Clinical implementation of endochondral bone regeneration (EBR) would benefit from the engineering of devitalized cartilaginous constructs of allogeneic origins. Nevertheless, development of effective devitalization strategies that preserves extracellular matrix (ECM) is still challenging. The aim of this study is to investigate EBR induced by devitalized, soft callus-mimetic spheroids. To challenge the translatability of this approach, the constructs are generated using an allogeneic cell source. Neo-bone formation is evaluated in an immunocompetent rat model, subcutaneously and in a critical size femur defect. Living spheroids are used as controls. Also, the effect of spheroid maturation towards hypertrophy is evaluated. The devitalization procedure successfully induces cell death without affecting ECM composition or bioactivity. In vivo, a larger amount of neo-bone formation is observed for the devitalized chondrogenic group both ectopically and orthotopically. In the femur defect, accelerated bone regeneration is observed in the devitalized chondrogenic group, where defect bridging is observed 4 weeks post-implantation. The authors' results show, for the first time, a dramatic increase in the rate of bone formation induced by devitalized soft callus-mimetics. These findings pave the way for the development of a new generation of allogeneic, “off-the-shelf” products for EBR, which are suitable for the treatment of every patient.

1. Introduction

Over the last years, an interest has grown in the use of decellularized and devitalized extracellular matrices as bioactive scaffolds for in situ tissue engineering (TE). These decellularization and devitalization processes entail the killing of resident cells while preserving the bioactive components of the native extracellular matrix (ECM). Yet, the main difference between the two methods is that with decellularization protocols, cellular debris is removed whereas this is not the case for devitalization strategies. The use of native or engineered decellularized and devitalized tissues as scaffolds for TE presents several advantages. The fundamental one is that the cells that are migrating into a decellularized or devitalized matrix, are surrounded by the ECM naturally present in the target tissue. Here, the biochemical cues present in the ECM promote cell attachment, migration, differentiation, and ultimately tissue repair. This offers a clear benefit from a regenerative perspective compared to using less instructive scaffolds. Besides their role in enhancing the regenerative capacity of implants, ECM-based regenerative strategies are also attractive from a translational point of view. In particular, the absence of living cells would simplify the regulations around the marketing and use of ECM-derived products. Furthermore, if all immunogenic components are removed, allogeneic tissues derived from non-immunologically matched donors could be used. Finally, the implementation of ECM-based products in clinical practice would be easier from a
logical point of view because, in contrast to living engineered tissues, as decellularized and devitalized constructs can be mass-produced, easily stored, and used when needed.\[2\]

In line with this trend, several decellularized or devitalized options have already been explored in the orthopedic field to promote fracture healing and bone regeneration.\[4b,5a\] Several examples of decellularized ECM-based scaffolds that mimic the hard callus, the late stage repair tissue of a bone fracture, have been reported in literature.\[6\] One of the most well-known examples is the demineralized bone matrix, an osteoinductive and osteoconductive biomaterial obtained after the removal of minerals from allogeneic bone.\[6,7\] On the contrary, a less explored area is the use of decellularized and devitalized tissues that mimic the soft, cartilaginous callus present in the early stages of fracture healing.\[5\] Evidence supporting the feasibility of using devitalized native cartilage for endochondral bone regeneration (EBR) has been available since 1920, when Asarni and Dock\[8\] implanted boiled ear and xiphoid cartilage subcutaneously in a rabbit and observed new bone formation. Recent studies further confirmed the feasibility of using decellularized or devitalized cartilaginous templates to trigger EBR ectopically\[9b,9\] and orthotopically in rodents.\[9c,10\] Nevertheless, unsatisfactory results in terms of bone regeneration were observed when the decellularized or devitalized cartilaginous constructs were compared to the respective living control.\[5b,9a,10a\] Furthermore, in some cases the addition of living progenitor cells to the decellularized cartilage-derived matrix before implantation was found to be necessary in order to observe new bone formation.\[9b,c\] All together, these results suggest that the applied decellularization or devitalization methods lead to suboptimal regeneration, potentially caused by the loss of bioactivity of the ECM components. This led to our hypothesis that milder devitalization methods are essential to preserve the structural and biochemical integrity of the tissue’s ECM. However, a downside of milder approaches is that cellular debris and DNA are retained within the implanted construct. This has shown to trigger an immune response that may hamper the regenerative process induced by non-autologous ECM-based scaffolds.\[9d,11\] Finally, contrasting evidence is present in literature regarding the optimal timing for devitalization (e.g., after chondrogenic differentiation or hypertrophic induction) in order to maximize the conversion of the cartilage template into new bone upon implantation in vivo.\[6,9a,12\]

The aim of this study was to investigate the regenerative potential of devitalized soft callus-mimetic cartilaginous spheroids for bone TE applications. To do so, the bone regeneration induced by devitalized ECM-based scaffolds produced after MSC chondrogenic or combined chondrogenic/hypertrophic stimulation was compared to the neo-bone formation induced by their living counterparts. In the context of the development of an off-the-shelf product, all cartilaginous constructs were produced from allogeneic MSCs. The use of allogeneic cells is a key element for the clinical translation of this approach, as allogeneic MSCs could be preselected for their high chondrogenic potential, overcoming the well-known hurdles of inter-donor variability and unpredictability of the differentiation potential of patient-derived cells.\[11c,13\] This would ensure the access to such a treatment for every patient, irrespectively of their own cells’ potential.\[11c,13,14\] Moreover, the use of allogeneic cells favors the potential storage of cells, upscaling, and clinical application since larger cell numbers can be obtained from an allogeneic cell source compared to autologous, especially when pooling such cells. This is also more cost-effective since large patient cohorts could be treated with such an allogeneic cell batch.\[13\] To mimic a relevant clinical scenario, the regenerative potential of the engineered constructs was evaluated in a challenging and fully immunologically mismatched setting (Figure 1).

2. Results

2.1. Effects of Devitalization on Human MSC-Derived Cartilage Constructs In Vitro

The efficacy of the devitalization procedure was investigated by evaluating resazurin reduction, which is caused by mitochondrial reductase and is a common marker for cell metabolic activity, and by calcein staining, which indicates cell viability.\[15\] The resazurin reduction in the chondrogenic and hypertrophic samples was significantly reduced following the devitalization treatment, to $4.9 \pm 2.6\%$ and $3.7 \pm 2.3\%$ of their vital counterparts, respectively (Figure 2A). After digestion of the spheroids, no calcein-positive (living) cells were detected in the devitalized groups (Figure 2B). On the contrary, viable cells were observed for both vital groups. Furthermore, no cells attached to tissue culture plastic after the digested constructs were re-plated, while they did for the living controls (Figure 2C). Overall, no differences in viability between the chondrogenically differentiated samples and the samples stimulated into hypertrophy were observed, although a lower number of viable cells was observed for Donor 2 in the vital hypertrophic group. After devitalization, cellular debris was still present in the devitalized construct, as confirmed by the unaffected DNA content (Figure S1, Supporting Information).

After assessing the effect of devitalization on cell viability, the retention of different ECM components was investigated by qualitative and quantitative analyses. Overall, ECM components were preserved after devitalization, as evaluated by histological analysis. No structural changes were apparent between vital and their respective devitalized groups. There was no evident reduction in GAGs, collagen type II, collagen type X, and calcium content in the constructs (Figure 3A). These observations were supported by the quantitative assays, where no statistically significant differences were found in total protein content (Figure 3B), GAG (Figure 3C), or hydroxyproline content (Figure 3D) between the devitalized and vital samples. Moreover, ALP activity, which is more likely to be affected by the devitalization than protein content, was not reduced after the devitalization procedure (Figure 3E).

The surface roughness and porosity were evaluated by scanning electron microscopy (SEM) analysis, which highlighted an increased porosity at the surface of the chondrogenic devitalized samples, compared to the vital chondrogenic samples (Figure 4A). This difference was less evident in the hypertrophic group due to the minerals present on the surface. The increased porosity in devitalized chondrogenic samples was indirectly confirmed by the highest diffusion of iodixanol (Figure 4B), a neutrally charged contrast agent, into the ROI selected at the center of the spheroids. Consistently, devitalized chondrogenic samples showed a higher degradation rate compared to other groups (Figure 4C,D). In particular, all samples from the devitalized chondrogenic group were completely degraded after a maximum of
Figure 1. Experimental outline. For the in vitro characterization, human MSCs were embedded in collagen hydrogels and differentiated either in chondrogenic medium (31 days) or in chondrogenic + hypertrophic media (21 + 10 days). After 31 days, half of the number of the constructs of each group was devitalized. The viability of the cells and retention of ECM components were evaluated. For in vivo implantation, rat MSCs were used and an identical differentiation schedule was followed. After 31 days, spheroids were assembled in multi-modular constructs and implanted either subcutaneously (2 spheroids per construct) or in a femur defect (8 spheroids per constructs). Carrier material control was included in the subcutaneous implantation. After 12 weeks, samples were explanted and new bone formation was evaluated as indicated.

Figure 2. Cell viability after devitalization of engineered cartilage spheroids. A) Resazurin reduction before and after devitalization and B) the number of living cells left as detected by calcein staining (green staining). C) Calcein-positive cells extracted from the digested constructs were stained with methylene blue after replating and culturing for 2 days. *** p < 0.001; ND: not detectable.
Figure 3. Qualitative and quantitative evaluation of the ECM preservation after devitalization of human MSC spheroids. A) Sections of spheroids from the four experimental groups were stained for GAGs (red staining) in the top row, for collagen type II and X (brown staining) and for mineralization in the bottom row (dark brown). Inserts: appearance of complete spheroids. Quantification of the B) total protein content, C) GAGs, and D) total hydroxyproline in the spheroids of the four groups before and after devitalization. E) ALP activity was quantified as an indication of the retention of the bioactivity after devitalization. ** p < 0.01, *** p < 0.001.
Figure 4. ECM porosity and degradation rate. A) SEM pictures showing the changes in surface porosity of the spheroids due to devitalization. B) Quantification of the changes in the average pixel intensity induced by the penetration of ioxanol in the selected ROI of the spheroids. C) Quantification and D) representative images of the degradation of the spheroids induced by a collagenase solution. 2/3 of the devitalized chondrogenic spheroids were already degraded after 18 h, whereas the last one was degraded in 24 h. The majority of the vital chondrogenic spheroids (2/3 samples) was degraded by 24 h, whereas 1/3 sample was degraded by 32 h. A slower degradation rate was observed for the hypertrophic groups. #: devitalized chondrogenic group significantly different compared to devitalized hypertrophic group.
24 h (degrading time 20 ± 3.5 h). This was 6.6 h faster than their vital counterparts (degrading time 26.6 ± 4.6 h). For the hypertrophic group, the spheroids were not fully degraded after 80 h, with no evident differences observed between the vital and devitalized groups.

2.2. Post-Surgery Observations

Prior to surgery, the mean body weight of the rats was 289 ± 19 g and increased throughout the entire period, reaching 358 ± 18 g after 12 weeks. No external signs of adverse reactions (i.e., swelling or redness) at the site of implant were observed in any of the rats during the course of the experiment. One animal, of the vital chondrogenic group, died prematurely 7 weeks post-surgery. As the cause of death was unknown, the retrieved samples were excluded from the analysis. In addition, one devitalized chondrogenic sample was excluded from the microCT analysis due to scattering from an inappropriately placed titanium screw. Nevertheless, this sample was still suitable for histological analysis.

2.3. Neo-Bone Formation Is Induced by Devitalized Constructs when Implanted Subcutaneously

All the collagen carrier controls were completely resorbed and could not be retrieved at the end of the study. No difference in mineralized tissue volume was observed in the microCT analysis of the subcutaneous implants, with roughly 1–3 mm³ mineralized tissue volume was observed in the microCT analysis could not be retrieved at the end of the study. No difference in mineralization volume of the devitalized constructs that could be prepared in advance from allogeneic cell sources and stored until needed.[5a–c] The devitalized status of the devitalized constructs that could be prepared in advance from allogeneic cell sources and stored until needed.[5b,17,18] Notably, the decrease of GAGs is of especial interest as cartilage decellularization processes often require aggressive treatments to degrade the dense ECM matrix surrounding chondrocytes.[5b,17,18] Our results show that devitalization does not affect the main ECM composition of samples of either the chondrogenic or the hypertrophic group. The retention of the collagenous and mineral component that we observed has been reported before using several decellularization or devitalization protocols.[17] However, the preservation of GAGs is a remarkable difference compared to what is commonly reported in the literature, especially as cartilage decellularization processes often require aggressive treatments to degrade the dense ECM matrix surrounding chondrocytes.[5b,17,18] Notably, the decrease of GAGs is often linked to the loss of bioactive proteins that are associated to...
Figure 5. Subcutaneous endochondral bone formation 12 weeks post implantation. A) Overview of the implanted constructs stained with H&E. The grey dotted lines indicate the edges of the constructs whereas the black boxes highlight the area depicted in the higher magnification pictures in the second row. New bone formation was observed in at least a few samples of all groups (bright pink in the H&E). Furthermore, Masson–Goldner trichrome staining highlighted the presence of newly deposited osteoid (orange staining). Non-remodeled cartilage was evident in all groups (red staining in the Safranin-O/fast green). B) Results of the histomorphometric analysis performed 12 weeks post implantation. b: bone; c: cartilage; m: bone marrow; arrows: osteocytes. *p < 0.05.
Figure 6. Micro-CT-based evaluation of bone formation in the femur defects. A) Quantification of mineralization over time for all groups. (*p < 0.05). B) Different percentages of defect bridging were observed between groups. C) 3D reconstructions and 2D images of the defects 4, 8, and 12 weeks post-implantation. White dots: titanium screws.
Figure 7. Endochondral bone formation in the femur defects 12 weeks post implantation. A) Overview of the femur defect area. The black dotted lines indicate the bone edges whereas the gray boxes highlight the magnified areas depicted in (B). B) High magnification images of H&E, Masson–Goldner trichrome, and Safranin-O/fast green staining. C) Results of the histomorphometric performed after 12 weeks. *p < 0.05; **p < 0.01; ***p < 0.001. b: bone; c: cartilage; s: spheroid; m: bone marrow.
them, such as growth factors and chemokines.[19] This loss often leads to reduced bone regenerative capacity of the devitalized cartilage-derived ECM compared to their respective vital counterparts when implanted in vivo.[5b,9a] Our data confirms the preservation of collagens and GAGs and also suggests that enzyme activity is preserved; a more in-depth characterization of structural preservation and the presence of ECM components following devitalization will be the topic of future studies.

Preserving biochemical integrity of a cell-derived ECM implies at the same time meager removal of cellular debris, especially in case of cartilage tissue. It has been shown that the presence of foreign DNA, mitochondria, and cellular membranes skew macrophage populations towards a pro-inflammatory M1 phenotype, both in vitro and in vivo.[11a,b] Consequently, this was reported to negatively affect tissue remodeling and the regenerative outcome in an abdominal wall defect in rats.[11a,b] Nevertheless, our recent work showed that EBR could be triggered by vital, allogeneic MSC-derived engineered cartilage, with no evidence of detrimental immune response.[11c] Thus, the presence of allogeneic cellular debris was not expected to impair the bone regenerative process. Consistent with this hypothesis, in the present study, we showed that the conversion of allogeneic, devitalized spheroids into new bone tissue was not hampered by the presence of residual DNA. On the contrary, in the devitalized chondrogenic group, bridging of the femur osteotomy gap was observed in all animals already after 4 weeks and the remodeling of the newly formed tissue into mature bone, characterized by a lamellar structure and a cortical and trabecular compartment, was achieved within 12 weeks. In contrast, defect bridging was observed in only 33% of the vital chondrogenic group, which was consistent with previous results.[11c] A similar trend was observed for the hypertrophic groups, where the devitalized group promoted a higher percentage of defect bridging compared to the vital hypertrophic group. The reasons why the devitalized groups outperformed their respective living counterparts could be multiple. The changes in microarchitecture induced by the devitalization process[20] might have played a role. The increase in porosity and degradation rate of the samples could have favored a faster host cell infiltration, growth factor exposure, vessel ingrowth, and ultimately implant remodeling into bone tissue. Nevertheless, further studies should elucidate whether the presence of allogeneic living cells actively hampered bone formation. Specifically, the changes in environment that occurred as a consequence of in vivo implantation (e.g., oxygen tension and pH) could have triggered the secretion of stress-response related signals (e.g., alarmins) by the implanted cells.[21] This could have influenced the local recruiting of immune cells and ultimately altered the wound healing process. Thus, a comparison between the immune response triggered by the vital and devitalized spheroids, especially in the early stages post-implantation, may lead to the identification of a specific branch of the immune response that accelerates or hinders EBR. In addition, future studies should investigate whether the newly formed bone shows adequate mechanical properties. This is clinically relevant to estimate when the newly formed bone is ready to withstand physiological load.

Several studies have shown that active enhancement of the hypertrophic phenotype in vitro preceding the devitalization[5b,9c,d] or decellularization[9b] improves the bone regenerative outcome in vivo. In the hypertrophically stimulated constructs, the secretion of pro-angiogenic and osteogenic growth factors, which enhance blood vessel invasion and osteogenesis in vivo, is promoted. Nevertheless, it has also been reported that ECM mineralization, as a result of the addition of β-glycerophosphate in the hypertrophic medium, could have an inhibitory effect on the release of VEGF and metalloproteinase.[22] This ultimately resulted in decreased pro-angiogenic and remodeling properties of the mineralized MSC pellets in an in vitro model[22] and in less bone formation in vivo.[23] In addition, it must not be neglected that, even upon induction of chondrogenic differentiation, MSCs already present hallmarks of hypertrophy.[16] Thus, here we evaluated whether the use of hypertrophic medium and matrix mineralization before in vivo implantation was required in order to observe the conversion of the devitalized cartilage into new bone tissue. MicroCT analysis and histological results suggest that chondrogenic differentiation of the samples—both vital and devitalized—was sufficient to achieve new bone formation in vivo ectopically and orthotopically. In particular, the chondrogenically differentiated, devitalized samples displayed enhanced bone formation at both implant locations, indicating that in our system active hypertrophy induction is not required. However, it should be noted that shorter or more prolonged hypertrophic stimulation periods could lead to other results.

All together, these results highlight the potential of a tissue engineered, allogeneic, devitalized MSC-based cartilaginous soft callus mimic as a powerful tool to increase the clinical translatability of EBR. Our system presents several advantages over alternative strategies that apply donor/engineered tissue devitalization or decellularization, or mimic the inorganic components of bone ECM (i.e., calcium phosphates). First, as it mimics the cartilaginous soft callus, which is a temporary matrix naturally present after bone injuries, its degradation and remodeling will follow the exact events and timing of physiological fracture healing, that is, the cartilaginous template will be progressively invaded by blood vessels and remodeled by the concert action of different types of host cells such as osteoclasts and osteoblasts.[13,24] In other words, synchronized degradation of the engineered construct and new bone formation will occur, overcoming the challenges associated with tailoring the resorption rate of biomaterials such as calcium phosphates.[25] Second, even if further analysis should be performed in order to confirm the absence of donor-derived cells in the neo-formed bone, in comparison to other systems, here the regenerated tissue is theoretically completely of host origin.[9b,26] This represents an advantage compared to engineered constructs containing living chondrocytes, as they can transdifferentiate into foreign resident osteoblasts and osteocytes.[27] In the presented devitalized implants, the full conversion of the implant into the patient’s own bone tissue would promote a solution to problems associated with the chronic immune response elicited by slowly degrading biomaterials or living allogeneic cell implants, potentially resulting in long-term graft rejection.[13,28] Third, this system will facilitate potential upscaling of the engineered constructs via modular assembly methods without encountering the problem of the formation of a necrotic core during the chondrogenic differentiation stage. The relatively small dimensions of the callus spheroids allow the optimal differentiation of the MSCs without encountering problems associated with the oxygen and nutrient diffusion.
limits prior to devitalization. The devitalized spheroids could then serve as building blocks to create larger constructs. Afterwards, different shapes and sizes could be achieved to perfectly match the patient’s defect. Nevertheless, additional studies need to be performed to confirm that upscaling to clinically relevant dimensions (from the millimeter cube to the centimeter cube range) still ensures uniform cell infiltration and tissue remodeling throughout the entire construct. Lastly, the absence of living cells increases the clinical translatability of this approach, as it potentially simplifies logistical and regulatory aspects.

4. Conclusion

In this study we present for the first time an allogeneic, MSC-derived devitalized soft callus mimic that goes beyond the state of the art and outperforms its living equivalent, in terms of accelerated bone regeneration and the quality of the newly formed bone. The development of these constructs paves the way for a next generation of EBR-based strategies, and to the potential generation of a scalable and off-the-shelf therapeutic product for bone restoration.

5. Experimental Section

Study Design and Overview: For in vitro characterization of the effect of the devitalization process on the engineered callus-mimetic spheroids, human MSCs were embedded in a collagen gel and chondrogenically differentiated or stimulated towards a hypertrophic state prior to devitalization. The ECM of the devitalized constructs was characterized and compared with the one of vital control samples.

For the in vivo studies, the same four groups (vital chondrogenic or hypertrophic, devitalized chondrogenic or hypertrophic) were used but with constructs derived from rat MSCs. Allogeneic rat MSCs were encapsulated in a collagen gel, differentiated, and implanted in a rat of a different strain, having a fully functional immune system. The constructs were implanted subcutaneously (n = 6 for each group) and in a critical size femur defect in rats. For the subcutaneous implantation, a carrier material (collagen) was added. For the surgical procedure and acute toxicity, five rats were used (three rats for each group). For devitalization, bioreduction of resazurin sodium salt (R7017; Sigma-Aldrich) was assessed. Briefly, the vital and devitalized chondrogenic and hypertrophic constructs were incubated for 18 h at 37 °C under humidified conditions and 5% carbon dioxide (CO2) in MSC expansion medium consisting of 10% heat-inactivated fetal bovine serum (5100831810, Biowest, Nuaille – France), 0.2 mM L-ascorbic acid 2-phosphate (A8960, Sigma-Aldrich, St. Louis, USA), 100 U/mL of penicillin and 100 mg/mL of streptomycin (15140, Invitrogen), and 1 ng/mL basic fibroblast growth factor (233-FB; R&D Systems, Minneapolis, USA).

Rat MSCs were isolated from 4-week old Dark Agouti rats (Envigo, Indianapolis, USA) with the approval of the Central Authority for Scientific Procedures on Animals (CCD, no. AVD1150020172465) and the animal ethical committee of the University Medical Center Utrecht. Briefly, the rats were euthanized through CO2 asphyxiation. After removal of the epiphysis, bone-marrow was obtained by flushing through the diaphysis with MSC expansion medium supplemented with 0.025% ethylenediaminetetraacetic acid (EDTA). Cells were allowed to adhere in a Petri dish overnight. Afterwards, StemX Vivo medium (CCM004, R&D Systems) was used for subculturing.

Both rat and human MSCs were passaged at 80% confluency until passage 4.

Generation of MSC Callus-Mimetic Spheroids: At passage 4, human or rat MSCs were chondrogenically differentiated. Human MSCs were used for the ECM characterization, whereas Dark Agouti rat MSCs were used for the in vivo experiments. Briefly, collagen spheroids were created by encapsulating MSCs (20 × 10^6 mL^-1) in 50 μL collagen type I gel droplets (4 mg mL^-1) (354249, Corning, New York, USA), according to the manufacturer’s instructions. After gelation, the samples were cultured in serum-free chondrogenic medium consisting of high glucose DMEM (31966, Invitrogen) with 1% insulin-transferrin-selenium (ITS) + premix (354352; Corning), 10^{-7} M dexamethasone (DB893; Sigma-Aldrich), 0.2 mM L-ascorbic acid 2-phosphate (A8960, Sigma-Aldrich), 100 U mL^-1 penicillin, and 100 mg mL^-1 streptomycin (15140, Invitrogen). To differentiate human MSCs, the medium was supplemented with 10 ng mL^-1 TGF-β1 (Peprotech, New Jersey, USA). For rat MSCs, also 100 ng mL^-1 BMP-2 (Induc-TOS, Wyeth/Pfizer, New York, USA) was added. Medium was refreshed daily for the first 4 days and afterwards three times per week. After 2 weeks of chondrogenic differentiation, half of the number of spheroids was subjected to hypertrophic medium, consisting of DMEM (31966, Invitrogen), 1% ITS + premix, 100 U/mL penicillin with 100 mg/mL streptomycin, 0.2 mM L-ascorbic acid-2-phosphate, 1 mM dexamethasone, 10 mM β-glycerophosphate (G9891; Sigma-Aldrich), and 1 mM 3,3’5-triiodo-L-thyronine (T2877; Sigma-Aldrich). Differentiation in chondrogenic or hypertrophic medium proceeded for 10 additional days till day 31.

Devitalization Procedure of the Spheroids and Viability Analyses: At 31 days, samples were harvested and devitalized by a mild procedure including lyophilization (European Patent Application no. 20 195 800.6). To confirm devitalization, bioreduction of resazurin sodium salt (R7017; Sigma-Aldrich) was assessed. Briefly, the vital and devitalized chondrogenic and hypertrophic constructs were incubated for 18 h at 37 °C in the dark with 500 μL of 10% resazurin sodium salt in chondrogenic medium without TGF-β1. Absorbance was measured on a spectrophotometer at 570 and at 600 nm for background correction (Versamax; Molecular Devices, Sunnyvale, USA). Data are presented as percentage, considering the resazurin reduction of the vital chondrogenic and hypertrophic groups as 100%. The values obtained from empty collagen controls were subtracted. To further confirm the absence of viable cells, constructs were digested using a 3 mg mL^-1 collagenase type II (LS004177, Worthington; Lakewood, NJ, USA) in phosphate-buffered saline (PBS) digest solution for a minimum of 2 h at 37 °C. The extracted cells were stained with 0.5 μg mL^-1 Calcein-AM (Molecular Probes, Thermo Fisher Scientific, Massachusetts, USA) for 30 min at 37 °C. Samples were excited at 495 nm and emission was registered at 515 nm (ASCENT Fluoroskan plate reader; Labsystem). For quantitative analysis, the signal was calibrated with known numbers of living MSCs to produce a standard curve. Images were acquired using an Olympus IX53 inverted fluorescence microscope. The spheroid digests were then re-plated in a 96-well plate and incubated with MSC expansion medium for 2 days to check for any remaining cell viability and the capacity to adhere to tissue culture plastic. Wells were washed with PBS, fixed in 10% neutral buffered formalin, and stained with methylene blue (341088-1G, Sigma-Aldrich) for 5 min. Images of the monolayers were taken with an Olympus IX53 inverted microscope. At least three constructs per condition for each donor were used.

Histological Analysis of Vital and Devitalized Human MSC-Derived Cartilage Constructs: After fixation, samples were dehydrated in a series of increasing ethanol solutions (70–100%) and cleared in xylene. Subsequently, the samples were embedded in paraffin and sliced into 5 μm thick sections.
To identify cell nuclei, collagenous fibers, and glycosaminoglycans (GAGs), sections were triple stained with Weigert’s hematoxylin (640:490, Klinpath BV), fast green (FN1066522, Merck), and Safranin-O (FN1164048213; Merck). To detect mineralization, von Kossa staining was performed by incubating the sections with 1% silver nitrate (209 139, Sigma-Aldrich) directly under a light bulb (Philips Master T5LSHO 54W 803, 1 m distance), for 1 h. The samples were subsequently washed with 5% sodium thiosulfate (A17629, Alta Aesar, Haverhill, USA) and counter-stained with haematoxylin.

For collagen type II (0.6 μg mL⁻¹, II-1683, Developmental Studies Hybridoma Bank) and collagen type X (10 μg mL⁻¹, I-CO097-05, clone X33, Quartett, Germany) endogenous peroxidase activity was blocked by incubating samples for 15 min with 0.3% H₂O₂. For collagen type II staining, antigen retrieval was done by a sequential treatment of 1 mg mL⁻¹ pronase (Sigma-Aldrich) and 10 mg mL⁻¹ hyaluronidase (Sigma-Aldrich) for 30 min each at 37 °C. For collagen type X staining, antigens were retrieved by sequential incubation with 1 mg mL⁻¹ pepsin (Sigma-Aldrich) at pH 2.0 for 2 h and 10 mg mL⁻¹ hyaluronidase for 30 min, both at 37 °C. Prior to primary antibody incubation, samples were blocked with 5% BSA/PBS for 30 min at room temperature. Samples were incubated with the primary antibody overnight at 4 °C. After 30 min of incubation with the secondary BrightVision antibody (VWR KDPVM110HRP, BrightVision, Quartett, Germany) endogenous peroxidase activity was blocked by incubating samples for 15 min with 0.3% H₂O₂. For collagen type II staining, antigen retrieval was done by a sequential treatment of 1 mg mL⁻¹ pronase (Sigma-Aldrich) and 10 mg mL⁻¹ hyaluronidase (Sigma-Aldrich) for 30 min each at 37 °C. For collagen type X staining, antigens were retrieved by sequential incubation with 1 mg mL⁻¹ pepsin (Sigma-Aldrich) at pH 2.0 for 2 h and 10 mg mL⁻¹ hyaluronidase for 30 min, both at 37 °C. Prior to primary antibody incubation, samples were blocked with 5% BSA/PBS for 30 min at room temperature. Samples were incubated with the primary antibody overnight at 4 °C. After 30 min of incubation with the secondary BrightVision antibody (VWR KDPVM110HRP, BrightVision, Quartett, Germany) endogenous peroxidase activity was blocked by incubating samples for 15 min with 0.3% H₂O₂. For collagen type II staining, antigen retrieval was done by a sequential treatment of 1 mg mL⁻¹ pronase (Sigma-Aldrich) and 10 mg mL⁻¹ hyaluronidase (Sigma-Aldrich) for 30 min each at 37 °C. For collagen type X staining, antigens were retrieved by sequential incubation with 1 mg mL⁻¹ pepsin (Sigma-Aldrich) at pH 2.0 for 2 h and 10 mg mL⁻¹ hyaluronidase for 30 min, both at 37 °C. Prior to primary antibody incubation, samples were blocked with 5% BSA/PBS for 30 min at room temperature. Samples were incubated with the primary antibody overnight at 4 °C. After 30 min of incubation with the secondary BrightVision antibody (VWR KDPVM110HRP, BrightVision, Quartett, Germany) endogenous peroxidase activity was blocked by incubating samples for 15 min with 0.3% H₂O₂.

To measure hydroxyproline content, 50 μL of the papain digests of all samples were freeze-dried overnight. Afterwards, samples were hydrolyzed by sequential incubation with 0.4 M NaOH at 108 °C and 1.4 M citric acid. Hydroxyproline contents were measured using a colorimetric method (extinction 570 nm), with chloramine-T and dimethylaminobenzidine oxidation. Sections were then counter-stained with haematoxylin, washed, dehydrated, and mounted with Depex mounting medium. Mouse isotypes (X0931, Dako, Santa Clara, USA) were used as negative controls at the same concentration as the primary antibodies.

Images were taken with an Olympus BX51 microscope (Olympus DP73 camera, Olympus, Hamburg, Germany). Histology of empty collagen control can be found in Figure S5, Supporting Information.

Biochemical Analysis: For total protein quantification, samples were digested with 0.5 mg mL⁻¹ collagenase II for 5 h at 37 °C. Protein concentration was determined using the Pierce BCA protein assay kit (23,225, Thermo Fisher Scientific) according to manufacturer’s instructions. Known concentrations of bovine serum albumin were used to create a standard curve. Absorbance was measured at 562 nm.

Samples for GAG and collagen analysis were digested overnight at 60 °C in papain digestion buffer (250 μg mL⁻¹ papain, 0.2 M NaH₂PO₄, 0.1 EDTA and 0.01 M DL-cysteine hydrochloride; all from Sigma-Aldrich). The total amount of GAGs was determined using the 1,9-dimethyl-methylene blue (DMMB pH 3.0; Sigma-Aldrich) assay.[29] Known concentrations of shark chondroitin sulfate C (Sigma-Aldrich) were used as standard. Absorbance values were detected at 525 and 595 nm.

To measure hydroxyproline content, 50 μL of the papain digests of all the samples were freeze-dried overnight. Afterwards, samples were hydrolyzed by sequential incubation with 0.4 M NaOH at 108 °C and 1.4 M citric acid. Hydroxyproline contents were measured using a colorimetric method (extinction 570 nm), with chloramine-T and dimethylaminobenzidine as reagents as previously described.[30] Hydroxyproline (Mercer) was used as a standard.

Alkaline phosphatase (ALP) activity was measured by using the p-nitrophenyl phosphate (pNPP) substrate system (N2765; Sigma). Different concentrations of ALP with a known activity (U per milliliter) were used as standard curve. The constructs and the standard series were incubated with the pNPP substrate at 37 °C for 8 min. Absorbance was measured at 405 nm with 655 nm as a reference wavelength. The DNA content was quantified using a Quant-IT Picogreen dsDNA assay (P11496, Thermo Fisher Scientific) according to the manufacturer’s instructions.

Four constructs were used per condition for each donor in all analyses. Three MSC donors were used for the analysis of GAGs, collagen, ALP, and DNA. Due to inferior proliferation capacity and shortage of primary cells obtained from one donor, only two out of three MSC donors were used for the total protein quantification.

Evaluation of the ECM Porosity and Susceptibility to Degradation: Fixed samples were dehydrated using a critical point dryer (CPD 030, Bal-Tec) for SEM. After gold sputtering (JEOL, JFC-1300, JEOL Ltd, Tokyo, Japan), samples were imaged using a SEM (JEOL JSM-5600, JEOL Ltd).

For the degradation study, samples were incubated with 10 U mL⁻¹ collagenase II (Worthington) in plain DMEM at 37 °C. Medium was collected and completely replaced after 1, 2, 4, 12, 18, 24, 36, 48, 60, 72, and 80 h. The collected medium were processed as described above to measure hydroproline content.

To indirectly measure the porosity of the constructs, samples were immobilized at the bottom of a custom-made mold of 3% agarose gel. The top of the sphereid was exposed to Visipaque solution (iodixanol, GE Healthcare) and microCT images (Quantum FX; PerkinElmer, Waltham, USA) were taken at different time points (20 μm resolution, voltage 90 kV, current 180 mA, field of view = 10 mm). For each sphereid, the diameter was measured and a ROI of 0.1 x 1.8 mm was selected at the center of the sphereid. The changes in average pixel intensity within the ROI due to the inward diffusion of the contrast agent were monitored over time using the image processing software Image-J (Java, Redwood Shores, USA).

One MSC donor was used to evaluate construct porosity and susceptibility to degradation. A triplicate was used for the quantitative measures whereas one sample per group was used for qualitative images.

Construct Preparation for In Vivo Implantation: Chondrogenic differentiation and metabolic activity of the Dark Agouti MSCs was verified prior to in vivo implantation (Figures S6 and S7, Supporting Information). For subcutaneous implantation, two chondrogenic spheroids per group (vital chondrogenic and hypertrophic, and devitalized chondrogenic and hypertrophic) were embedded in collagen (4 mg mL⁻¹) and cast in custom-made square cuboid molds (3 mm x 3 mm x 2 mm). Gelation was allowed for 45 min at 37 °C according to manufacturer’s instructions. Empty collagen controls were included as controls. For the orthotopic defects, eight chondrogenic spheroids were encapsulated in collagen gel in 3.5 mm x 3.5 mm x 6 mm custom-made molds, as described above. The constructs were prepared the day before implantation and incubated overnight in a chondrogenic differentiation medium without TGF-β1 and BMP-2.

Animal Experiment and Surgical Procedures: The animal experiments were performed with the approval of the Central Authority for Scientific Procedures on Animals (Dutch national CCD) and of the local animal welfare body (2465-2-01) in accordance with the ARRIVE guidelines for animal experimentation.[32] The power analyses used to determine the number of samples required per group are presented in the Supporting Information. Twenty-four male Brown Norway rats of 11 weeks old (Envigo) were randomly housed in pairs at the Central Laboratory Animal Facility of the University of Utrecht, the Netherlands, on a standard food pellets and water ad libitum, under climate-controlled conditions (21 °C; 12 h light/12 h darkness). After 7 days of acclimatization, subcutaneous pockets were created under general anesthesia from 5 mm dorsal incisions and blunt dissection as previously described[33] (1-3.5% isoflurane in oxygen, AST Farma, Oudewater, the Netherlands). In each pocket, one construct of either group (collagen control, vital chondrogenic, vital hypertrophic, devitalized chondrogenic, or devitalized hypertrophic) was implanted (n = 6 per group). The skin was closed transcutaneously with Vicryl Rapide 4-0 sutures (VIB 2297; Ethicon). Each animal received a maximum of 2 subcutaneous pockets. For implantation of the construct in a femur defect, a 6-mm critical-size segmental bone defect was created as previously described[33] (n = 8 for the devitalized chondrogenic and hypertrophic experimental groups and n = 4 for the vital chondrogenic and hypertrophic controls). Briefly, the right hind leg was shaved and carefully disinfected. A lateral skin incision was made and soft tissue was dissected in order to expose the right femur. After the peristium removal, three proximal and three distal screws were used to stabilize a 23 x 3 x 2 mm polymer ether ketone (PEEK) plate to the femur in the anterolateral plane. After fixation, a saw guide and a wire saw (RISystem, Davos, Switzerland) were used to remove a 6-mm cortical bone segment. The collagen constructs were press-fit into the defect and a single dose of antibiotic (Duplicolin LA, 22.000 IE/kg, MSD Animal Health, Boxmeer, the Netherlands) was locally injected intramuscularly. The fasi
implants, a region of interest (ROI) of 6.5

throughout the entire construct area was quantified. For the orthotopic

and orthotopic samples after H&E staining. Briefly, an overview of the

staining. H&E, Masson–Goldner trichrome staining and Safranin-O/fast green

ethanol solutions (100–70%). New bone formation was evaluated us-

paraffinized with xylene and gradually rehydrated through decreasing

into 5

lutions (70–100%), cleared in xylene, embedded in paraffin and sliced

calcified for 6 weeks in a 10% EDTA-phosphate buffered saline solution

×

plugin Reorient3 TP (Image-J 2.0.0; Java, Redwood Shores, CA, USA). A

21 mm). All scans were oriented in the same fashion using the ImageJ

size of 42

}[34] (13 of 14)

was selected in the

μ

voltage

90 kV, current

= 180 mA, field of view = 10 mm). After segmentation with a global threshold, the mineralized vol-

umes (MV) for both the subcutaneous and femur implants were measured in millimeter cube using the image processing software plugin BoneJ [34] (ImageJ). 3D reconstructions of the femur defect were based on the microCT data and created using ParaView (ParaView 5.3.0, Kitware Inc., USA).

Histological Analysis of the In Vivo Samples: All specimens were fixed in a 10% neutral buffered formalin solution for 1 week and thereafter de-

calcified for 6 weeks in a 10% EDTA-phosphate buffered saline solution (pH 7.4). After decalcification, samples were additionally fixed for 2 days, dehydrated in a Leica ASP300S tissue processor in graded ethanol solu-

tions (70–100%), cleared in xylene, embedded in paraffin and sliced into 5 μm thick sections (Microm). Before staining, samples were de-

paraffinized with xylene and gradually rehydrated through decreasing ethanol solutions (100–70%). New bone formation was evaluated using H&E, Masson–Goldner trichrome staining and Safranin-O/fast green staining.

Histomorphometric analysis was performed for both the subcutaneous and orthotopic samples after H&E staining. Briefly, an overview of the whole sample was made by merging images into a panoramic image in Adobe Photoshop CS6. For the subcutaneous implants, bone formation throughout the entire construct area was quantified. For the orthotopic implants, a region of interest (ROI) of 6.5 × 5 mm² was selected in the center of the defect. The titanium screw holes present on each side of the defect were used as reference points in order to ensure an equivalent position-

ing of ROI in all samples. Three different areas were manually selected for each ROI: bone, hypertrophic cartilage, and bone marrow. The number of pixels for each area was quantified via the function “recording measure-

ment” and expressed as a percentage of the total construct area for the ectopic implants and of the ROI area in the orthotopic ones. The sections were scored independently by two scientists and the results are presented as an average.

Statistics: A randomized block design with Bonferroni’s post hoc correc-

tion was applied for the in vitro data to accommodate donor variation, including viability and biochemical analyses (protein, CAG, hydroxypro-

line and DNA content and ALP activity). A linear mixed model followed by a Bonferroni’s post hoc correction was used to compare mineralization in the femur defect over time and to evaluate statistical differences in the Visiapake diffusion test (IBM SPSS 22.0, New York, USA). For the histo-

morphometric measures, when data were normally distributed, a one-way ANOVA test was performed, followed by Tukey post-hoc test (GraphPad Prism 6, San Diego, CA, USA). When the condition of normality was not satisfied, a Kruskal–Wallis test, followed by a Dunn’s post hoc test was performed. Differences were considered to be statistically significant for p < 0.05.

Supporting Information

Supporting Information is available from the Wiley Online Library or from

the author.

Acknowledgements

Project no. S-16-130G was supported by the AO Foundation. The antibody against collagen type II (III-16B3), developed by T. F. Linsenmayer, was ob-

tained from the DSHB developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA52242. R. L. acknowledges funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement No. 949806). Finally, the authors would like to thank Anja van der Sar for her gracious support during the animal ex-

periments.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the cor-

responding author upon reasonable request.

Keywords

allogeneic, devitalization, endochondral bone tissue regeneration, mes-

enchymal stromal cells, orthotopic bone defect

Received: July 29, 2021

Revised: November 12, 2021

Published online: December 28, 2021

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