Original Article - *In vitro* Study

Three-Dimensional Bioprinting Using a Coaxial Needle with Viscous Inks in Bone Tissue Engineering - An *In vitro* Study

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Abstract

Introduction: Vascularized autologous tissue grafts are considered "gold standard" for the management of larger bony defects in the craniomaxillofacial area. This modality does however carry limitations, such as the absolute requirement for healthy donor tissues and recipient vessels. In addition, the significant morbidity of large bone graft is deterrent to fibula bone flap use. Therefore, less morbid strategies would be beneficial. The purpose of this study was to develop a printing method to manufacture scaffold structure with viable stem cells. **Materials and Methods:** In total, three different combinations of ground beta tri-calcium phosphate and CELLINK (bioinks) were printed with a nozzle to identify a suitable bioink for three-dimensional printing. Subsequently, a coaxial needle, with three different nozzle gauge combinations, was evaluated for printing of the bioinks. Scaffold structures (grids) were then printed alone and with additional adipose stem cells before being transferred into an active medium and incubated overnight. Following incubation, grid stability was evaluated by assessing the degree of maintained grid outline, and cell viability was determined using the live/dead cell assay. **Results:** Among the three evaluated combinations of bioinks, two resulted in good printability for bioprinting. Adequate printing was obtained with two out of the three nozzle gauge combinations tested. However, due to the smaller total opening, one combination revealed a better stability. Intact grids with maintained stability were obtained using Ink B23 and Ink B42, and approximately 80% of the printed stem cells were viable following 24 hours. **Discussion:** Using a coaxial needle enables printing of a stable scaffold with viable stem cells. Furthermore, cell viability is maintained after the bioprinting process.

Keywords: Adipose stem cells, bioinks, CELLINK, *in vitro*, viability

Introduction

Many strategies have been proposed for the management of craniomaxillofacial congenital bony defects or those defects following avulsive trauma or ablative surgery;[1] nevertheless, autogenous bone grafts are still considered "gold standard" for reconstructive bone surgery.[2]

Small-sized mandibular defects ranging from less than 4 to 6 cm can be managed with nonvascularized corticocancellous grafts harvested from different sites, for example, the anterior or posterior iliac crest.[2-4] Larger defects are commonly treated with vascularized grafts such as the fibula,[2,5,6] which offer a long pedicle, wide vessel diameter, and the possibility to incorporate skin, muscle, and bicortical bone components if such tissues are required.[7] However, the use of vascularized autologous tissue grafts requires healthy donor tissues and vessels, as well as recipient vessels. The significant morbidity of large bone graft harvesting is deterrent to fibula bone flap use.[8-11] Less morbid treatment strategies would be advantageous,[12] although, in a longer perspective, the quality of life among these patients seems to be acceptable.[13]

Bone tissue engineering aims to regenerate osseous tissue by combining biomaterials and stem cells.[12-17] One reliable source of stem cells for bone regeneration is autologous fat tissue that provides an abundant amount of adipose stem

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cells (ASCs).[17,18] ASCs are capable of multiple lineage differentiation to adipocyte chondrocyte and osteoblast pathways.[17] Harvesting fat tissue is simple and causes far less morbidity in comparison to traditional bone harvesting techniques.[17-21] Another advantage of ASCs compared to bone marrow stromal/stem cells (BMSCs) is that BMSCs are present in low frequency in the bone marrow, whereas ASCs can be retrieved in high numbers from adipose tissue and can easily be expanded *in vitro*.[16,22] Using the tissue engineering model,[14] it has been possible to harvest autogenous ASCs from patients and to use the cells to seed a resorbable scaffold.[23-26]

Biomaterials of various compositions and physical forms can serve as scaffolds in bone tissue engineering, including granular forms and solid blocks. Although they have no load bearing ability compared to solid blocks, granules present more surface area for cellularization. In addition, granules are more quickly incorporated into host tissues than block forms, which remodel more slowly.[27] Granular calcium phosphate ceramics and related biomaterials such as hydroxyapatite possess macro- and micro-structural properties that make them useful biomaterials in tissue engineering. Such properties affect cell attachment, survival, signaling, growth, and propagation.[27,28] While some inorganic ceramics may also bind directly to bone,[29] the surfaces of other biomaterials may affect differentiation of stem cells to osteoblasts.[30]

One obstacle in planning complex reconstructive surgery using either autologous bone or tissue-engineered constructs is the inability to produce complex facial contours in a predictable manner, using commercially available reconstruction plates and meshes.[31] One solution to this problem is to use computer-guided surgical planning and additive manufacturing technology to produce a passive, but fitting implant, designed for patient-specific anatomical needs.[32,33] Yet, complex defects are difficult to restore with existing three-dimensional (3D) technology. The development of simultaneous 3D printing of scaffolds with cells for medical use is one technique that shows great promise.

A variety of 3D printing processes are currently being used. These include, inkjet, orifice free, and extrusion bioprinting.[33] Many of these approaches allow for the printing of supportive scaffold structure, as well as the simultaneous printing of different types of cells. However, there are several problems related to the use and the composition of the specific bioink, printing technology, and the type of cell laden structure to be produced. These include the primary stability, the dispersion of cells, and the specific combination of different biomaterials used in producing a viable implant. Another well-known problem is the viscosity versus clogging and pressure effects on the printed cell populations together with mechanical stability of the printed construct.[34] Combining stem cells, growth, and linking factors together with a stable scaffold material to replace complex-shaped bony resection defects is the primary goal of bioprinting.

Different combinations of materials have been used in additive manufacturing from cellulose to tri-calcium phosphate (TCP) and hydrogel-based compounds.[34] The addition of a sacrificial or resorbable material used initially for extra stability has been advocated, and cross-linking hydrogel is the most commonly used technique. In large defects, a more rigid and stable material such as TCP would be of benefit.

The aim of the present study was to develop a printing method to achieve a stable scaffold structure with viable stem cells.

Materials and Methods

The current *in vitro* study was performed within August 2018 to October 2019. In total, four independent experiments were carried out to address the aim of the present study. An initial experiment compared bioinks regarding printability; the second experiment investigated a coaxial needle for printing resolution, using different nozzle gauge configurations, and the third experiment measured the stability of printed grids [Figure 1]. In the fourth experiment, cell viability of the printed grids comprising human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) was evaluated. In addition, cell viability of the same grids but at two different pressures was assessed.

Inks and bioinks

To identify suitable bioinks for extrusion printing, three different combinations (Ink B23; Ink B42, and Ink B48) of ground β-TCP (ChronOS Bone Graft, Synthes, West Chester, PA, USA) and nanofibrillated cellulose/alginate (NFC-A; CELLINK AB, Gothenburg, Sweden) were evaluated. The different composition of the evaluated bioinks is listed in Table 1. Printability tests were performed with a nozzle using a 3D printer (Inkredible; CELLINK AB, Gothenburg, Sweden) and a curvy bracelet model. Printing parameters were adjusted to give a smooth outflow of the bioink, and a total of five samples were printed for each composition. The samples were cross-linked with a 100 mM calcium chloride solution for 6 min.

Nozzle design

Coaxial needles (Ramé-Hart Instruments Co., Succasunna, NJ, USA) [Figure 2a] using three different nozzle gauge combinations (22–18, 22–16, and 22–14) were tested for extrusion printing of the bioinks. Figure 2b illustrates a

Table 1: Composition of the three bioinks (Ink B23, Ink B42, and Ink B48) evaluated for printability using a nozzle. Beta tri-calcium phosphate, nanofibrillated cellulose/alginate (CELLINK)

	Amount β-TCP (weight %)	Amount Cellink (weight %)
Ink B23	23%	77%
Ink B42	42%	58%
Ink B48	48%	52%

schematic of the nozzle configuration, and Figure 2c presents the measurements of the different gauge combinations tested. Distance A, i.e., the inner diameter of the coaxial needle, was maintained at 0.406 mm throughout all the printing procedures in the present study. Distance B, i.e., the outer diameter, was set to 0.129, 0.479, and 0.889 mm for the respective nozzle gauge combination tested.

Cell culture preparation

The hAD-MSCs used for printing were RoosterVial hBM-10M (MSC-001) (RoosterBio Inc., Frederick, MD, USA) purchased in aliquots of 10×10^6 cells. Cell expansion was performed in accordance with the manufacturer's protocol. In detail, an expansion medium was prepared by mixing RoosterBio media booster and basal medium, respectively. The cell aliquot was brought to 37°C and transferred to a 10 ml centrifuge tube. Four milliliters of expansion medium was added drop-wise to the cell suspension. The solution was then centrifuged at 200 *g* for 5 min. The supernatant was aspired, and the cell pellet re-suspended in 2 ml expansion medium and incubated for 5 days at 37°C and 5% CO_2. The cell medium was changed at day 3. A concentration of 4×10^6 cells/ml ink yielded a total number of 148 constructs with 3.3 ml ink during one print process.

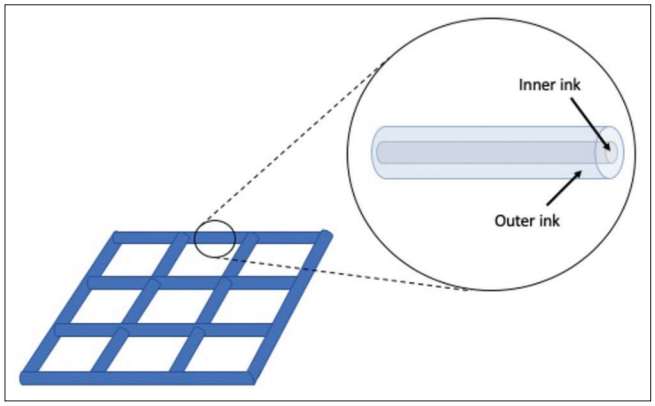


Figure 1: Schematic illustration of a printed grid

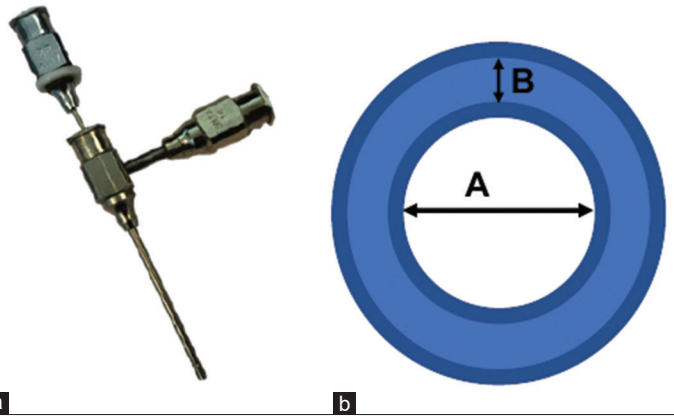
Three-dimensional printing

Printing without adipose stem cells

Grids were printed using the 22–16 gauge combination previously described and a combination of Ink B23 or B42 as an inner ink and CELLINK as an outer ink. The mineral ink was inserted to printer head 1 (PH 1) and the CELLINK was connected to printer head 2 (PH 2). Grids in two layers, 9 mm × 9 mm, were printed using a pressure of 105 kPa for PH 1 and 55 kPa for PH 2. Completed grids were cross-linked with $CaCl_2$, 100 mM (Sigma-Aldrich, Stockholm, Sweden) for 6 min before being transferred into Hanks balanced salt solution (HBSS; GIBCO) and incubated at 37°C in 5% CO_2 overnight. Following incubation, stability of the printed grids was evaluated by assessing the degree of maintained grid outline.

Bioprinting with adipose stem cells

Printing was done within 2 h after cell preparation, using the 3D printer (Inkredible; CELLINK AB, Gothenburg, Sweden). Before mixing the hAD-MSCs with Ink B23 or CELLINK, cell confluence was evaluated by confocal light microscopy. Confluent cells were washed with 6 ml Dulbecco's phosphate-buffered saline (GIBCO) and incubated with 6 ml TrypLE™ (GIBCO) at 37°C for 1 min. Cell de-attachment was the verified by confocal light microscopy, and TrypLE™ activity was quenched by adding 6 ml spent expansion media. For the printing, the mineral ink was inserted to PH 1 and the bioink containing cells to PH 2. Grids in two layers, 6 mm × 6 mm, were printed using the combinations of inks and bioinks previously evaluated. A pressure of 105 kPa (PH 1) and 55 kPa (PH 2) was then applied. For the evaluation of printing pressure effects on the cells in the Ink B23, two different pressures on PH 2 were tested, 75 kPa and 85 kPa, respectively. Completed grids were cross-linked with $CaCl_2$, 100 mM (Sigma-Aldrich, Stockholm, Sweden) for 6 min before being transferred into expansion medium and incubated at 37°C in 5% CO_2.



Nozzle configuration measurements from the different gauge combinations tested

	Distance A (mm)	Distance B (mm)
22-18	0.406	0.129
22-16	0.406	0.479
22-14	0.406	0.889

c

Figure 2: (a) Image of coaxial needle (b) Description of nozzle configuration. Arrow A represents the inner diameter and arrow B represents the outer diameter of the respective nozzle (c) Table of combinations tested; remake according to the information provided by Ramé-Hart Instrument Co. (www.ramehart.com/pdf/needles.pdf)

Viability control

Cell viability was measured 24 h after printing using a Olympus Microscope with ×10 objective. The cells were stained using a Live/Dead Cell Imaging Kit (Invitrogen), a two-component imaging kit which stains viable cells with calcein-AM and dead cells with BOBO-3 Iodine. The staining was performed in accordance with the manufacturer's protocol. In brief, the printed constructs were washed with HBSS for 30 min at 37°C. The two different components in the live/dead kit were mixed together to make a 2 × working solution. The solution was mixed with an equal volume of HBSS and added to cover the printed constructs. The constructs were incubated for 1 h at 37°C. The staining solution was removed and washed with HBSS for 1 h at 37°C before imaging. Viable cells were imaged by exciting at 488 nm and the dead cells at 570 nm. Images were processed using the image processing software Image J (NIH, LOCI; University of Wisconsin, WI, USA).

Results

Inks and bioinks

Among the three evaluated combinations of bioinks (Ink B23, Ink B42, and Ink B48), two combinations of bioinks resulted in good printability for extrusion bioprinting, Ink B23 (23% ground β-TCP; 77% CELLINK) as well as Ink B42 (42% ground β-TCP; 58% CELLINK) [Figure 3a]. The remaining combination, Ink B48 (48% ground β-TCP; 52% CELLINK) [Figure 3b], caused clogging and negative pressure effects on the prints; hence, this combination was excluded from further tests in this study. Ink B23 and Ink B42 were both considered suitable for further testing.

Nozzle design

The suitable bioinks from the printability tests, i.e., Ink B23 and Ink B42, were further evaluated using a coaxial needle. Out of the three nozzle configurations available only two were successful in printing an adequate amount of β-TCP + CELLINK. Printing with the 22-18 gauge combination was unsuccessful due to the narrow path for the outer ink (B distance) [Figure 2b], which means that a much higher pressure would be needed for printing than is currently allowed by the 3D printer (Inkredible). The remaining gauge combinations, 22–16 and 22–14, both proved to be good for printing. However, due to the smaller total opening, the 22–16 gauge combination was shown to give a better printing resolution. Furthermore, when the 22–16 and the 22–14 gauge coaxial needles were compared with regard to printing of β-TCP + CELLINK, the former revealed a better stability and was used for further experiments.

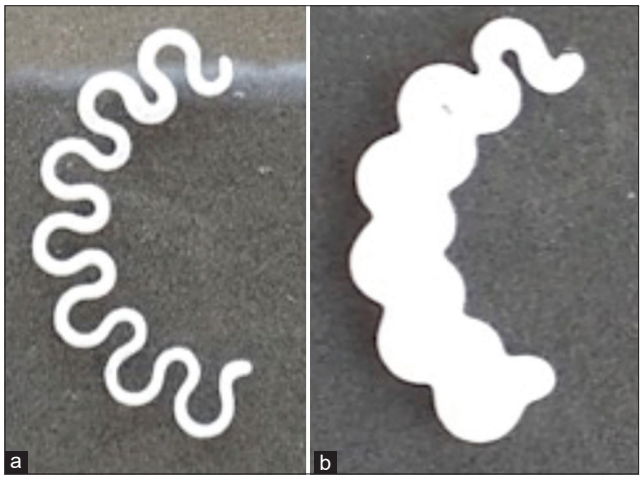


Figure 3: Printability test of (a) Ink B42 and (b) Ink B48 using three-dimensional printer (Inkredible) and curvy bracelet model for the two bioink compositions

Bioprinting

Stability tests were performed by printing grids with the 22–16 gauge nozzle combination. This was carried out in two different settings, either by using the two bioinks previously determined best for printing, namely Ink B23 or Ink B42 and CELLINK [Figure 4a and d] or by using the two bioinks without any additional CELLINK [Figure 4g]. Intact grids were possible to print using the Ink B23 or Ink B42 as the inner ink and CELLINK as the outer ink [Figure 4b and e]. Stable grids were also possible to print without using CELLINK, with Ink B42 as the inner ink and Ink B23 as the outer ink [Figure 4h]. All these combinations were shown to be printable with a maintained grid outline and stability after one night in HBSS [Figure 4c, f, and i]. Hence, all combinations were used for further cell viability experiments.

Viability control

hAD-MSCs were mixed together with CELLINK and printed as an outer ink with either the Ink B23 or the Ink B42 as an inner ink. hAD-MSCs were also mixed together with the Ink B23 and printed as an outer ink (at two different printing pressures) using B42 as an inner ink. Cell viability was evaluated after 24 h. The cell viability test following printing revealed a higher viability in the grids enclosing Ink B23 as the inner ink and CELLINK + hAD-MSCs as the outer ink as compared to the grids with B42 as the inner ink and CELLINK + hAD-MSCs as the outer ink. Nearly 80% of the cells were viable in the grids comprising Ink B23 whereas the corresponding figure was 65% for the grids with Ink B42 [Figure 5a]. Grids comprising Ink B42 as an inner ink and hAD-MSCs mixed with Ink B23 as an outer ink had a higher cell viability when printed at 75 kPa compared to 85 kPa [Figure 5b]. The grids printed with lower pressure retained a cell viability of about 65% which was similar to the grids enclosing Ink B42 as an inner ink and CELLINK and hAD-MSCs as an outer ink. Higher pressures for printing the cells resulted in lower viability, with a viability of <60%.

Discussion

The primary aim of the present study was to identify and develop a bioprinting process with a predictable outcome, producing a stable scaffold with viable cells. These factors are important when introducing bioprinting into the clinical setting, such as to use for reconstructing major skeletal defects

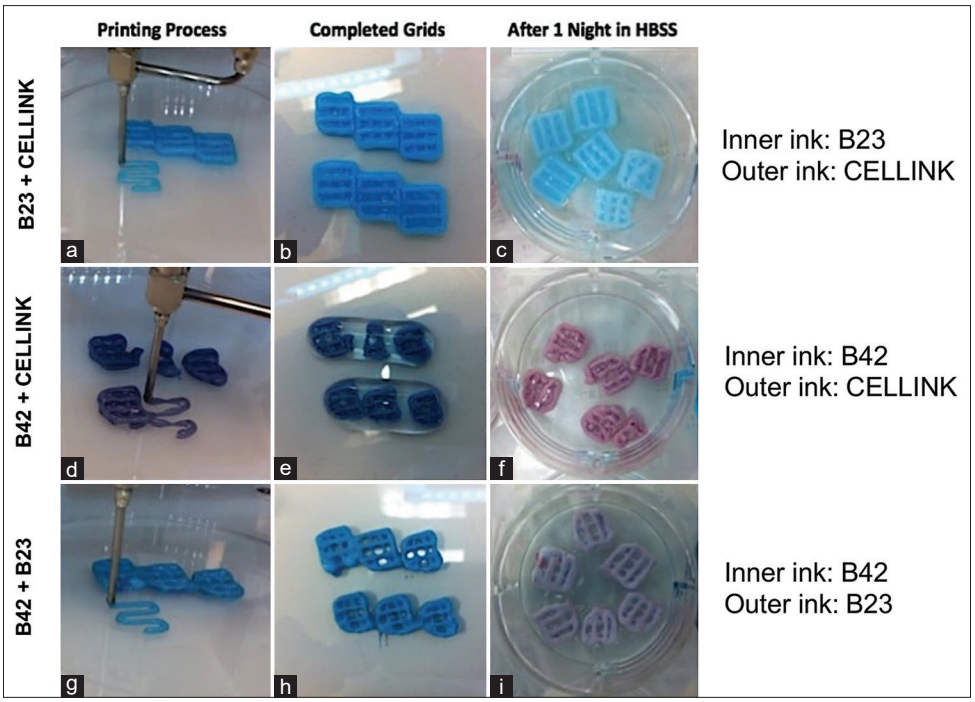


Figure 4: Printing process, completed grids, and stability test after one night in Hanks balanced salt solution, using either Ink B23 or Ink B42 as inner ink and CELLINK as outer ink (a-f) or by using the two bioinks, Ink B42 and Ink B23 as the inner and outer ink, respectively (g-i)

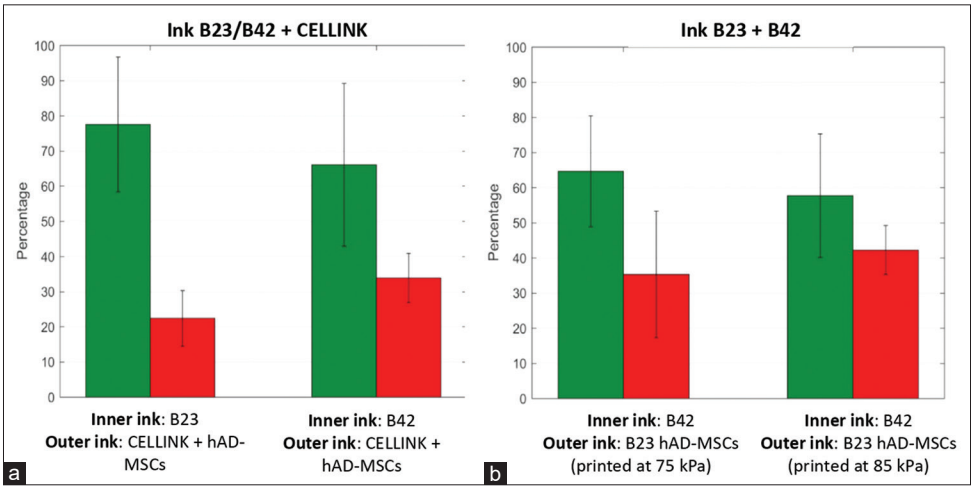


Figure 5: Cell viability test of printed grids comprising human adipose tissue derived mesenchymal stem cells. (a) Ink B23 or Ink B42 as the inner ink and CELLINK + human adipose tissue-derived mesenchymal stem cells as the outer ink. (b) Ink B42 as the inner ink and CELLINK + human adipose tissue-derived mesenchymal stem cells as the outer ink, at 75 kPa and 85 kPa, respectively

in the head-and-neck area. The craniomaxillofacial skeleton is load bearing, implying that the printed construct must be stable. While hydrogels are commonly used materials in bioprinting, they are not inherently load bearing. Cross-linking the hydrogel is a common procedure to increase its stability. However, cross-linking is not sufficient to fulfill the needs for a skeletal construct. In the present study, a synthetic material, β-TCP, was chosen as the scaffold to help achieve a stable printed construct. β-TCP is today used as a bone substitute to restore minor and intermediate-sized defects in the maxillofacial area with encouraging results.[24-26] However, it must be noted that, in larger defects, β-TCP without cells is not sufficient for predictable bone healing.[26] Therefore, this study evaluated three different compositions of β-TCP and CELLINK and found that bioinks, comprising β-TCP of 23% and 42%, respectively, were printable with a stable structure using an extrusion 3D printer. Another issue of importance is the printing process itself. When a construct with the purpose to both deliver cells and acting as a scaffold to bridge a defect, both the cells and the scaffold must be printed concurrently,

and their cellular viability must be maintained. The type of bioprinter may be essential; however, the 3D printer used in this study fulfilled the requirements and produced constructs at both 75 kPa and 85 kPa.

Earlier studies using mono-needles revealed substantial limitations, especially regarding the time required for printing.[35] Thus, to overcome these problems, the authors used a coaxial needle, making it possible to print both the cells as well as the scaffold simultaneously. Evaluation of different nozzle gauge combinations of the coaxial needle revealed that a coaxial needle with an inner diameter of 0.406 mm and an outer diameter of 0.479 mm, especially the 22–16 nozzle configuration, was most suitable for the purpose of our study. This as the 22–18 nozzle configuration resulted in a poorer printing resolution where the structure of the grid was not visible and the structural properties were insufficient. In addition, the cell laden layer was lost during movement of the grid. Furthermore, although the 22–14 gauge nozzle combination proved to be suitable for printing, it resulted in decreased stability of the printed grids when compared to the 22–16 coaxial needle. Hence, the authors decided to use the 22–16 gauge nozzle combination for subsequent experiments of this study.

Finally, the problem to maintain cell viability of the printed grids was studied. Our results indicate that the number of human adipose stem cells in the preparation was sufficient, which has been concluded by other studies.[26] However, the results with an increased number of cells to determine their optimal number in the print have yet to be investigated. Another finding was that the printing pressure was of importance. A higher pressure resulted in an increased number of dead cells after 24 h. In addition, the viscosity of the bioink used for printing the scaffold seemed to be important since the ratio of living versus dead cells decreased with higher viscosity of the scaffold ink. However, the effect of the dead versus living cells needs to be further explored since cell degradation may release intracellular products that may both stimulate and hamper the activity of the remaining living cells. In the present series of experiments, the size of the printed grids was small. However, cell viability in larger grids is of importance to further investigate as these may be used to replace complex-shaped bony defects in the facial skeleton. Combinations using other printing materials or processes such as a sacrificial matrix material which is intended to stabilize the larger construct during a predefined period may be a possibility. Different printing pressures and printing speed are other important aspects to consider for further research. Taken together, although the results in this study look promising, the authors want to further develop the technique for better dimensional stability, cell viability, and reproducibility so it can be put to use *in vivo* and then in clinical practise.

Conclusions

This study indicates that using a coaxial needle enables printing of a stable scaffold with viable cells. Furthermore, cell viability is maintained after the bioprinting process.

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Conflicts of interest

There are no conflicts of interest.

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