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Hypoxia in Static and Dynamic 3D Culture Systems for Tissue Engineering of Bone

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ABSTRACT

Tissue engineering of sizeable cell-scaffold constructs is limited by gradients in tissue quality from the periphery toward the center. Because homogenous delivery of oxygen to three-dimensional (3D) cell cultures remains an unsolved challenge, we hypothesized that uneven oxygen supply may impede uniform cellular growth on scaffolds. In this study we challenged static and dynamic 3D culture systems designed for bone tissue engineering applications with a well-growing subclone of MC3T3-E1 preosteoblasts and continuously measured the oxygen concentrations in the center of cell-seeded scaffolds and in the surrounding medium. After as little as 5 days in static culture, central oxygen concentrations dropped to 0%. Subsequently, cells died in central regions of the scaffold but not in its periphery, where oxygen levels were ~4%. The use of perfusion bioreactors successfully prevented cell death, yet central oxygen concentrations did not rise above 4%. We conclude that 3D culture *in vitro* is associated with relevant oxygen gradients, which can be the cause of inhomogeneous tissue quality. Perfusion bioreactors prevent cell death but they do not entirely eliminate 3D culture–associated oxygen gradients. Therefore, we advise continuous oxygen monitoring of 3D culture systems to ensure tissue quality throughout engineered constructs.

INTRODUCTION

AUTOLOGOUS BONE GRAFTING is limited by its biological properties and accompanied by a significant extent of donor site morbidity.[1] Engineering bone tissue *in vitro* has the potential to provide an unlimited supply of bone substitute material.[2–4] Yet up to now, scaffold-based tissue engineering of clinically relevant extents of bone tissue failed. With increasing size of a cell-seeded scaffold, gradients in tissue quality emerge, including inhomogeneous cellular proliferation and differentiation from outer areas of the scaffolds toward the center.[5–9] These tissue quality gradients most likely reflect uneven nutrient and oxygen supply within the three-dimensional (3D) culture, and they point out the current dilemma in scaffold-based tissue engineering: the bigger the cell-seeded scaffolds become the more difficult it is to supply all cells equally with nutrients and oxygen.[6]

In vivo, the distance between cells and capillaries, which provide nutrients and oxygen and at the same time account for waste elimination, ranges from 20 to 200 μm.[10] *In vitro*,

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sufficient nutrition and oxygenation of cells by diffusion is limited to a distance of 100–200 μm.[11,12] Because oxygen has only a poor diffusion capacity and solubility in aqueous solutions, it has been hypothesized that hypoxia is the limiting factor in scaling up 3D cultures *in vitro*.[6,13]

The oxygen concentration affects various cellular mechanisms, including cell cycle, cell proliferation, apoptosis, and glucose metabolism.[6,14] Several recent studies provided proof that the process of osteogenic differentiation is also highly dependent upon the oxygen level,[7,15,16] and hypoxia was shown to have an impact on bone development.[14] In consequence, maintenance of stable and even oxygenation in bone tissue engineering is a prerequisite for generating homogeneous tissues. In marked contrast to its potential impact, almost no information is available concerning the oxygen performance of 3D culture systems designed for bone tissue engineering. This holds especially true when it comes to clinically relevant construct sizes exceeding several millimeters of thickness.

To overcome diffusional limitations of static culture, bioreactors were introduced to improve the quality of different types of engineered tissues, including bone and cartilage.[17,18] However, only few studies directly addressed whether these culture systems truly improve the oxygen delivery to cells within the scaffold. Studies on chondrocytes and cardiomyocytes in static 3D culture revealed that the oxygen concentration decreases from the periphery toward the center of the scaffolds,[5,19] and these oxygen gradients correlate with cell density and cell viability.[8] Medium flow around the scaffolds mitigated oxygen gradients and enhanced cell survival.[8] In most of these experiments, however, extremely thin scaffolds were used. This leaves open the possibility that within thicker, clinically more relevant constructs, the central oxygen concentration may be lower, especially because cell growth and matrix production seem to deteriorate nutrient delivery to central regions of 3D constructs.[6,17]

In our study we therefore addressed the question whether 3D culture systems commonly used for bone tissue engineering are generally subjected to oxygen gradients, and whether these gradients have an impact on cell survival within the construct.

MATERIALS AND METHODS

Cell culture

A well-growing subclone of the murine preosteoblast cell line MC3T3-E1 (DSZM, Braunschweig, Germany) was expanded in minimum essential medium alpha with L-glutamine (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Sigma, Munich, Germany) and 40 IU/mL penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria). This medium was used throughout all experiments of the study except that in the case of dynamic cell culture, 25 mM of HEPES buffer (PAA) was added. Cells were kept in a humidified atmosphere of 95% air with 5% carbon dioxide (CO_2) at 37°C and supplied with fresh medium three times a week.

3D culture

Cylindrical, sterilized, bovine demineralized bone matrix (DBM; Tutogen Medical, Neunkirchen, Germany) scaffolds of 9 mm in diameter and 5 mm in height served as matrix in all experiments. Prior to seeding, the DBMs were centrifuged in medium for 5 min at 500 *g* to remove all air within the scaffold. For 3D culture, cells were trypsinized, centrifuged, and resuspended in medium to achieve a final concentration of $\sim 7.5 \times 10^4$ cells per mL. Each matrix was transferred to one well of a 48-well dish (NUNC, Wiesbaden, Germany), and 666 μL of the cell suspension (5×10^4 cells) was pipetted onto the scaffolds. During the first 2 h, the scaffolds were turned around and reseeded with the cell suspension in 20-min intervals to ensure a maximum number of cells attaching to the matrix rather than to the surface of the culture dish. At last, the seeded constructs were either transferred to 24-well plates for static culture or to bioreactors with cylindrical flow chambers (MINUCELLS and MINUTISSUE GmbH, Bad Abbach, Germany) and sealed. With this technique, a seeding efficiency of ~90% was reached.

For static subculture, the seeded scaffolds were kept in a standard cell culture incubator in 24-well plates. Medium was changed three times weekly throughout the culture period.

Dynamic culture was performed in bioreactors as described previously.[3] In brief, bioreactors were connected to both fresh medium containing 25 mM HEPES buffer (PAA) and to waste reservoirs by gas-permeable silicone tubes. Multichannel roller pumps (Ismatec, IPC, Glattbrugg, Switzerland), with each flow chamber being connected to its own independent pumping system, guaranteed flow through the scaffold from bottom to top at a flow rate of 18 μL/min. Tilting of the reactors prevented air trapping within the reactors. All perfusion culture systems were assembled under sterile conditions. Systems were placed on a heating plate (37°C) and covered with an atmosphere heating unit set to 37°C.

Two setups of perfusion bioreactor culture were used in this study: as the flow chamber of the bioreactors is bigger in diameter than the scaffolds (13 vs. 9 mm), medium was able to flow around the scaffolds. This setup was termed standard perfusion setup (Fig. 1A). To force the medium flow exclusively through the scaffolds, ring-shaped carrier cassettes of polycarbonate were constructed, which carry the scaffold in a press-fit fashion. The cassettes fit exactly into the perfusion chamber of the bioreactors, leaving no space for medium to flow around the scaffolds (forced perfusion setup; Fig. 1B).

Oxygen measurements

In two-dimensional (2D) culture, oxygen was measured in sterile polystyrene O_2 sensor microtiter 24-well plates

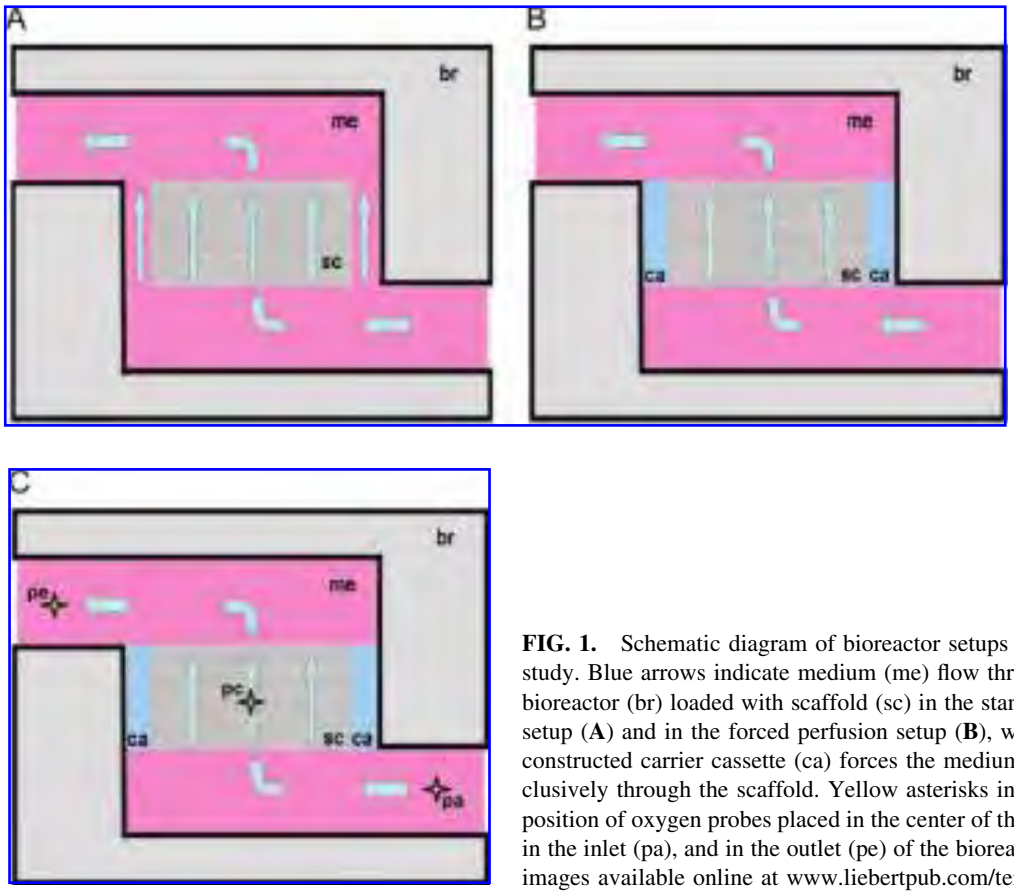

FIG. 1. Schematic diagram of bioreactor setups used in this study. Blue arrows indicate medium (me) flow through perfusion bioreactor (br) loaded with scaffold (sc) in the standard perfusion setup (**A**) and in the forced perfusion setup (**B**), where a self-constructed carrier cassette (ca) forces the medium to flow exclusively through the scaffold. Yellow asterisks indicate the tip position of oxygen probes placed in the center of the scaffold (pc), in the inlet (pa), and in the outlet (pe) of the bioreactor (**C**). Color images available online at www.liebertpub.com/ten.

(OxoPlate® OP24F; PreSens, Regensburg, Germany). A nontoxic O_2 sensor fixed on the bottom of each well can be read out from the bottom side with a commercially available reader and the according software (PreSens). The detailed mechanism is described elsewhere.[20,21] In brief, the sensor contains two different dyes, one of which is the O_2 indicator. Its phosphorescence intensity depends on the concentration of O_2 in the sample within the well, while the fluorescence of the other dye is not dependent on the O_2 concentration. Using the luminescence intensities, a ratio can be calculated, which corresponds to the concentration of O_2 in the sample. The OxoPlate is calibrated ex factory by a two-point calibration.

For 2D measurements, cells were seeded into the wells of the OxoPlate at the indicated densities and cultured under standard cell culture conditions. Special care was taken to seed the cells homogeneously within the wells by gently shifting the plate back and forth and to the sides for at least 20 times. The measuring device was placed inside the incubator and connected to the computer. Oxygen was measured in 1-h intervals over a period of 7 days. For illustration purposes, all measurements of 1 day were pooled and a standard deviation was calculated.

Oxygen measurements in 3D culture were performed using needle-type oxygen microsensors with fixed sensor tip (NFSx; PreSens), which function analogously to the OxoDish® sensors. These oxygen sensors are mounted on optic fibers with a tip diameter of 50 µm. To protect these fragile sensors, they are fixed within a standard hollow needle of 0.4-mm diameter.

To continuously monitor oxygen partial pressure in statically cultured scaffolds, the needle sensor was passed through a self-made hole in the lid of the culture plate and inserted in the center of the freshly seeded scaffold. A self-constructed aiming device ensured introduction of the tip exactly in the geometric center of the circle-shaped surface of the cylinder at a depth of 2.5 mm from the top in a rectangular fashion. Oxygen was measured every hour over a period of 7 days.

Oxygen measurements in the standard perfusion setup were performed analogously to static culture measurements, except that the probe was inserted from the side of the scaffold. Using another aiming device, the probe was rectangularly inserted halfway from the top to a depth of 4.5 mm. To measure oxygen within the forced perfusion setup, a 0.4-mm hole was drilled in the side of the polycarbonate carrier cassette through which the oxygen probe was inserted into the center of the scaffold as described above.

Oxygen measurements in the medium were carried out by inserting a probe in the medium next to the scaffold

(static culture) or by inserting additional oxygen probes directly in the bioreactor, one in the afferent and one in the efferent medium reservoir (Fig. 1C).

A two-point calibration was performed before each measurement using ambient air as the 21% oxygen reference and a 100% CO_2 atmosphere as the 0% oxygen reference.

Live-dead assay

To assess survival of cells, fluorescence microscopy based on incubation of cells with fluoresceindiacetate (FDA) and propidium iodide (PI), both from Fluka/Sigma, Munich, Germany, was performed. After double staining with FDA and PI, viable cells appear green, while nonviable cells are red. Upon staining, the scaffolds exhibited a weak red fluorescence. A stock solution of FDA was prepared by freshly dissolving 10 mg of FDA in 2 mL of pure acetone and by diluting this mix 1:500 with phosphate-buffered saline (PBS). To obtain the final FDA/PI staining solution, the two components were mixed at a ratio of 1:1.

Prior to evaluation by fluorescence microscopy, scaffolds were cut in halves with a scalpel using a self-designed cutting fixture. For staining, medium was removed and samples were washed with 0.5 mL PBS. Each sample was then incubated for 1 min with 500 μL FDA/PI staining solution. After discarding the dye, the wells were again washed with 0.5 mL PBS. Subsequently, samples were analyzed by fluorescence microscopy using an Axiovert 100 microscope equipped with a 75 W mercury lamp (Zeiss, Munich, Germany). To detect red and green fluorescence, the Zeiss filter sets #10 and #15 were used. Pictures were taken with a Zeiss black and white digital camera (AxioCam MRm) and processed with the Zeiss Axiovision software (AxioVs40 V 4.5.0.0).

Samples were photographed in a standardized manner. First, the bisected scaffold was photographed in total from all sides using a 1.25× magnification. Then, with a 5× magnification objective and later with a 10× magnification objective, a specific set of pictures was taken from the inner side of the scaffold, which included all corners, borders, and central regions of the matrix. Finally, five shots were taken from central regions of the scaffold, one of which was taken in the exact center of the scaffold using a 20× magnification objective. This picture was used for quantification of cells. To this end, a 500×500 μm section of the picture was divided in 16 high-power fields (HPF), and vital (green) cells were counted by three independent observers.

Statistical analysis

Statistical analysis was performed using SigmaPlot version 8 (SPSS, Munich, Germany). Significances were calculated using Student's *t*-test. A value of $p < 0.05$ was considered significant. For all experiments, a minimum of two independent experimental runs were performed.

RESULTS

Oxygen concentration in the center of cell-seeded scaffolds cultured under static conditions falls to 0%

To uncover oxygen gradients in statically cultured cell-seeded scaffolds, we continuously measured the oxygen concentration in the geometric center of the constructs and the surrounding medium. After as little as 5 days, the oxygen concentration in the center of the scaffold dropped to 0% (Fig. 2A, solid line). In contrast, the oxygen concentration measured in the medium around the scaffold declined to approximately 4% revealing an oxygen gradient from the surface of the 3D construct toward its center (Fig. 2A, dashed line).

So far, almost all information available on cell metabolism has been acquired from 2D experiments. To be able to compare the oxygen consumption of cells in 3D culture to that of cells in 2D culture, we seeded different amounts of cells in 24-well dishes (2D) in comparison to 50,000 cells per scaffold (3D) and continuously measured the oxygen concentration in the medium. Seeding 100 MC3T3 cells per well did not result in measurable oxygen depletion within 7 days (data not shown). Seeding 1000 cells per well resulted in a slight decrease of oxygen in the medium (Fig. 2B, squares). Whenever 10,000 cells (Fig. 2B, triangles) or more cells (data not shown) were seeded per well, the oxygen level remained virtually constant at ~8–10% once cells reached confluence. The oxygen concentration measured in the medium surrounding the cell-seeded scaffold fell below the level of that reached by confluent cells (Fig. 2B, diamonds).

Prolonged hypoxia leads to cell death in 3D culture

To assess the effect of the 3D culture–associated hypoxia on cell survival in our 3D constructs, we performed a live-dead assay after statically culturing the scaffolds for periods of 3, 5, and 7 days. After 3 days (Fig. 3A), virtually all cells within the scaffold were viable (green). After 5 days (Fig. 3B), most of the cells were viable even though the oxygen concentration was already as low as 1%. Yet in central and bottom areas of the scaffold evaluated on day 5, some dead (red) cells appeared. After 7 days, the core and the basis of the scaffold were filled with dead cells, while viable cells were only left on the top and side margins of the scaffold (Fig. 3C).

The use of perfusion bioreactors partially rescues 3D-associated hypoxia and concomitant cell death

In an attempt to overcome 3D culture–associated hypoxia, we cultured seeded scaffolds in the standard perfusion bioreactor setup. Because cells had died in the center of the statically cultured scaffold after 7 days, we chose to compare this time point across all cell culture settings. As we

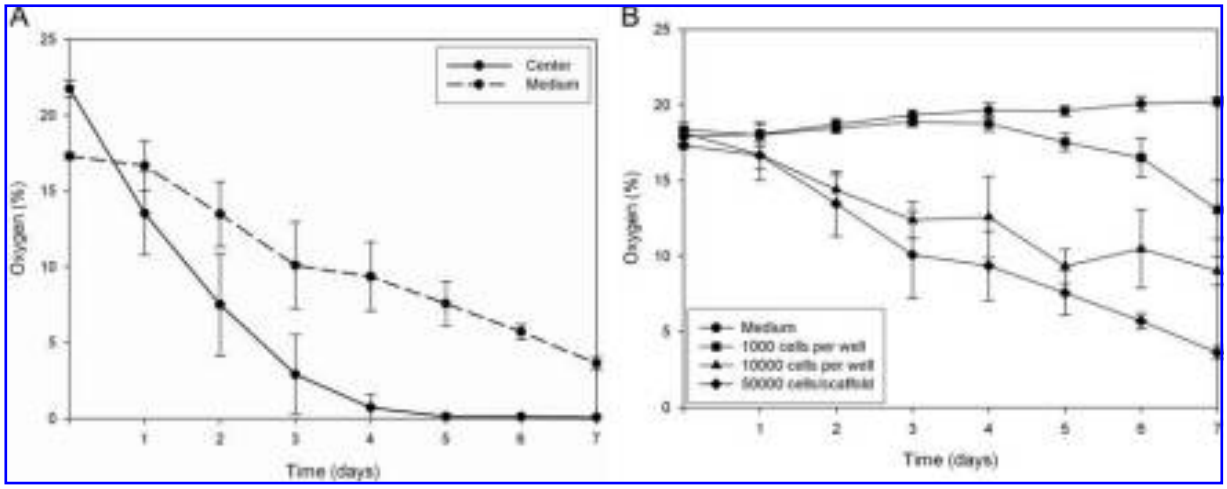

FIG. 2. Oxygen concentration in static culture. Oxygen concentration in the center of MC3T3-seeded DBM scaffold falls to 0% after 5 days (**A**; solid line). Oxygen concentration measured in the medium around the scaffold dropped to approximately 4% revealing an oxygen gradient from the surface of the 3D construct toward its center (**A**; dashed line). Oxygen concentration in the medium decreases depending upon seeding density (**B**). Seeding 1000 cells per well (**B**; squares) results in moderate oxygen depletion as compared to medium control (**B**; circles). Seeding 10,000 cells or more per well results in confluent 2D layers after 5 days. Accordingly, oxygen concentration levels out at $\sim$10% (**B**; triangles). Seeding 50,000 cells on a scaffold results in further depletion of oxygen because of an increased surface (**B**; diamonds). Error bars represent SD of pooled values of 1 day of two independent experiments.

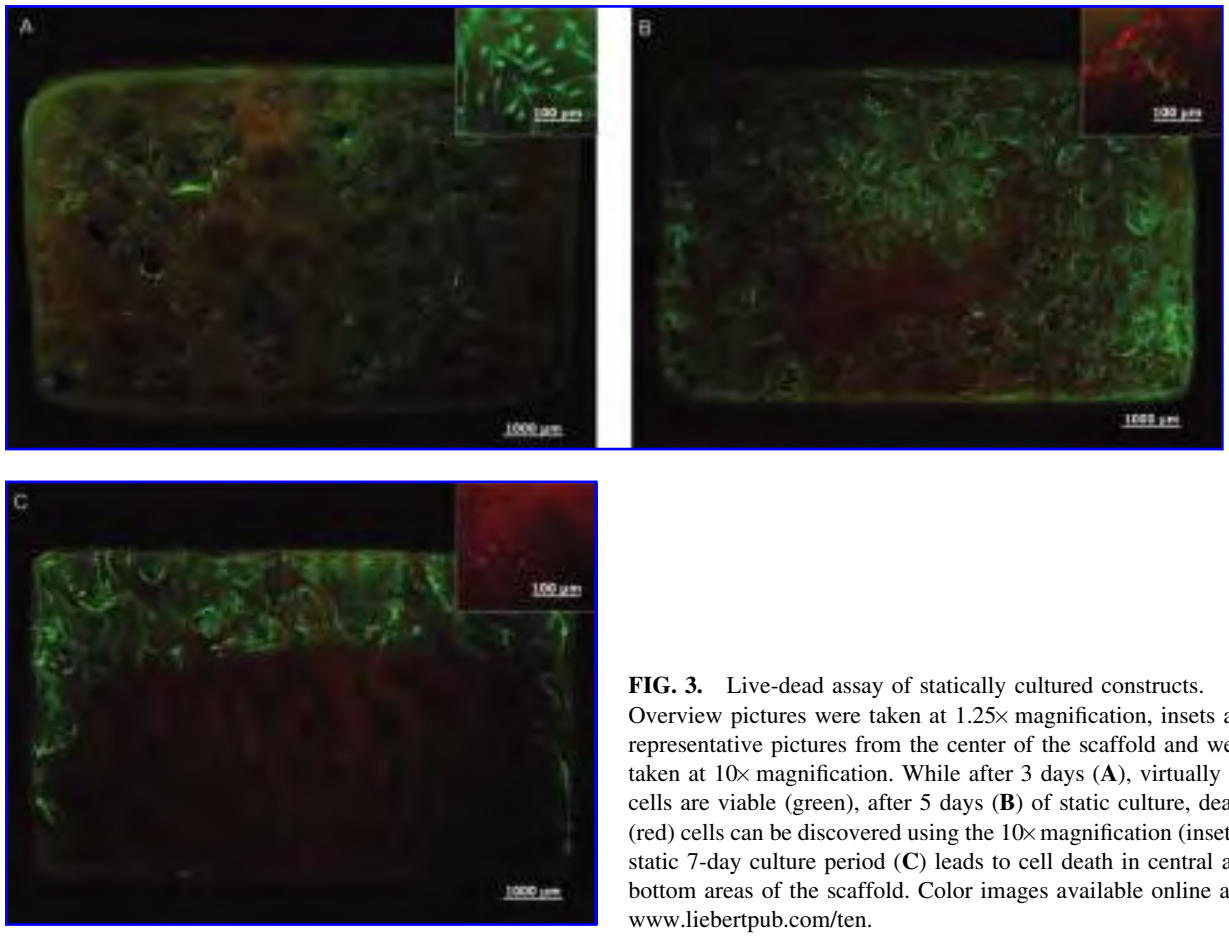

FIG. 3. Live-dead assay of statically cultured constructs. Overview pictures were taken at 1.25× magnification, insets are representative pictures from the center of the scaffold and were taken at 10× magnification. While after 3 days (**A**), virtually all cells are viable (green), after 5 days (**B**) of static culture, dead (red) cells can be discovered using the 10× magnification (inset). A static 7-day culture period (**C**) leads to cell death in central and bottom areas of the scaffold. Color images available online at www.liebertpub.com/ten.

expected, less cells had died in central regions of the scaffold after 7 days of dynamic culture compared to static culture conditions (Fig. 4A). Using the forced perfusion bioreactor setup, we repeated the experiments under otherwise identical conditions. The viability-stain performed after 7 days revealed that even more cells had grown within the scaffold's center and periphery (Fig. 4B). Only in the top region of the scaffold, less cells seemed to have grown as compared to the rest of the scaffold. Higher magnification of the respective area, however, revealed decreased but satisfactory cell proliferation.

Nonetheless, the central oxygen concentration of both dynamic culture setups decreased in an almost identical manner (Fig. 4C). Measuring the oxygen concentration at the medium inlet in the forced perfusion bioreactor as well as at its outlet revealed that the inflowing medium had an oxygen concentration around 20%, while in the outflowing medium the oxygen declined to $\sim$12.5%. In sharp contrast, the oxygen concentration in the center of the scaffold was as low as $\sim$4% (Fig. 4D).

Dynamic 3D culture increases the area under the oxygen curve and promotes cell survival

To be able to quantitatively compare the oxygen concentrations in the center of differently cultured constructs

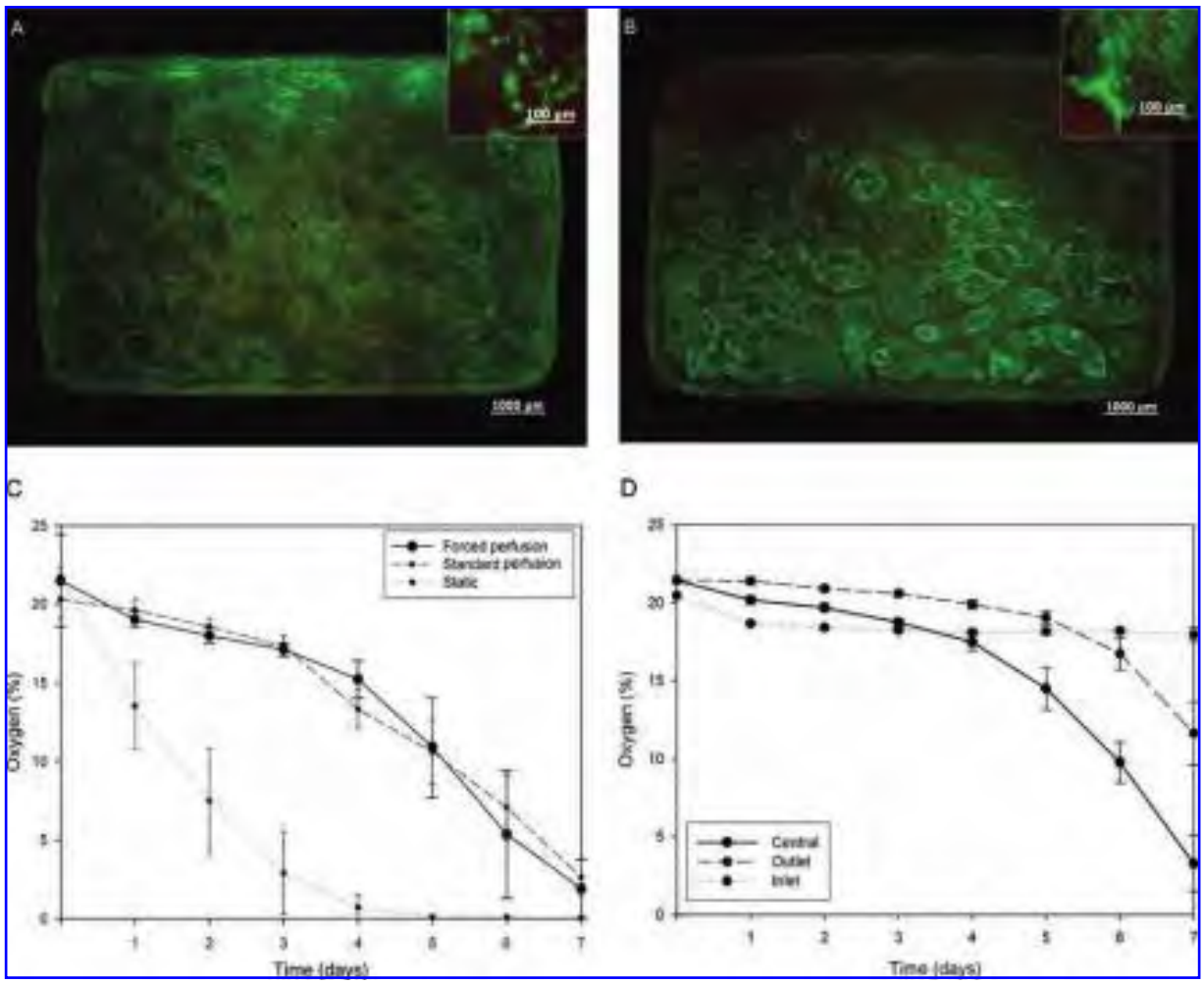

FIG. 4. Dynamic culture rescues cell survival and mitigates hypoxia in the center of the scaffold. The overview pictures of the live-dead stain were taken at 1.25× magnification, insets are representative pictures from the center of the scaffold and were taken at 10× magnification. Cells in the center survive after 7 days in the standard perfusion setup (**A**) in contrast to cells in static culture. Cell proliferation is further enhanced using the forced perfusion setup (**B**). Comparison of the oxygen curves derived from the center of statically and dynamically cultured cells reveals a superior oxygen performance of dynamic culture systems over static culture but no differences between the two dynamic systems (**C**). Measurement of oxygen concentrations at the inlet (dotted line), the outlet (dashed line), and the center of a construct cultured in the forced perfusion setup shows the expected differences at the inlet and outlet (**D**). Central oxygen concentrations, however, do not correlate with the outlet measurements. Color images available online at www.liebertpub.com/ten.

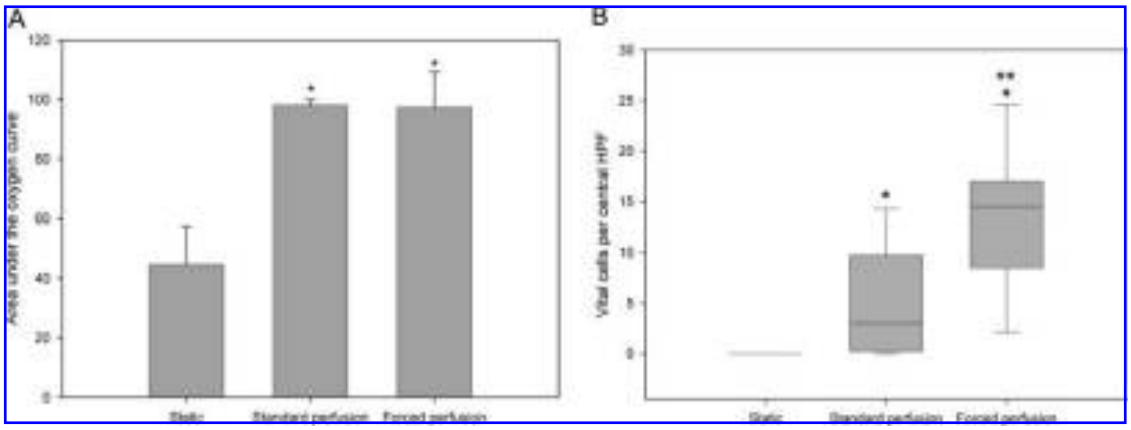

FIG. 5. Comparison of the area under the oxygen curves (AUCs) and cell growth from the different culture setups. (**A**) The AUCs derived from the forced perfusion setup and the standard perfusion setup are both significantly greater than that derived from the static culture. The asterisk (*) indicates a p-value of <0.01 ($p = 0.008$ and $p = 0.005$, respectively) compared to static culture. (**B**) Quantification of living cells within a 500×500 µm section acquired from the true center of each type of scaffold illustrates that in the center of the static setup no vital cells were left. In the standard perfusion setup, significantly more cells per HPF were found and similarly, the forced perfusion setup yielded significantly more cells per HPF when compared to the static setup. The asterisk (*) indicates a p-value of <0.01 ($p = 0.00126$ and $p = 0.0000000413$, respectively) compared to the static setup. There were also significantly more cells in the forced perfusion setup when comparing it to the standard perfusion setup. The asterisks (**) indicate a p-value of <0.01 ($p = 0.000419$) compared to the standard perfusion setup. Box plot ($n = 6$): percentiles, 5 and 25; median, 75 and 95.

over time, we determined the area under the curve (AUC) of the oxygen curves derived from three different culture setups. There was a significant difference between the AUC of the static culture and the AUC of the standard perfusion setup ($p = 0.005$), as well as between the AUC of the static culture and the AUC of the forced perfusion setup ($p = 0.008$; Fig. 5A).

To compare the amount of vital cells in the center of the scaffolds across the different setups, we quantified the cells within a 500×500 µm section acquired from the true center of each type of scaffold (Fig. 5B). While in the center of the static setup no vital cells were found, the standard perfusion setup yielded significantly ($p = 0.00126$) more cells per HPF. Similarly, the forced perfusion setup yielded significantly ($p = 0.0000000413$) more cells per HPF when compared to the static setup, and at the same time, there were significantly ($p = 0.000419$) more cells in the forced perfusion setup when comparing it to the standard perfusion setup.

DISCUSSION

The goal of this study was to reveal oxygen gradients in 3D culture systems designed for bone tissue engineering, to unmask their impact on cell survival, and to assess whether dynamic culture systems are suitable to overcome potential oxygen gradients. Limitations in scaffold-based tissue engineering arise from an uneven nutrient and oxygen supply of the cell–matrix constructs, which is reflected by significant gradients in tissue quality. Due to its poor diffusion capacity and solubility coefficient in aqueous solutions, oxygen is a limiting factor in scaling up 3D cultures.[6,13]

Static culture

We demonstrated that static 3D culture of bone precursor cells is associated with central hypoxia and a marked oxygen gradient toward the surface of the scaffold, similar to that noted in cardiac and cartilage cultures.[6,8,19] Malda *et al*. measured minimal central oxygen levels in static 3D culture of chondrocytes around 5%, and Kellner *et al.*, who used thicker scaffolds (4×4 mm vs. 5×2 mm), reported that oxygen concentrations in static 3D cultures of chondrocytes even fell below 1%.[5,19] Although there is evidence that chondrocytes tolerate or even require low oxygen concentrations down to 1%,[6,22] optimal oxygen levels for culturing osteoblastic cells are unknown. Generally, mammalian cells are supplied with blood that carries approximately 12% (arterial blood) to 5% (venous blood) of oxygen.[23] Oxygen levels in the medullar cavity of rabbit and porcine long bones range from 4% to 5%.[24–26] The oxygen tension is as low as 2% in the murine metaphyseal bone and reaches 0.8–1% in cortical bone as well as in hematopoietic stem cell niches of the murine bone marrow.[27] While 20% of oxygen may therefore be excessive for *in vitro* cell culture, hMSCs were shown to die when exposed to prolonged anoxia, even more so if nutrient supply was also perturbed.[28] The cells in our experiments did obviously not

tolerate extended exposure to 0% of oxygen either and died when exposed to anoxia.

As a general rule, it can be stated that the more cells proliferate, the more oxygen they consume. It was even suggested that the oxygen consumption rate may be used as a monitoring tool to assess proliferation.[29,30] Although the data are somewhat contradictory, it seems that MC3T3 cells cultured in 2% oxygen show reduced cell proliferation compared to cells grown at 21%, and similarly, cells cultured in 5% oxygen show a trend toward a decrease in proliferation.[23] Our results indicate that three-dimensionally cultured cells with a high proliferative potential create oxygen gradients within the scaffold, which prevent uneven cell proliferation and may even cause cell death. We therefore believe that it is important to monitor the oxygen levels within 3D cell cultures in order to assess the quality of the cell-seeded construct.

We further showed that in 3D culture more oxygen was consumed than in 2D. This is likely due to the fact that in 2D culture, cells reach confluence and thus stop to proliferate due to contact inhibition, whereas in 3D culture, cells can further proliferate given that the scaffold provides a larger surface.[30] It is especially important to guarantee sufficient oxygen supply during the early time of proliferation, as cells seem to consume more oxygen in this phase when compared to late-stage 3D culture.[8,31]

Dynamic culture

Dynamic culture systems for tissue engineering of bone were developed to improve nutrient delivery and waste removal from 3D tissue cultures.[17,18] Flow perfusion bioreactor systems are considered especially valuable for bone tissue engineering as they impose mechanical stimulation by shear stress and, in parallel, mitigate perfusional limitations of other 3D tissue culture systems.[32–35]

When analyzing the oxygen performance of perfusion bioreactors, we found that flow perfusion improves oxygen delivery to the cells seeded on a scaffold. This is evidenced by a more parabolic oxygen curve and a remarkably enhanced cell proliferation in the dynamic settings when compared to static culture. However, we noted that even under dynamic culture, the oxygen concentration decreased significantly in the center of the scaffolds within only 7 days, independently of whether the medium flow was forced through the scaffold or not. These data demonstrate that flow perfusion bioreactors do not necessarily warrant homogeneous oxygen supply in 3D culture. This hypothesis is supported by the finding that in the top region of the scaffold grown in the forced perfusion setup, less cells seemed to have grown as compared to the rest of the scaffold. This may be a result of the fact that in the forced perfusion system, cells at the top part of the scaffold are provided with the medium that had previously perfused underlying parts of the scaffold. Accordingly, the medium may not carry as many nutrients as it does at the lower parts of the scaffold. Further, it contains waste products of cells in lower regions of the scaffold, possibly resulting in reduced cell proliferation in this specific region. Although it is tempting to speculate that an increased medium flow rate may further improve oxygen supply to central regions of the scaffolds, washout of cells due to excessive shear stress has been observed.[36] Therefore, the optimal medium flow rate has to be titrated for each cell–scaffold–bioreactor setup individually.

It is known that many primary cells adapt to low oxygen concentrations by switching to an oxygen-saving metabolism.[37] The well-growing cells used in our experiments, however, reduced proliferation too slowly and thus suffocated in central regions of the statically cultured scaffold. It is obvious that data obtained from a fast-growing mouse cell line must not be directly transferred to human cells. However, our results indicate that oxygen gradients may occur in 3D culture. In consequence, individual monitoring of oxygen is advised for every cell–scaffold–bioreactor system, independent of whether mouse or human cells are used. We selected MC3T3 cells for two main reasons: firstly, they are preosteoblasts and therefore they are commonly used as a model cell line to assess bone cell metabolism; secondly, they have a tremendous proliferative potential as one would expect from cells used for tissue engineering. We believe that challenging the systems with this specific cell type revealed weaknesses, which may otherwise not have been discovered. For example, one may have assumed that the oxygen gradients noted in static culture would resolve using bioreactors. Conversely, the central oxygen concentration was significantly lower than the oxygen concentration at the inlet and the outlet of the bioreactor (Fig. 4D). This suggests that sufficient oxygenation of the culture is not necessarily guaranteed if outlet measurements are within a tolerable range, possibly due to medium bypasses inside the bioreactor. In other cell–scaffold combinations, the oxygen gradients may not be as steep as in our system, yet they may still be existing, potentially resulting in tissue quality gradients as differentiation processes are highly dependent on oxygen levels.[7,15,16]

In summary, 3D culture of osteoblastic precursor cells is associated with significant oxygen gradients in static culture as well as under dynamic culture conditions. While static 3D culture is an unacceptable method to generate tissue constructs in the range of millimeters, bioreactors promote cell proliferation and mitigate oxygen gradients *in vitro*. However, engineering of sizeable tissues requires careful monitoring of oxygen levels within the cell–scaffold constructs and measuring the central oxygen concentration is a minimum requirement for all scaffold-based tissue engineering activities. Because oxygen gradients, after application *in vivo*, will not decrease until sufficient vascularization is achieved, strategies will have to be developed to provide sufficient nutrient supply of engineered constructs after implantation.[6,13]

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