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Oxygen consumption, acidification and migration capacity of human primary osteoblasts within a three-dimensional tantalum scaffold

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Abstract A major clinical problem within synthetic, large-scaled scaffolds is the insufficient nutrient supply resulting in inhomogeneous cell proliferation and differentiation. The aim of this study was to analyse pH value, oxygen consumption and migration of human osteoblasts within a 3D tantalum scaffold, clinically used for larger bone defects. After 24 h the oxygen concentration within the scaffold decreased significantly and remained low during incubation. Monitoring of the pH value inside the tantalum scaffold showed a slightly acidification under static culture conditions. However, cell migration within the 3D scaffold was detected. Hence, in clinical application it can be assumed that porous tantalum scaffolds can be settled by osteoblasts under critical oxygen and nutrient supply. In general, monitoring of cell migration, oxygen consumption and acidification can be a suitable instrument for creating advanced 3D bone scaffolds.

1 Introduction

Large bone defects can be the consequence of trauma, tumours or infections. There are more than 1.5 million bone-grafting procedures worldwide every year [1] which have a market volume of approximately one billion US dollars [2]. In current therapeutic strategies, bone defects are filled up by bone auto- or allografts [3–5] but there are disadvantages to these methods such as limited availability, donor site morbidities, immunological reactions or risk of infections [5–7]. Hence, research concentrates on new alternatives, such as synthetic, porous three-dimensional (3D) scaffolds, which can be adapted to the bone defects. Therefore, cells are seeded on scaffolds which mimic an artificial extracellular matrix (ECM). On these structures, cells are able to migrate, proliferate and differentiate within the scaffold [8]. With increasing volume of the scaffolds, gradients in cellular proliferation and differentiation within the three-dimensional culture have been reported [9–11]. These gradients mostly reflect an uneven nutrient and oxygen supply within the 3D scaffold [11]. In particular, oxygen is a critical substrate for eukaryotic cells. Variations in oxygen concentrations have far-reaching consequences for cell behaviour in vivo as well as in vitro. Hyperoxia is connected with cell toxicity [12]. In contrast, hypoxia resulted in a limitation of many signalling pathways, which regulate survival, proliferation and differentiation [10, 12]. Another consequence of hypoxia in vivo is acidosis, caused by the anaerobic cell metabolism as well as the reduced perfusion [13]. It is well known, that bone cells react extremely sensitive to direct pH effects [14]. So, the interaction of both, hypoxia and acidosis, leads to osteoclast formation and inhibits mineral deposition by osteoblasts [13, 14].

Nutrients, oxygen supply and also waste disposal will be kept constant over a distance of 20–200 μm in living tissue [10, 15]. In vitro, a sufficient supply of nutrients and oxygen by diffusion processes is limited to a distance of 100–200 μm [10]. Furthermore, differences in environmental conditions predominate between the periphery and the core of tissue-engineered constructs. Thus, cells within a 3D scaffold are dependent on the diffusion of nutrients,

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oxygen and also the removal of waste products which is the major challenge in the development of large-scaled 3D scaffolds [10].

There are several materials used for bone reconstructions in orthopaedic surgery. Porous tantalum metal (Trabecular Metal™, Zimmer, Freiburg, Germany) has proved to be a convenient synthetic material [16]. The tantalum scaffold is characterised by a high porosity, sufficient strength, low stiffness and good biocompatibility, so that it offers a lot of advantages compared to other implant materials used [16–18]. Additionally, this biomaterial generates bone ingrowth and mechanical attachment [19]. In animal experiments, bone ingrowth was shown up to a depth of 2 mm [20].

Porous tantalum scaffolds are used for various applications in orthopaedic surgery [19], which include covering of implant surfaces or refilling of bone defects. There are some studies about the interactions of osteoblasts with this material [19]. But so far, there is no data about the supply of oxygen within a porous 3D tantalum scaffold. Additionally, there are no data about pH monitoring within 3D scaffolds. Hence, in the present study, two porous tantalum discs with 26 mm diameter, which were placed on top of each other, were used. Thus, we obtained a 3D construct with a height of 10 mm and four distinct planes (Fig. 1a). For the in vitro investigations, we seeded human osteoblasts on these discs and analysed the migration of cells within the 3D scaffold. In order to obtain more information about the oxygen consumption and the acidification within the tantalum scaffold, we measured the oxygen concentration and the pH value in the core and the periphery using optical microsensors. Furthermore, the migration capacity of human osteoblasts into the core of the 3D tantalum scaffold and their ability to synthesise collagen type 1 should be analysed.

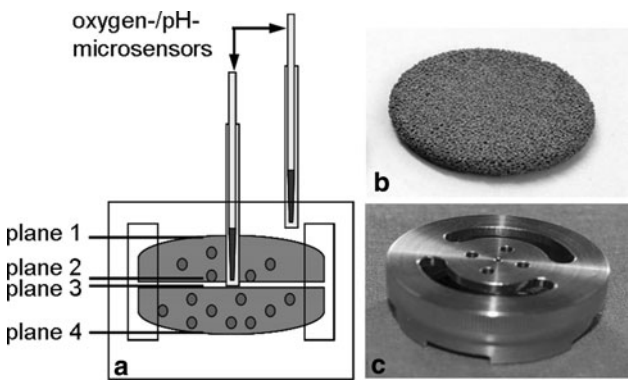

Fig. 1 Schematic design of the 3D tantalum scaffold with four different planes (**a**), Trabecular Metal™ disc (**b**) and clamping ring (**c**)

2 Materials and methods

2.1 Isolation and cultivation of human primary osteoblasts

Human femoral heads were obtained from patients undergoing primary total hip replacement. The bone samples were collected after patient's agreement and approval by the Local Ethical Committee (registration number: A 2010-10).

Cancellous bone was carved from the inside of femoral heads, washed three times with PBS (PAA, Cölbe, Germany) and cut into small pieces. Afterwards, the cancellous bone was treated with Dulbecco's Modified Eagle Medium (DMEM, Gibco® Invitrogen, Darmstadt, Germany) containing collagenase A (0.125 U/ml; Roche, Mannheim, Germany) and dispase (1.5 U/ml, Roche, Mannheim, Germany) at a ratio of 1:2:1 at 37°C for 3 h. The cell suspension was filtered through a cell strainer (pore size: 70 µm; Nunc, Wiesbaden, Germany) and centrifuged at $118 \times g$ for 10 min. The remaining cell pellet was resuspended in complete medium containing 10% FCS, 1% amphotericin B, 1% penicillin/streptomycin and 1% hepes-buffer (all: Gibco® Invitrogen, Darmstadt, Germany).

Freshly isolated osteoblasts from 2 to 3 g wet weight cancellous bone were plated in a 25 cm^2 culture flask with 8 ml complete medium and ascorbic acid (final concentration: 50 µg/ml), β-glycerophosphate (final concentration: 10 mM) as well as dexamethasone (final concentration: 100 mM) (all: Sigma, Seelze, Germany). Cells were incubated in a humidified atmosphere of 5% CO_2 and 37°C. The cell culture medium was changed every second day to remove non-adherent cells. Cell proliferation was determined by microscopical control. As cells reached 90% confluency, they were trypsinized and splitted at a ratio 1:6.

2.2 Cell settlement on tantalum scaffold

Human osteoblasts were seeded on porous cylindrical tantalum discs (Trabecular Metal™, Zimmer, Freiburg, Germany) which posed a diameter of 26 mm and a height of 5 mm (Fig. 1b). Typically Trabecular Metal™ has a porosity of 80% relative to solid tantalum and a pore size of 550 µm in diameter, as previously described [21]. The pore size is suitable for osteoconductivity [19]. To distinguish between different depths, the scaffold sample was composed of two different discs which were placed in a clamping ring (Fig. 1a). The lower part of the clamping ring consisted of a small border, so that the lowest plane (plane 4) had no contact to the bottom of the six-well plate.

Before cell seeding, the tantalum discs were incubated in complete medium to remove air bubbles within the pores. A cell amount of 3×10^5 was seeded on the first and the third plane by dropping 10 µl cell suspension pointwise on the discs. After 45 min, complete medium was refilled and the discs were incubated at 37°C and 5% CO_2 for 72 h. Afterwards, the discs were arranged on top of each other into the clamping ring (Fig. 1c) and incubated for 8 days. Medium was completely changed every second day.

2.3 Oxygen measurement

For the oxygen measurements, needle-type oxygen microsensors (Oxygen Micro-Optode, Type PSt1; Presens, Regensburg, Germany), mounted on optic fibres with a 140 µm tip, were used. For protection of the fragile sensors, they were fixed within a standard hollow needle of 0.4 mm diameter. Standard hollow needles with a diameter of 1.02 mm were used to get more stability during measurement (Fig. 1a). A two-point calibration was performed before measurement using oxygen-free water (0% air saturation) and water vapour-saturated air (100% air saturation). Oxygen consumption was measured both in the periphery and in the core of the 3D scaffold every 24 h over a period of 30 min for 8 days.

2.4 pH monitoring

In order to monitor the pH values within the 3D cell culture, needle-type pH microsensors (pH microsensor; Presens, Regensburg, Germany), mounted on optic fibres with a tip of less than 150 µm were used. These microsensors provided a high spatial resolution and were optimised for culture media. For protection of the fragile sensors, they were fixed within a standard hollow needle of 0.4 mm diameter. Standard hollow needles with a diameter of 1.02 mm were used to get more stability during measurement (Fig. 1a). Before measurement a calibration with buffer solutions of pH 4–7 (all: Roth, Karlsruhe, Germany) was performed. The pH value was monitored both in the periphery and in the core of the 3D scaffold every 24 h over a period of 30 min for 8 days.

2.5 WST-1 assay

The mitochondrial activity of osteoblasts on the tantalum scaffold was analysed by using a WST-1 assay (Roche, Mannheim, Germany). The background of this assay is, that cells with a high metabolism activity cleave the tetrazolium salt WST-1 to formazan dependent on their metabolic activity [22]. Therefore, the tantalum module was disconnected and both discs were covered with assay reagent (ratio between complete medium to WST-1 reagent of 10:1). Additionally, a medium control was used. After an incubation time of 30 min under cell culture conditions, 200 µl aliquots of assay reagent were transferred to a 96-well format. Absorbance was measured at 450 nm (reference 630 nm) using an Opsys MR™ microplate reader (Dynex Technologies, Denkendorf, Germany).

2.6 Pro-collagen type I quantification

Synthesis of pro-collagen type I (Metra™ CICP EIA Kit, Quidel, Marburg, Germany) was determined by an enzyme-linked immunosorbent assay (ELISA). For the analysis, 500 µl medium were removed from the core and the periphery of the 3D scaffold. The assay was performed according to the manufacturer's instructions. Absorbance was measured at 405 nm using the Opsys MR™ microplate reader (Dynex Technologies, Denkendorf, Germany).

2.7 LIVE/DEAD® assay

By means of the LIVE/DEAD® viability/cytotoxity kit (Invitrogen, Darmstadt, Germany), cell viability was determined. Hence, both discs were incubated in LIVE/DEAD® assay reagent containing calcein AM and ethidium homodimer. Calcein AM is the acetoxymethyl ester derivative of the fluorescent dye calcein. Calcein AM is membrane-permeant and thus is taken up by cells incubated with PBS supplemented with calcein AM. Once inside the cells, calcein AM is hydrolyzed by endogenous esterases into the polyanionic non-membrane permeant green fluorescent dye calcein. As a result, viable cells with intact membranes produce an intense uniform green fluorescence (ex/em 495/515 nm). Ethidium enters cells with damaged membranes and produces a bright red fluorescence (ex/em 495/635 nm) indicating dead cells. After 30 min of incubation at room temperature with PBS containing both dyes, images of the cells were taken using a fluorescence microscope with a fourfold magnification object lens (Nikon ECLIPSE TS100; Nikon GmbH, Düsseldorf, Germany).

2.8 Statistical analysis

Data are presented as mean values ± standard deviation. Statistical significance between groups was calculated by an ONEWAY ANOVA test (Post Hoc LSD) or Mann–Whitney-*U*-test using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered significant. A minimum of three independent experimental runs was performed for all analysis.

3 Results

3.1 Human osteoblasts migration within the 3D tantalum module

To investigate whether cells were able to migrate into the tantalum discs, the primary osteoblasts were seeded on planes 1 and 3. After one day under static culture conditions, cells were observable on all four planes, whereby only few cells were found on planes 2 and 4. Furthermore, some dead cells (red) were observed on the third plane (Fig. 2).

After 8 days of incubation, all four planes were occupied by osteoblasts. Moreover, cells appeared larger and formed filopodia for cell connections over the period of time. Compared to day one, more dead cells were found on plane 3 (Fig. 2).

3.2 Oxygen measurement within a porous tantalum scaffold

To obtain data about the oxygen content within the 3D tantalum scaffold, measurement of the oxygen concentration was done in the core of the cell-free as well as cell-settled tantalum scaffold and in the surrounding medium. Directly after assembling of the cell-free module the oxygen concentration in the core was approximately 16% and in the surrounding medium 17.4%. During the first 24 h the oxygen concentration increased to 18.8% for both measure points. For the next 7 days, oxygen concentration was about 19.5% with no differences between core and periphery (Fig. 3a).

In contrast, in the cell-seeded tantalum module, the level of oxygen within the scaffold decreased significantly from approximately 18.5% to below 3.0% during the first 24 h of incubation. Over the period of the following 7 days oxygen concentrations remained low (<0.6%) and were not affected by the addition of fresh medium. The oxygen concentration in the surrounding medium only decreased from approximately 19.0 to 14.0% during the 8 day of incubation period (Fig. 3b).

We also performed an oxygen measurement when cells were seeded only on plane 3 (amount of 3,00,000 cells). During incubation, a decreasing oxygen concentration from 18.8 to 7.2% within the tantalum module was observed. In contrast, the oxygen concentration in the surrounding medium varied between 18.3% on day one and 15.0% on day seven (data not shown).

3.3 pH monitoring within the 3D tantalum scaffold

To get information whether osteoblast cells accumulated metabolic waste products within the 3D scaffold, we monitored the pH value during incubation in the core as well as in the periphery of the scaffold. Immediately after assembling of the two tantalum discs, we could demonstrate a difference of the pH, i.e. we measured pH 7.1 in the periphery and pH 6.9 in the core of the scaffold. After 24 h the pH switched to 7.04 within the scaffold. For the next 7 days, there was an increasing, but non-significant acidification both in the periphery and in the core (Fig. 4).

3.4 Cell survival and pro-collagen type I expression

In order to measure the viability of cells seeded on the 3D tantalum scaffold, the WST-1 assay was performed after 8 days of cultivation under hypoxic conditions. There was no difference in the metabolism activity of cells between both discs (Fig. 5).

Over the period of time, the synthesis of pro-collagen type I clearly increased. Furthermore, there were only little differences in pro-collagen type I concentrations in the core of the scaffold compared to the periphery (Fig. 6).

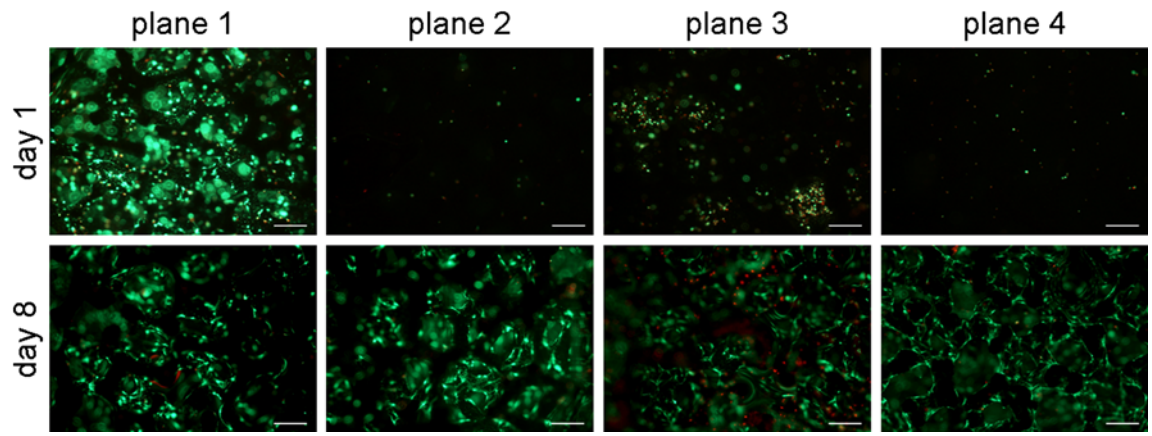

Fig. 2 Migration capacity and viability of primary human osteoblasts on the different planes of the 3D tantalum scaffold on day one and after 8 days of cultivation under static culture conditions ($n = 3$; living cells = *green*, dead cells = *red*; original magnification: ×40; *bar* 500 µm)

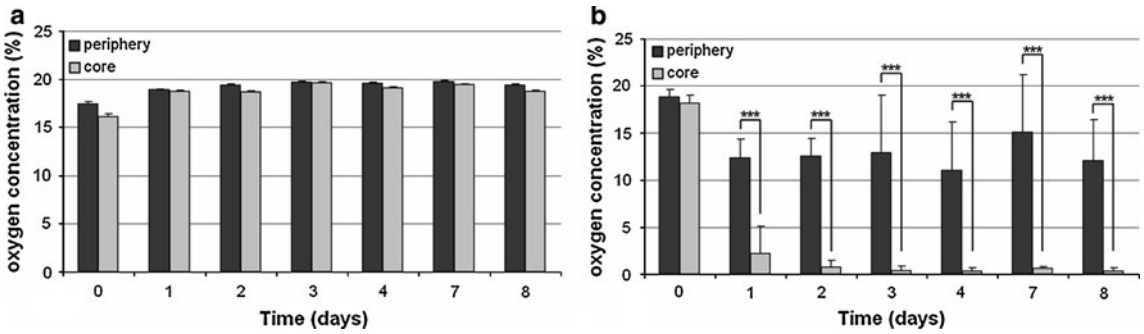

Fig. 3 Oxygen concentration in a cell-free tantalum scaffold (**a**; $n = 1$) and in a static 3D cell culture of human osteoblasts (**b**; $n = 3$). Measurement was done in the periphery as well as in the core of the tantalum scaffold. Data are demonstrated as mean values ± standard deviation. Statistical analysis was done with ONEWAY ANOVA (Post hoc LSD). Significance is based on the respective oxygen concentration of the periphery (***$P < 0.001$)

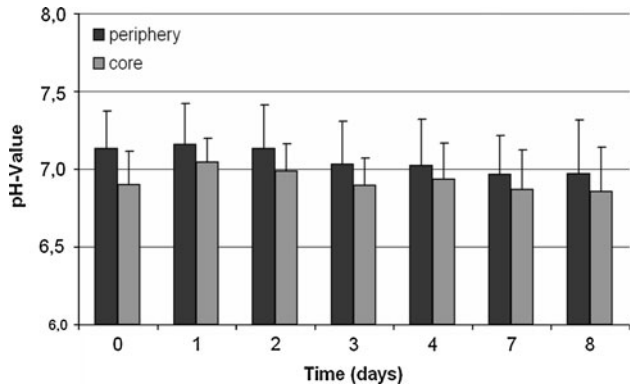

Fig. 4 Monitoring of the pH value in a static 3D culture of human osteoblasts ($n = 3$). Measurement was done in the core and in the periphery of the tantalum scaffold over a period of 8 days. Data are demonstrated as mean values ± standard deviation. Statistical analysis was done with ONEWAY ANOVA (Post hoc LSD). Significance is based on the respective pH value of the periphery

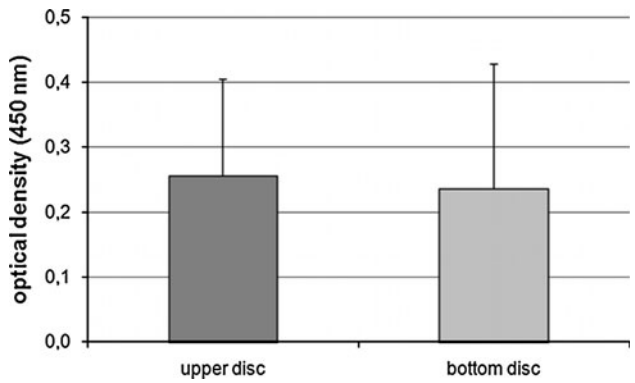

Fig. 5 Metabolic activity of human osteoblasts seeded on the 3D tantalum scaffold after 8 days of cultivation ($n = 4$). Data are presented as mean value ± standard deviation. Statistical analysis was done with a Mann–Whitney-*U*-test

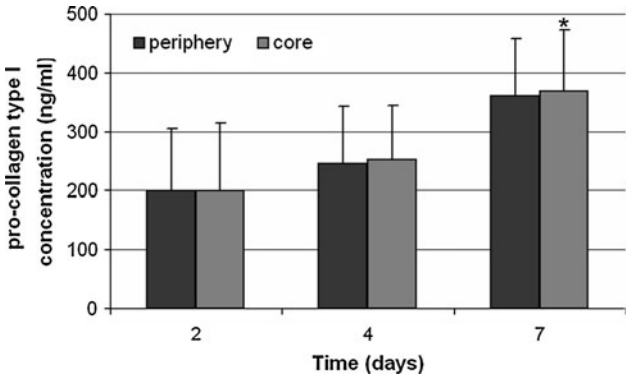

Fig. 6 Synthesis of pro-collagen type I of human osteoblasts seeded on the 3D tantalum scaffold. Supernatants were collected after 2, 4 and 7 days of cultivation and analysed by ELISA ($n = 5$). Data are presented as mean values ± standard deviation. Statistical analysis was done with ONEWAY ANOVA (Post hoc LSD). Significance is based on the pro-collagen type I concentration of the periphery on day two (*$P < 0.05$)

4 Discussion

In orthopaedic surgery, the availability of bone substitutes (e.g. auto- and allografts) for the treatment of large bone defects is limited. Therefore, alternatives such as synthetic, porous 3D scaffolds are becoming more relevant for the therapy. However, the limitation of these scaffolds is the non-uniform supply of nutrients and oxygen within the construct [10, 11]. For large-scaled constructs, in particular oxygen supply is the limiting factor because of its low solubility coefficient and diffusion capacity in aqueous solutions [10].

Porous tantalum, such as Trabecular Metal™, was introduced as a new material for orthopaedic reconstruction. Trabecular Metal™ has several advantages, e.g. its geometry, which allows an excellent ingrowth of bone cells. Other important aspects are good mechanical

properties (high strength and low stiffness) as well as a superior biocompatibility [17, 18].

The objective of the present study was to analyse the consumption of oxygen and the pH value within a 3D tantalum scaffold which consisted of two porous tantalum discs and a clamping ring. Human primary osteoblasts were seeded on these discs and the migration potential of the cells was analysed. Furthermore, the oxygen content within the scaffold was measured and the synthesis rate of pro-collagen type I was determined.

In accordance to Welldon et al. [19], it was demonstrated that human osteoblasts were able to attach to the surface of the tantalum discs. Furthermore, the cells migrated a distance of 5 mm downwards through the pores of the tantalum discs. After 8 days of incubation cells were detected on all four planes over a total distance of 10 mm. Since osteoblasts were able to survive in a large-scaled tantalum scaffold for 8 days, longer incubation times on cell survival will be examined in subsequent studies.

By means of the oxygen measurements, a central hypoxia within the tantalum scaffold as well as oxygen gradients between the core and the periphery of the construct could be shown. In reference to Volkmer et al. [11] the oxygen concentration in the core of a large-scaled scaffold clearly decreased in the static cell culture, so that a critical oxygen undersupply during the first 24 h was found. However, even though the medium was changed every second day, the oxygen concentration within the scaffold did not increase. Nevertheless, it is important to know that we measured the oxygen concentration every day on the same time point, so medium exchange was done after measurement. Therefore, we have no information about oxygen concentrations in the cell culture directly after exchange because we did not measure the oxygen level again. According to Volkmer et al. [11], the proliferation rate of cells is considerably higher in 3D cell culture systems than in 2D cultures because of the larger scaffold surface area. Hence, our results indicate that the low oxygen concentration in the core of the scaffold could be a result of an increasing cell density, which leads to a higher oxygen consumption within the scaffold. This could be supported by our first results of oxygen measurement, when human osteoblasts (3,00,000 cells) were seeded on the third plane only. Here, we observed a slowly but a noticeable decrease of the oxygen level during incubation. We assume that osteoblasts can be supplied with oxygen in a better way when there were no cells on the top of the scaffold. Thus, the decrease of oxygen concentrations within the scaffolds indicated an increase of cell number which resulted in a higher oxygen consumption.

Data on optimal oxygen concentrations in patients bone tissue is unknown, but the mean tissue levels of oxygen are between 1 and 9% [23]. It was demonstrated, that the oxygen concentration in static 3D cultures of chondrocytes was around 5% [9] and drop below 1% using thicker scaffolds [24]. In contrast to bone tissue, cartilage is characterised by avascularity [25], therefore chondrocytes are not supplied with blood as an oxygen carrier. Hence, chondrocytes tolerate low oxygen levels. However, oxygen toleration levels for osteoblasts are unknown and dead cells were detected on plane 2 and especially on plane 3, which was seeded with cells, there seems to be an undersupply of oxygen for the osteoblasts in the static 3D cell culture. Nevertheless, the inner structures of the tantalum module were settled by a large number of cells, so that cell survival could be supported within the scaffold.

Although hypoxia is able to cause a lethal environment due to a limited cellular growth and respiration, this can also increase the production of ECM components [10]. In bone tissue, collagen type I is the primary component of the ECM and plays an important role in osseous repair [26]. In the present study, it could be demonstrated that the synthesis of pro-collagen type I increased within the scaffold under hypoxic conditions. This observation was in contrast to Utting et al. [13], who determined a decrease of collagen type I expression in rat osteoblasts under hypoxic conditions. However, Warren et al. [26] demonstrated an up-regulation of collagen type I mRNA in response to hypoxic culture conditions over a period of 48 h.

In addition, higher oxygen consumption by proliferating cells in the core of the scaffold can lead to an increase in matrix production which seems to reduce the diffusion of oxygen within the 3D construct. It was reported that matrix formation within the pores of scaffolds could hamper the diffusion of nutrients and also of oxygen [10]. Consequently, the supply with oxygen and nutrients as well as waste removal is not guaranteed in the core of a static 3D scaffold. Moreover, in a cell-free tantalum scaffold we could not show any differences in oxygen concentrations between core and periphery. Therefore, we conclude that there are no diffusion problems within the tantalum disc. This aspect also implies that cell settlement as well as matrix formation could influence diffusion processes within a scaffold negatively.

One important aspect of hypoxia is the local decrease of tissue pH in vivo, which is caused by anaerobic metabolism and a reduced perfusion [13]. Furthermore, hypoxia and a decreased pH value could stimulate the formation of osteoclasts and their resorptive activity in vivo [13]. Utting et al. [13] showed that small pH reductions inhibited mineralisation of organic matrix which was deposited by osteoblasts. In our experiments we could show a slightly increasing acidification during the incubation. The pH difference between core and periphery was approximately 0.1. Frick et al. [27] reported, that in mouse osteoblast cultures only mild changes in the pH value resulted in a

decreased expression of collagen type I. In contrast, Brandao-Burch et al. [28] demonstrated that collagen synthesis on rat osteoblasts were not affected by pH in the range of 7.4–6.9. Our results revealed an increase of procollagen type I expression, thus collagen synthesis was also unaffected by pH ranges. Furthermore, cell migration within the core of the 3D tantalum scaffold was detected. Hence, in clinical application it can be assumed that porous tantalum scaffolds can be settled by osteoblasts under critical oxygen and nutrient supply in case of large segmental bone defects. Nevertheless, in terms of insufficient tissue vascularisation a long-lasting hypoxia as well as acidosis within a 3D scaffold could result in osteoclast formation and ECM degradation. In general, monitoring of osteoblast migration, oxygen consumption and acidification seems to be helpful for creating advanced 3D bone scaffolds in terms of sufficient ingrowth in synthetic bone substitutes.

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Conflict of interest None.

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