fe-HA) release considerably less iron ions than unmodified foams (500 µg/g iron per day for DMEM and 250 µg/g iron per day for McCoys), while almost no corrosion can be detected on brushite coated foams (Fe-brushite).

The appearance of Fe-HA and Fe-brushite after incubation in the two different cell culture media DMEM and McCoy's is illustrated in Fig. 2b. In both cell culture media (McCoys and DMEM) heavy corrosion of Fe-HA was observed already after three days, being more intensive in DMEM than in McCoys.

No corrosion was detected for Fe-brushite. The brushite coating proved to be highly protective and even after 28 days of incubation only minimal concentrations of iron corrosion products in the medium can be detected.

Fig. 3 shows oxygen saturation and hydrogen peroxide formation to characterise the corrosion behaviour of Fe-HA and Fe-brushite compared to the unmodified reference (Fe). For Fe the highest oxygen depletion is observed (Fig. 3a) which is in agreement with its highest iron release (Fig. 2). The oxygen consumption of Fe-HA is lower than for Fe, but still a significant decrease of the oxygen saturation in incubation solution is observed. Incubation of Fe-brushite does not result in a change of the oxygen saturation compared to the control (McCoys only).

Our results correspond to those published by Huang [24] who described oxidative stress as being caused by iron degradation and

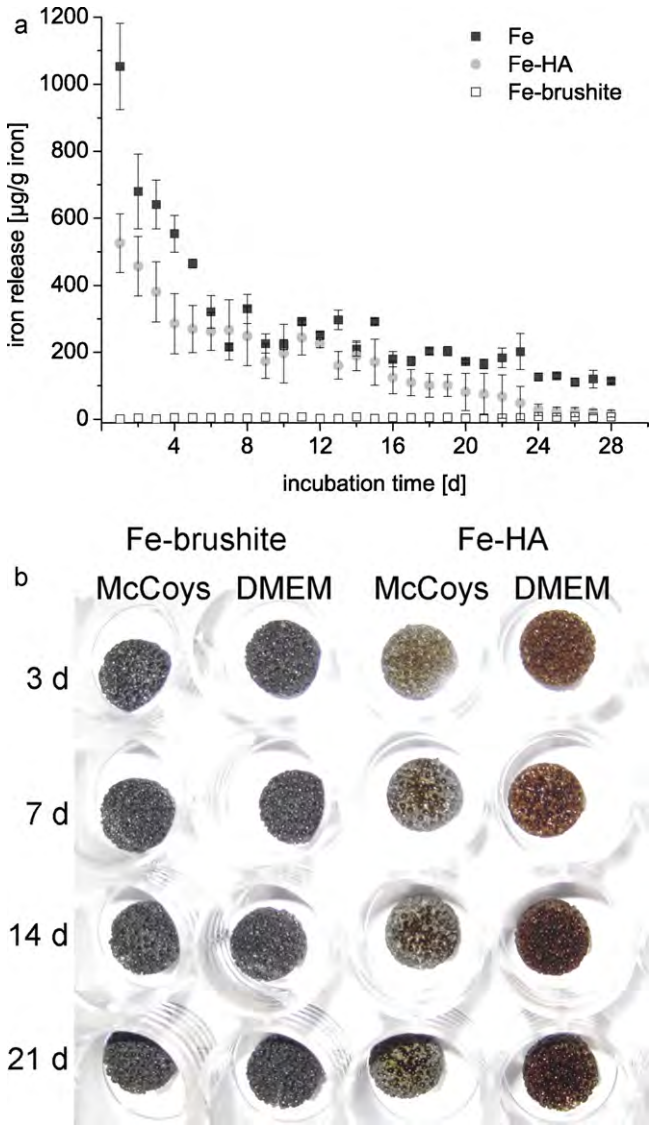

Fig. 2. (a) Preincubation of differently modified iron foams for 28 days with daily medium exchange (McCoys + 15% serum) and measurement of iron ion release in the supernatant and (b) appearance of Fe-brushite and Fe-HA coated foams after 3 days, 7 days, 14 days and 21 days incubation in different cell culture media (McCoys and DMEM).

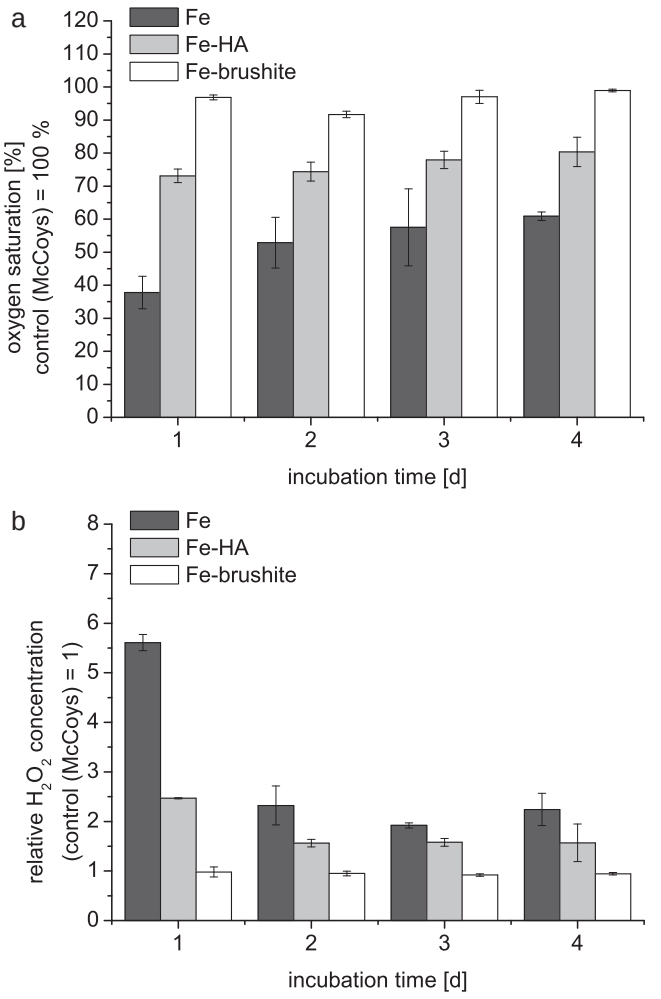

Fig. 3. Measurement of (a) oxygen saturation in the supernatant and (b) relative H_2O_2 in the supernatant after preincubation of differently modified iron foams with daily medium exchange (McCoys + 15% serum).

the resultant formation of reactive oxygen species. This is illustrated for the ferrous iron mediated autoxidation reactions:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -} \quad (2)$$

$$Fe^{2+} + O_2^{\bullet -} + 2H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (3)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH- + OH^{\bullet} \quad (4)$$

$$Fe^{2+} + OH^{\bullet} \rightarrow Fe^{3+} + OH^- \quad (5)$$

According to the Eqs. (2)–(5), the self-quenching reaction can be summarized as follows:

$$4Fe^{2+} + O_2 + 2H^+ \rightarrow 4Fe^{3+} + 2OH- \quad (6)$$

In addition, it is known from electrochemical investigations on the mechanism of the oxygen reduction [25] that the cathodic reduction according to Eq. (11) is split into 4 single electron transfer reactions ((7a)–(10)) resulting in radicals and H_2O_2 as intermediates (Eqs. (7)–(10)):

$$O_2 + e^- + H_2O \rightarrow HO_2^{\bullet} + OH^- \quad (7a)$$

$$HO_2^{\bullet} + H_2O \rightarrow H_2O_2 + OH^{\bullet} \quad (7b)$$

$$OH^{\bullet} + e^- \rightarrow OH^- \quad (8)$$

$$H_2O_2 + e^- \rightarrow OH^{\bullet} + OH^- \quad (9)$$

$$OH^{\bullet} + e^- \rightarrow OH^- \quad (10)$$

$$O_2 + 4e^- + 2\,H_2O \rightarrow 4\,OH^- \quad (11)$$

Generation of H_2O_2, one of the oxidative stress intermediate products, was observed during the corrosion of the different modified iron foams (Fig. 3b). For unmodified Fe the highest concentrations of hydrogen peroxide were detected. Initial H_2O_2 formation was 5.5 times higher than for the control (McCoys only).

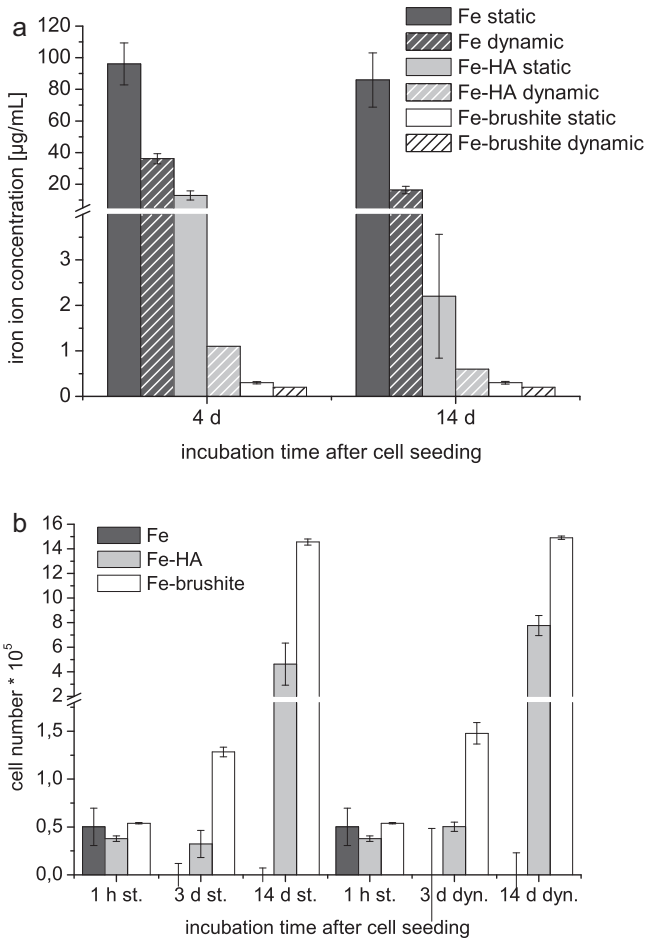

Fig. 4. (a) Iron-ion concentration in the supernatant (McCoys) after 4 days and 14 days incubation of SaOs-2 cells on different (bioactivated) iron foams and (b) adhesion and proliferation (LDH activity) of SaOs-2 cells incubated on different (bioactivated) iron foams.

Continued incubation resulted in considerable reduction of H_2O_2 formation compared to the initial 24 h, which agrees with the decrease of the corrosion rate as detected by the release of iron ions. While for Fe-brushite no H_2O_2 formation could be detected, initial H_2O_2 formation on Fe-HA was more than two times higher than for the control. Continued incubation resulted in minor reduction of H_2O_2 formation.

In summary, unmodified iron foams showed by far the highest corrosion accompanied by high concentrations of H_2O_2. Coating the iron foams with different calcium phosphate phases diminished the corrosion in a material dependent manner. While the brushite layer proved to be highly protective with almost no detectable corrosion, the HA coated iron foams showed a significant iron release, hydrogen peroxide formation and oxygen depletion, although lower than for unmodified iron foams. Fe-HA has a coating of hydroxyapatite, the thermodynamically stable phase under the given conditions. Fe–B on the other hand still has a brushite coating, and it is expected that the brushite transforms into HA during the exposure to the test media. This offers the chance to repair weak areas in the coating and intercept corrosion, which is not possible for the coating on Fe-HA as here the transformation from brushite into HA is already complete.

Corrosion behaviour of different iron foams was further influenced by the type of the incubation medium, with DMEM resulting in more intensive corrosion than McCoys. Major reason for this finding is expected to be the presence or absence of ascorbic acid.

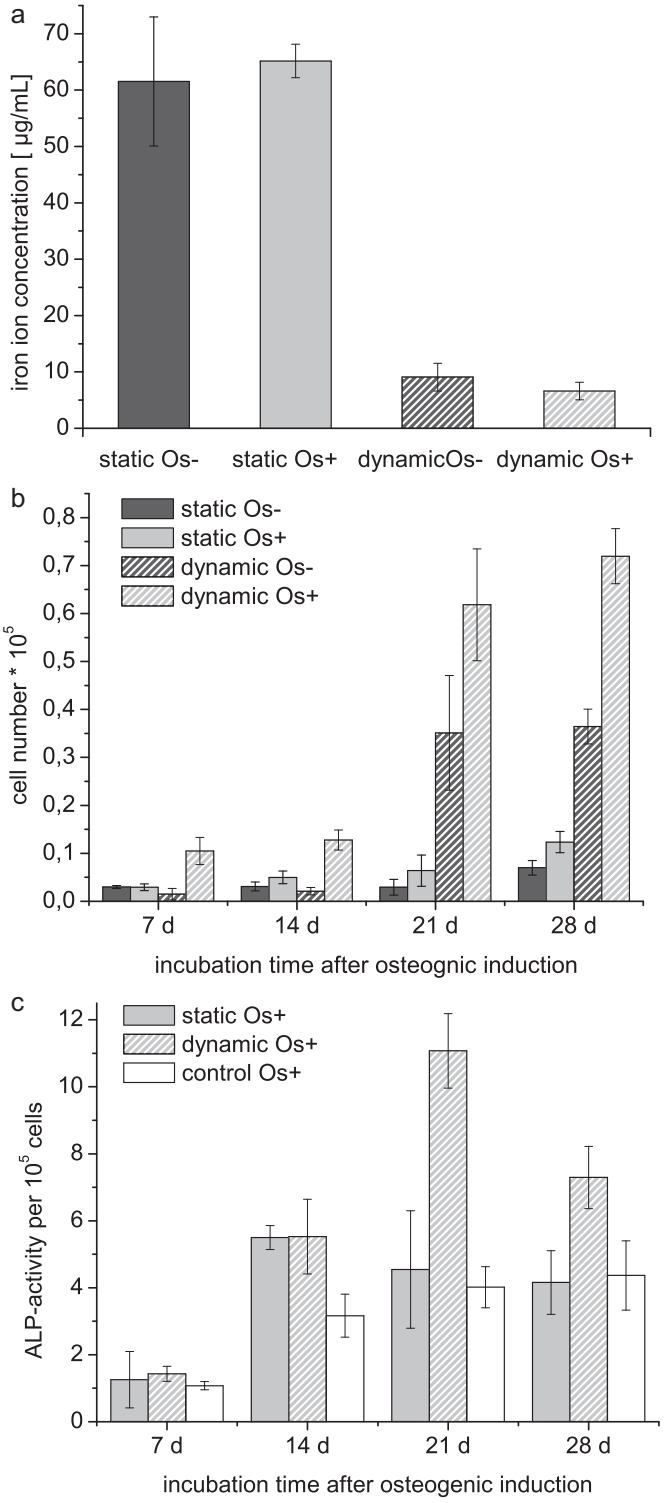

Fig. 5. (a) Iron determination in the supernatant (DMEM) after 14 days incubation of hMSCs on Fe-HA for static vs. dynamic cell culture conditions; (b) proliferation (determined by LDH-measurement) of hMSC incubated on Fe-HA for static vs. dynamic cell culture conditions and (c) Alkaline phosphatase activity of hMSCs on Fe-HA for static vs. dynamic cell culture conditions.

3.2. *Perfusion vs. static cell culture – SaOs-2 cells*

The degree of corrosion of Fe, Fe-HA and Fe-brushite had a significantly influence on the cellular response of SaOs-2. Under static culture conditions cells could proliferate only on Fe-brushite, while on Fe and Fe-HA the accumulation of cytotoxic corrosion products

lead to a strongly diminished cell viability (Fe-Ha) or even to cell death (Fe) (Fig. 4b).

To simulate the dynamic character of a biological system, a perfusion culture system (Fig. 1) was set up. For both Fe and Fe-HA iron ion concentration in the medium decreased in the dynamic system (Fig. 4a). The continuous supply of fresh medium and removal of toxic corrosion products and metabolites in the perfusion system thus resulted in more moderate conditions for cell culture. On Fe-HA SaOs-2 cells showed a better proliferation as a consequence than under static culture conditions, but for Fe there was no increase in cell number after 3 days under static or dynamic conditions. Obviously the local concentrations of toxic corrosion products were still too high in this case.

Fe-brushite released almost no corrosion products even under static conditions, therefore a high increase of cell number was observed for both static and dynamic cell culture.

3.3. *Perfusion vs. static cell culture – hMSC*

The effect of the higher corrosion of Fe-HA in DMEM (cell culture medium for hMSC) than in McCoys (cell culture medium for SaOs-2) as shown in Fig. 2b could also be compensated by using dynamic culture. After 14 days of statically or dynamically incubating hMSCs on Fe-HA, the iron ion concentration in the supernatant was found to be approximately 100 μg/mL for static cell culture, and less than 10 μg/mL for perfusion cell culture. Using perfusion culture conditions an accumulation of high amounts of corrosion products could be avoided, allowing hMSC to proliferate and differentiate on Fe-HA, too (Fig. 5).

Fig. 5c shows the level of alkaline phosphatase activity as a marker for osteogenic differentiation. The results illustrate that the application of a perfusion culture system during incubation of hMSCs on bioactivated porous iron foams is beneficial to osteogenesis. Due to the continuous supply of fresh differentiation medium the ALP activity in the dynamic system was significantly enhanced. After 21 days and 28 days cells dynamically cultured on Fe-HA showed a two to three times higher ALP activity compared to cells which were incubated statically either in cell culture well plates on polystyrene or on HA coated iron foams.

In summary, the permanent exchange of the medium offers a number of benefits. Metabolites and corrosion products are drained with the medium flow so that they do not accumulate and a more constant physiological environment in the perfusion chamber is achieved [26]. Therefore dynamically cultured hMSCs proliferated (Fig. 5b) and differentiated (Fig. 5c) on Fe-HA much better than under static cell culture conditions where medium exchange took place only twice a week.

4. Conclusions

We demonstrate that the degradation behaviour of biocorrodible iron foams can be varied by different calcium phosphate coatings. Brushite coating proved to be a significantly more effective means of corrosion control than HA coating. The results further suggest that perfusion cell culture can be used to enhance proliferation and osteogenic differentiation of hMSCs on CPP-bioacitvated iron foams due the continuous supply of fresh medium and removal of cytotoxic corrosion products.

Further studies will focus on the influence of the differently modified iron foams on the expression of oxidative stress enzymes. Additional information about *in vivo* reactions and remodelling behaviour are expected from testing in implantation studies.

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