The osteogenic capacity of a novel bone graft substitute

Abstract

Purpose

Iliac crest bone graft is considered the gold standard for bone grafting. However, this method has its drawbacks, an alternative is therefore desired. Recently, a novel surgical suction filter device was introduced to the market which can generate a bone graft substitute. The osteogenic capacity of this novel bone graft has not yet been determined.

Materials and Methods

BoneFlo (BF) samples were obtained during non-union surgery. The BF was loaded with a collagen based β-TCP containing foam sponge and fitted to the surgical suction device upon opening the intramedullary canal. As a control for the BoneFlo, whole blood was used. Samples were compared to cells from the bone marrow (BMAC) and to RIA samples, which is another alternative bone graft. On day 0, BF samples were analysed for expression of osteogenic marker genes and cell composition was investigated. Additionally, samples were cultured and analysed on day 7, 14 and 21 for ALP activity, matrix mineralization and expression of osteogenic marker genes.

Results

In total, 13 BF, 3 BMAC and 1 RIA sample were used for the experiments. The BF samples showed distinct differences in comparison to the control samples in gene expression and ALP activity. In addition, the dynamic expression profile of the BF samples is comparable to the expression profile of the BMAC and RIA group.

Conclusion

This study shows that the BoneFlo can create an osteogenic bone substitute as it enhances the expression of osteogenic marker genes and has a higher ALP activity in comparison to the control groups. It may therefore be a promising tool to generate a less invasive bone graft substitute.

Introduction

Bone tissue has several functions, including providing strength and rigidity to the body, protection of internal organs, and it functions as a mineral reservoir. In addition, bone is regarded as one of the few organs with a high regenerative capacity, as reflected during fracture healing. (1) Typically, fracture healing occurs in the first 6-8 weeks after an injury but can be delayed by several known risk factors, including excessive movement of the fracture site, aging, alcohol and tobacco use, medical conditions, infections etcetera. (2) If 9 months have elapsed since the injury and the fracture doesn't show visible progressive signs of healing for 3 months, the FDA defines the fracture as a non-union. (3) Non-union rates are variable in the literature, reported from 18.5% in the tibia to 1.7% in the femoral shaft. (4)

Bone grafting is commonly performed during non-union surgery to augment bone healing. Different kinds of bone grafts are used although autologous bone grafting is considered the gold standard. The reason autologous bone grafts are considered the gold standard is because it combines all the required properties for fracture healing; osteoinduction is induced by the bone morphogenetic proteins and other growth

factors, osteogenesis is acquired by the osteoprogenitor cells and osteoconduction is provided by the tissue structure. (5) In addition to autologous bone grafts, allografts and synthetic graft substitutes are of interest. Allografts are easier to obtain but are susceptible to graft rejection and disease transmission while synthetic grafts are more likely to wear and tear and can cause a foreign body reaction. (2,5)

Mesenchymal stem cells (hMSC) play a crucial role in the healing and reconstruction of bone tissue. They are found in many tissues of the body, but bone marrow has high abundance and is easily accessible. Therefore, they are applied for bone reconstruction in clinical scenarios. Currently, autograft is mostly obtained from the iliac crest, however, the iliac crest bone graft (ICBG) also has its drawbacks like the limited amount of bone that can be harvested and the morbidity of the procedure to the patient. (6)

Because of the complications with the use of ICBG, there is a demand for alternative bone grafts. Bone marrow aspirate (BMA) is a popular source of hMSCs and can be used by surgeons as bone graft. Bone marrow aspirate concentrate (BMAC) is an attempt to improve the healing capacity of the BMA by increasing the recovery of the nucleated cells from the marrow aspirate while decreasing the recovery of the non-nucleated cells such as red blood cells. Through centrifugation of the BMA, the cell concentration and amount of growth factors are increased. (7) BMAC, however, only consists of osteogenic cells and shows high patient variability. (8)

Another reliable and efficient source of autograft is obtained by the Reamer-Irrigator-Aspirator (RIA) system (Johnson and Johnson, New Brunswick, USA). Originally designed to reduce intramedullary canal pressure and thereby fat emboli, the RIA system reams the intramedullary canal while aspirating the intramedullary contents containing irrigation fluid, bony fragments and marrow. It was later discovered that the intramedullary contents were a viable source of autologous (bone) graft. (9) Multiple studies have shown that the RIA system yields a graft material containing osteoblasts, osteocytes, hMSCs and exhibited higher levels of bone morphogenic proteins (BMPs) and vascular endothelial growth factor (VEGF) receptors than ICBG of the same patient. (10-12)

When treating larger bone defects, the restricted volume and the osteogenic capacity of the autografts are regarded as limiting factors. In addition to this, the complications during harvesting autografts are also a drawback. BMAC yields quite a low volume. It also only harvests cells and no osteoconductive material. The RIA is effective in harvesting larger quantities of bone graft and yields more osteogenic cells. However, it is invasive, costly and bears the risk of fracturing the healthy bone. In addition to this, RIA is not always feasible, for example, when intramedullary nailing is present or when smaller volumes of bone graft are needed. (13,14) Therefore, alternative methods to harvest osteogenic graft material are still desired.

During the non-union surgery, significant amounts of bony fragments, bone marrow and other tissue are aspirated from the surgical field. This mixture is normally aspirated by the surgical vacuum system and discarded afterwards. Recently, a novel surgical vacuum filter device was introduced to the market which can be fitted with a beta-tricalcium phosphate (β-TCP) containing collagen sponge (Cersorb Foam®, Curasan AG, Kleinostheim, Germany). This device, the BoneFlo (BF) (TissueFlow GmbH, Essen, Germany), claims to harvest a material which can be used as an osteogenic autologous bone graft.

One study done by Henze et al. showed that the stromal cells harvested by this device are superior in proliferation in comparison to cells harvested via bone marrow aspiration. (15) However, the osteogenic capacity and

osteogenic potential of this graft material is yet unknown. During osteogenic differentiation, hMSCs show a certain mRNA profile (17,18), secrete extracellular matrix (ECM) molecules, and enable calcium deposition. (16) Therefore, by showing the osteogenic mRNA profile and the secretion of the ECM and calcium deposition, the osteogenic potential can be quantified.

This study aims to investigate the osteogenic capacity of the bone graft obtained by the BF by determining the osteogenic mRNAs, alkaline phosphatase (ALP) activity and the cell composition by means of Flow Cytometry.

Materials and methods

Human samples

BF samples were collected during non-union surgery. Inclusion criteria were age ≥ 18 years and a posttraumatic, non-infected, non-union of a long bone. Exclusion criteria were infected non-unions and patients undergoing bone marrow suppressive treatment. This study was approved by the Institutional Review Board of the Maastricht University Medical Center and performed according to the Geneva Convention and Good Clinical Practice guidelines. All procedures were carried out in accordance with the Declaration of Helsinki in its latest amendment.

Immediately upon opening the intramedullary canal, the BF was fitted to the surgical suction device and loaded with a collagen based, β-TCP containing foam sponge. The vacuum device was used until the filter was fully saturated with bone marrow, blood and bony fragments from the surgical field, this was visually confirmed by the senior consultants performing the surgery.

Autograft was harvested from the ipsilateral or contralateral femur using the RIA system according to the manufacturer's instructions. In short, a longitudinal incision cranial to the major trochanter was made and the tip of the trochanter was identified. The medullary canal was opened and the system was introduced over a guide wire. The size of the reamer head was determined using fluoroscopy and the bone graft was harvested in a single pass under fluoroscopic control. For the harvesting of the BMAC samples, a small incision was made to expose the left or right iliac crest. Subsequently, bone marrow was drawn using a syringe. The BMA was processed using the Arthrex Angel system in order to obtain BMAC (Arthrex Inc, Naples, USA). Inclusion and exclusion criteria for the BMAC and RIA samples were the same as for the BF samples. The RIA was, however, used for bone defects larger than 2cm while the BF was used for defects smaller than 2cm. The samples were not necessarily harvested from the same patients.

As a control for the BF (BF-C), 5ml of whole blood was drawn from 5 healthy volunteers and used to completely soak the Cerasorb. The samples were left to incubate for 20 minutes at room temperature to closely mimic the circumstances during surgery. All the experiments in this study were performed in triplicates.

Cell culture

All samples were processed as soon as possible after the samples were harvested. For the osteogenic assay of the BF samples, a 6 mm biopsy punch was used to create uniform 3D sponges. For the osteogenic assay of the BF, the sponges were placed in a Nunc™ Cell-Culture Treated 24-well plate (Thermo Fisher Scientific, Waltham, USA) and cultured in mineralization medium containing αMEM, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Pen/Strep), 1% Ascorbic Acid 2-phosphate (ASAP), 1% GlutaMAX, 1% β-Glycerophosphate (BGP) and 0.1% Dexamethasone for 7, 14 and 21 days. After each endpoint, the experiments described below were performed.

The BMAC samples were homogenized, counted and transferred to a T75 cell culture flask (VWR, Radnor, USA). They were

expanded in expansion medium containing DMEM and GlutaMAX (Thermo Fisher Scientific, Waltham, USA) and 10% FBS until they were fully confluent.

The RIA samples were put into T25 cell culture flasks and expanded in expansion medium. When they reached confluency, they were transferred to a T75 flask. Samples were expanded until passage 2, after which they were used for the experiment or frozen for further experiments.

For the osteogenic assay of the BMAC and RIA samples were seeded at a density of 10.000 cells/cm^2 in a Nunc™ Cell-Culture Treated 24-well plate. All samples were cultured until fully confluent after which half of the samples changed to mineralization medium (mineralization group), the other half was kept on expansion medium (expansion group). Samples were cultured for 7, 14 and 21 days. After each endpoint, all the experiments described below were performed.

Sample processing and RNA extraction

To examine the gene expression of the BF on day 0, the BF and BF-C samples were cut into small fragments using a sterile scalpel and transferred to 2ml microcentrifuge tubes (Biotix, San Diego, USA) using sterile tweezers directly after receiving the samples from the operating room. After the samples were snap frozen in liquid nitrogen, they were disrupted by adding 1 ml of TRIzol Reagent (Thermo Fisher Scientific, Waltham, USA). The mixture was lysed for 5 minutes with a Qiagen TissueLyser LT (Qiagen, Venlo, The Netherlands).

To examine the gene expression of the cultured samples, the BF, RIA and BMAC samples from the cell culture from day 7, 14 and 21 were disrupted by adding 1 ml of TRIzol. The mixture was transferred to a 2 ml microcentrifuge tube and, after incubating on ice for 15 minutes, transferred to -80°C until further analysis.

RNA isolation was performed using the chloroform phenol method, using GlycoBlue co-precipitant (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. The Biodrop µLITE (Biochrom, Cambridge, UK) was used to determine mRNA concentration.

Quantitative PCR for osteogenic genes

The acquired mRNA was transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA) according to the manufacturer's instructions. Depending on the initial concentration of mRNA, either 200 or 500 µg of RNA was transcribed to cDNA. For the quantitative PCR, the following osteogenic marker genes were used: runt related transcription factor 2 (RUNX2), osterix (OSX), osteopontin (OPN) and osteocalcin (OCN). As a housekeeper gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. The specific primers are listed in table 1, all of which were validated beforehand.

PCR measurements were performed using the CFX96 Real-Time PCR system (Bio-Rad, Hercules, USA) and the gene expression was determined by the cycle number (Ct). The quality of the experiments was assessed by a melt curve analysis. A Ct cut-off value of 35 was chosen and any mRNAs with a Ct value of over 35 were not included in further analysis due to negligible or absent presence.

Table 1: Human quantitative PCR primers osterix (OSX), runt related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Primer	**Forward Primer 5'→3'**	**Reverse Primer 5'→3'**
OSX	CCTCTGCGGGACTCAACAAC	AGCCCATTAGTGCTTGTAAAGG
RuNX2	TCAACGATCTGAGATTTGTGGG	GGGGAGGATTTGTGAAGACGG
OPN	CTCCATTGACTCGAACGACTC	CGTCTGTAGCATCAGGGTACTG
OCN	CCAGCGGTGCAGAGTCCAGC	GACACCCTAGACCGGGCCGT
GAPDH	GGCTGAGAACGGGAAGCTTGTCAT	CAGCCTTCTCCATGGTGGTGAAGA

ALP assay

Medium was aspirated from the wells at the designated observation times. All cells and sponges remained in the wells until the incubation was finished. The wells were washed twice with phosphate-buffered saline (PBS). 500µl of AP substrate solution (4-Nitrophenyl phosphate di-sodium salt hexahydrate and AP buffer) was added to each well. As a negative control, 500µl of AP substrate solution was added to empty wells. The mixture was incubated for 30 minutes at 37°C after which 100µl of the reaction mixture was transferred to a 96-well plate. The absorbance was measured at a wavelength of 405nm with a CLARIOstar platereader (BMG LABTECH, Ortenberg, Germany) and the results were normalized to a standard curve.

Alizarin Red staining

The Alizarin Red staining was only performed on the BMAC and RIA samples. Medium was aspirated from the wells and the cells were gently washed once with PBS. To fix the samples, 1 ml of 4% formaldehyde was added to the wells. After incubating for 15 minutes at room temperature, the fixative was removed and the cells were washed 3 times with distilled water. Subsequently, 1ml of 40 mM Alizarin Red solution (Alizarin Red S (Sigma-Aldrich, Saint Louis, USA) with distilled water) was added to each well. The mixture was incubated at room temperature for 15 minutes while gently shaking. After incubation, the dye was removed and the cells were washed with distilled water until the washing liquid was colourless. After removing all the water, pictures were taken. The plates were stored at 4°C with distilled water until the staining was quantified.

For the quantification of the staining, 143.3 mg of sodium phosphate (Sigma-Aldrich, Saint Louis, USA) was dissolved in 100ml of distilled water. To this solution, hexadecyl pyridinium chloride (HPC) was added to acquire a 10% HPC solution. Water was removed from the stained wells and 400µl of the HPC solution was added. The mixture was incubated for 20 minutes or until all the staining was dissolved. 100µl of the dissolved solution was transferred to a 96-well plate and the absorbance was measured at a wavelength of 562nm on the CLARIOstar platereader and the results were normalized to a standard curve.

Flow cytometry

The BF and BMAC samples (before and after centrifugation with the Arthrex system) were processed as fast as possible after receiving the samples from the operating room. The strip of Cerasorb was transferred to a petri dish and the same 6mm biopsy punch as used for the cell culture was used to create uniform sponges. The sponges were washed twice with PBS to remove non-adherent cells and other debris. To each 15ml tube, 3 sponges were transferred after which they were completely covered with 2.5mg/ml Collagenase D solution (Collagenase D powder (Sigma-Aldrich, Saint Louis, USA) and PBS) to detach the cells from the sponges. After gently shaking up and down to make sure the whole sponge was covered, the tubes were transferred to a 37°C waterbath to incubate for 30 minutes. The tubes were centrifuged for 5 minutes at 300rcf and the supernatant was carefully removed. The detachment protocol was performed twice.

After the second detachment protocol, the pellet was carefully resuspended in PBS and ran through a 100µm cell strainer. The cell strainer was flushed twice with 10ml of PBS into a 50ml tube. The filtrate was run through a cell strainer again and the tubes were centrifuged at 300rcf for 5 minutes. The pellet was resuspended in 7.5 ml 1X Red Blood Cell Lysis Buffer (10X Red Blood Cell Lysis Solution (Miltenyi Biotec, Bergisch Gladbach, Germany) with Cell culture water (Westburg, Leusden, Netherlands)) and after incubating for 10 minutes at room temperature, the mixture was centrifuged for 8 minutes at 300rcf. The supernatant was removed and the cells were resuspended in 1ml of FACS buffer, finally, cell counting was performed.

For the staining of the cells, the Human Mesenchymal Stem Cell Verification Flow Kit (R&D systems, Minneapolis, USA) was used according to the manufacturer's instructions. In short, cells were transferred to a 5ml round bottom tube and stained with either 10µl of all 3 of the positive markers (CD90, CD73 and CD105), 10µl of the negative marker cocktail (CD45, CD34, CD11b, CD79A and HLA-DR), 10µl of all of the positive isotype controls, 10µl of the negative isotype control cocktail or left unstained. The samples were incubated for 45 minutes at room temperature in the dark after which they were centrifuged and washed once with staining buffer. After washing, the cells were resuspended in staining buffer containing 1% formaldehyde to fix the samples. 24 hours later, the fixed samples were washed again and resuspended in 300µl of staining buffer. The samples were stored on 4°C in the dark until the measurements were performed. The flow cytometry measurements were performed on a BD FACSCanto (BD Biosciences, Franklin Lakes, USA).

Statistical analysis

All data are expressed as ± standard error of the mean. Because of the low sample size, only descriptive statistics are provided. Because of a lack of control for the qPCR, the qPCR data are represented as ΔCt ($Ct_{gene\ of\ interest}$ -$Ct_{housekeeper\ gene}$) values where the lower values represent a higher expression and vice versa. To compare the different groups, the dynamic gene expression is examined.

Results

Sample processing and cell culture

In total, 10 BF samples were collected during non-union surgery. Of these 10, 5 were used for the gene expression on day 0 and 5 samples were used for the cell culture and analysed on day 7, 14 and 21. For the control, blood from 8 healthy volunteers was used. 3 of which was used for the ALP assay on day 7, 14 and 21 and 5 samples were used for PCR on day 0. 2 BMAC samples (BMAC 1 and BMAC 2) and 1 RIA sample were used for cell culture and analysis on day 7, 14 and 21. 1 of the BF samples was used for the Flow Cytometry measurement together with one BMAC sample.

Quantitative PCR for osteogenic genes

BoneFlo vs BoneFlo control samples, day 0

None of the BF-C samples (N=5) displayed any osteogenic marker gene expression. In the BF samples (N=5), in comparison to the housekeeper gene, an expression of all the osteogenic marker genes was observed. As shown in figure 1, the lowest expression (shown as ΔCt) was for RUNX2, the other genes showed similar higher levels of expression.

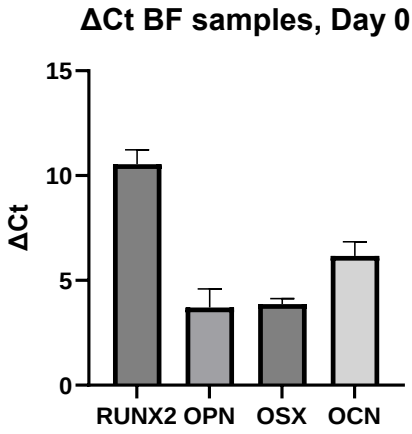

Figure 1: ΔCt values of the BF samples on day 0, the lower values represent a higher expression

BoneFlo samples vs BMAC and RIA

The dynamic gene expression was investigated and displayed in figure 2. A few observations can be made about the dynamic expression profile. Most importantly, all markers showed expression when normalized to the housekeeper gene in all the groups at all timepoints. The RUNX2 expression in all the groups remained consistent during the entire experiment. The ΔCt values of the relative gene expression of RUNX2 in the BF group and the RIA group were similar. The OSX expression showed a similar expression profile in all the groups with a decreased expression on day 14. There was a difference between

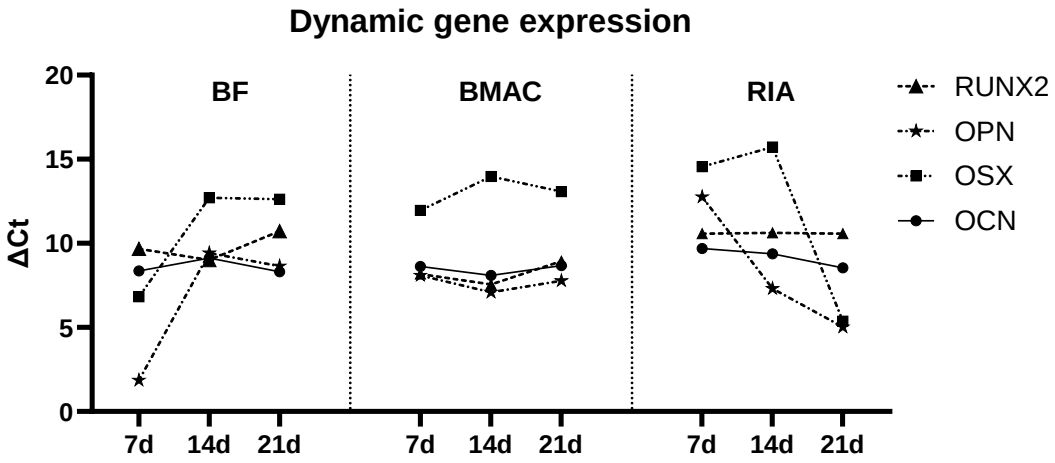

Figure 2: the mean dynamic gene expression of the BF, BMAC and RIA groups, the lower values represent a higher expression

BMAC and RIA, the BF expression was in between those. OCN showed an expression in the same order of magnitude in all groups. Overall, the expression of OSX was lowest in the BMAC group when compared to the other genes, whereas the OSX expression in the BF and RIA group showed an expression similar to the other genes. OSX and OPN showed a peak in expression on day 7 in the BF group, the RIA group showed a peak on day 21. The BMAC group stayed consistent overtime.

ALP assay

5 BF samples and 3 BF-C samples were used for the ALP assay (figure 3). On average, the ALP concentrations in the BF-C group were 54.2±5.7, 54.6±5.3 and 28.7±0.8µM on day 7, 14 and 21 respectively. The concentrations in the BF group were much higher: 145.4±85.8, 250.9±77.6 and 404.0±107.9µM respectively.

ALP assays were also performed on 2 BMAC samples and 1 RIA sample. The ALP concentrations in the BMAC group were 402.6±26.4, 351.7±17.8 and 322.2±18.3µM for the expansion group on day 7, 14 and 21 and 417.9±19.3, 507.5±77.4 and 845.1±46.0µM in the mineralization group respectively. For the RIA expansion group, the concentrations were 54.4±1.3, 70.4±1.6, 116.0±6,4µM and for the mineralization group 243.0±32.5, 488.5±60.2 and 1003.8±44.3µM for day 7, 14 and 21 respectively.

Alizarin Red staining

Two BMAC samples and one RIA sample were used for the Alizarin Red staining. The staining was successful and overall, the staining seemed to visually increase each week on all the samples. Pictures were taken with 4x magnification some are shown in figure 4. The

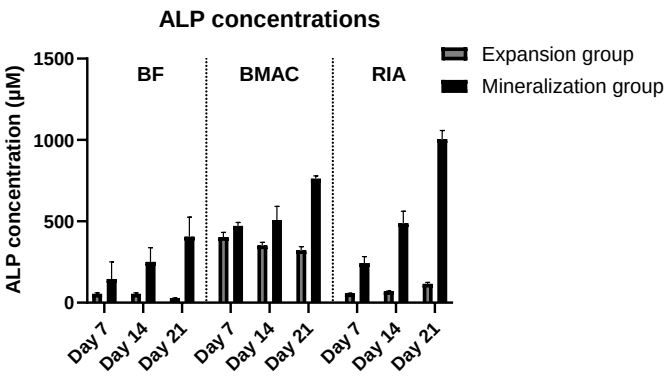

Figure 3: the ALP concentrations in µM of the BF, BMAC and RIA group on all the different endpoints. The ALP activity was measured in the expansion group and the mineralization medium group.The BMAC group starts the highest and stays the most consistent. The RIA group increases the most and is the highest on day 21. The BF group shows the lowest expression

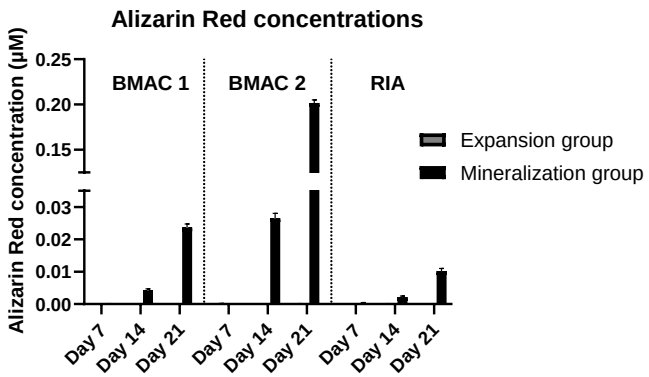

Figure 5: The Alizarin Red concentrations in µM of the 2 BMAC (BMAC 1 and BMAC 2) and RIA samples. The BMAC samples display a way higher concentration than the RIA sample. Measurements were performed on the expansion group and the mineralization group.The BMAC 2 sample shows the highest concentration, the RIA sample the lowest. All values on day 7 are similar.

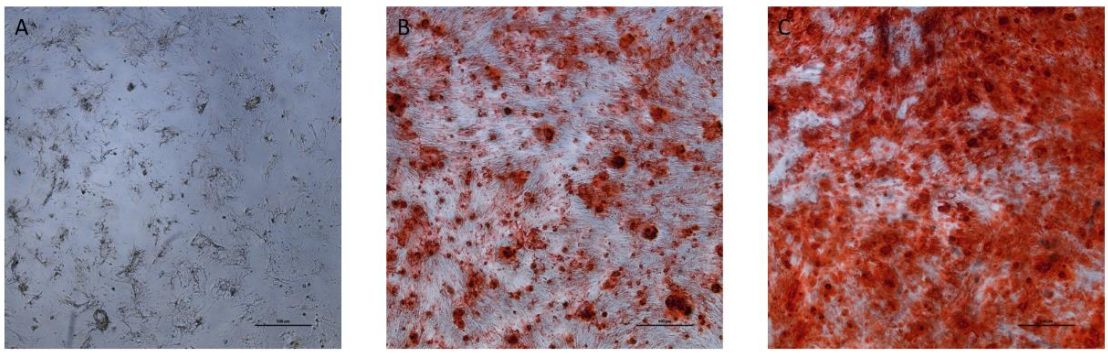

Figure 4: Pictures of the Alizarin Red staining of different groups. A is BMAC 1 on day 7, B is RIA on day 14 and C is BMAC 2 on day 21

Alizarin Red concentrations were examined for 2 BMAC groups and 1 RIA group (figure 5). The average Alizarin Red concentrations are displayed in table 2 to give a clear overview of the values. Both BMAC groups display a higher concentration than the RIA group. In all the groups, the mineralization groups has a higher concentration than the expansion groups on day 14 and 21. The concentrations on day 7 are similar in all groups. The BMAC 2 group has the highest concentration. In all the mineralization groups, the concentration keeps increasing overtime.

Flow Cytometry

The cell isolation was successful. Before the staining, a count was performed on the BF samples. In total, from 9 sponges, 550.000 cells were counted. The staining was performed on one BMAC samples (before and after centrifugation) and one BF sample. None of the positive markers (CD73, CD90, CD105) showed expression in any of the samples. In all the samples, the negative marker control cocktail was expressed.

Discussion

This study examined the osteogenic capacity of a graft material obtained with a novel surgical suction filter device by determining the gene expression, ALP activity, mineralization and cell composition. The increased expression when compared to the control samples shows that this device may be a promising method of acquiring a bone graft substitute that has a considerable osteogenic capacity.

Cell culturing

Medium and passage number

First of all, the mineralization medium contained αMEM while the expansion medium contained DMEM. Research done by Coelho et al. showed that osteoblastic bone marrow cells cultured in αMEM were superior in ALP activity and the ability to form mineralized deposits than cells cultured in DMEM. The ALP activity was dependent on the type of medium and the serial subculturing. (20) This study also shows the importance of passaging the cells before using them for the experiments. Cells with a higher passage number showed significantly lower osteogenic potential. A

Table 2: The Alizarin Red concentrations on day 7, 14 and 21 day in µM

	BMAC 1		BMAC 2		RIA	
	Expansion group	Mineralization group	Expansion group	Mineralization group	Expansion group	Mineralization group
Day 7	1.0×10^{-4} $\pm2.0\times10^{-5}$	1.0×10^{-4} $\pm2.0\times10^{-5}$	2.0×10^{-4} $\pm3.0\times10^{-5}$	4.0×10^{-5} $\pm4.0\times10^{-6}$	8.0×10^{-5} $\pm1.0\times10^{-5}$	3.0×10^{-4} $\pm2.0\times10^{-4}$
Day 14	3.1×10^{-5} $\pm1.0\times10^{-5}$	4.3×10^{-3} $\pm4.0\times10^{-4}$	1.0×10^{-4} $\pm2.0\times10^{-5}$	2.7×10^{-2} $\pm1.5\times10^{-3}$	1.5×10^{-4} $\pm2.0\times10^{-5}$	2.2×10^{-3} $\pm3.0\times10^{-4}$
Day 21	1.0×10^{-4} $\pm1.0\times10^{-5}$	2.4×10^{-2} $\pm1.1\times10^{-3}$	1.0×10^{-4} $\pm4.0\times10^{-5}$	2.0×10^{-1} $\pm3.4\times10^{-3}$	1.6×10^{-4} $\pm2.0\times10^{-5}$	1.0×10^{-2} $\pm8.0\times10^{-4}$

study by Yang et al. also showed the influence of aging on the differentiation potential. The osteogenic potential significantly decreases in passage 8 cells in comparison to passage 4 cells. The use of a αMEM based medium might yield a larger osteogenic potential in comparison to a DMEM based medium. (21) Another study performed by Sotiropoulou et al. also found that a αMEM based medium was more suitable for the expansion of hMSCs derived from the bone marrow compared to a DMEM based medium. (22)

2D vs. 3D culture

In this study, the BF samples were cultured in Cerasorb, a collagen based, β-TCP containing sponge. The BMAC and RIA samples, however, were cultured in 2D on a treated well plate. While 2D cell culturing has predominated in the past, 3D cell culturing is upcoming in the literature because it represents a more realistic biochemical and biomechanical environment. Increasing the dimensionality of the ECM significantly impacts the cell proliferation, differentiation, mechano-response and cell survival. (23) A study by Anerillas et al. showed that 3D culturing in a collagen-based hydrogel promotes MMP13 expression, thus suggesting that 3D culturing increases osteogenesis in comparison to 2D cultures. (24)

Seeding density

In this study, the BMAC and RIA samples were seeded at a density of 10.000 cell/cm^2. A study by Zhou et al. explored the influence of seeding density of umbilical cord stem cells on a scaffold and found that a higher seeding density leaded to increased cell proliferation, osteogenic differentiation and bone mineral synthesis. When the seeding density was too high, the bone marker gene expression and mineralization slightly decreased however. (25) Other studies also use a seeding density of 10.000 cells/cm^2 for osteogenesis or seed the cells at lower density in expansion medium and change to mineralization medium when the cells reach confluency. (31,32)

Recommendations

This study had a lot of variables in the cell culturing. When undergoing further research on this topic, a few specifications should be made to enhance the scientific integrity. A control medium with αMEM would make for a better control group. Another important improvement would be to culture the RIA and BMAC samples in Cerasorb with the same seeding density as the BF group. In such manner, the osteogenic potential of the BF samples could better be compared to the other groups.

ALP and Alizarin Red staining

As stated above, the proliferation, ALP deposition and matrix mineralization strongly depend on the culturing conditions. When making sure the conditions in all the groups are more alike, better ALP and Alizarin Red experiments can be performed. In this study, however, Alizarin Red quantification could also have been performed on the BF samples. Due to a lack of time and expertise, this was not done. Further research should focus on the mineralization of the BF group.

Osteogenic gene expression

Because of a lack of comparability in the BF group vs the RIA and BMAC group due to the different culturing methods and different amounts of cells, we looked at the dynamic expression profile of the osteogenic genes instead of looking at the relative gene expression. It is important to know which genes show expression during which phase of osteogenesis to compare the groups to any extent. RUNX2 is considered a master transcription factor and is upregulated in preosteoblasts and reaches optimal expression in immature osteoblasts. Afterwards, it is downregulated in mature osteoblasts. It is thus essential in the early differentiation. (26,28) OSX (also known as SP7) is a downstream target of RUNX2 and is known to mediate both the commitment of hMSCs to the osteoblast lineage and the further differentiation with the expression of OCN and Col1α1. (27) OPN and OCN are major non-collagenous proteins (NCP's) involved in matrix organization and deposition (29) The levels of OPN and OCN are highest closest to the mineralized ECM since they are important components of the developed bone ECM. (30)

Generally, the expression RUNX2 increases during the formation of bone. In this study, this is not visible, the RUNX2 expression remains the same during the 3 weeks of this experiment. However, this is observed in all the groups which leads us to believe that the BF samples are indeed undergoing osteogenesis. In all the groups, the OSX expression is the lowest in day 14, which similar as what is described in the literature. It is shown to be expressed during the commitment of hMSCs to the osteogenic lineage and the further mineralization of the matrix. This suggests that all of the samples undergo differentiation at the start of the experiment and are mineralizing at the end, this is in line with the results of the ALP assay and Alizarin Red staining. The RIA group is the only group that shows an increase in OPN expression, suggesting that this group is furthest with the matrix formation. An increase in OCN expression was expected in all the groups, this is not the case. An explanation for this could be that the 21 days is too early to detect any significant increase in OCN expression. The divergent expression patterns in the BF group could be explained by the fact that these cells are obtained at the site of surgery, of which, not much is found in the literature.

Recommendations

Firstly, more should be known about the contents of the samples, especially the BF samples, to further understand the exact ongoing mechanisms in the samples. Due to the different culturing conditions and a lack of control due to a shortage of time, the samples are not strictly comparable. To enhance comparability, it would be a good option to culture the BMAC and RIA samples in the same sponge as the BF samples. This way, the samples would be cultured exactly the same and a direct comparison between the BF, RIA and BMAC can be made. Longer culture time could also show a larger difference in the OCN and OPN expression between the groups since these genes come up during a later part of the mineralization process, this could tell us something about the healing capacity of the materials during the later stages of the recovery.

Flow Cytometry

Isolation of the hMSCs from the Cerasorb was successful, as indicated by the fact that a cell count could be performed and the presence of the hMSCs was visually confirmed. However, most Flow Cytometry protocols advise to use fresh samples in order to optimize the use of surface markers for cell quantification and identification. However, due to time restrictions, the use of fresh samples was not possible in this study. If fixation is needed, the protocols advise to fixate the samples shorter than 24 hours and use them within 1 week after fixating. In addition to this, more washing steps are also advised. Although this is not yet proven, we suspect that due to the fixation step, the expression of the surface markers was influenced. The expression of the negative markers suggests the presence of leukocytes and hematopoietic stem cells. The exact cell type is unknown. The reason why the negative markers did show expression and the positive markers did not is unknown and needs further investigation.

Recommendations

A lot of optimizations have to be done to perfect the flow cytometry measurements. It would be preferred to do the measurements as soon as possible after staining the samples without fixating. The length of the fixation and the extraction methods need to be optimized. Shorter fixation time, different concentrations of PFA offer the most promising solution.

Conclusions

This study has shown a clear osteogenic capacity in the BoneFlo-derived graft material. In comparison to the BF-C, the BF samples express elevated levels of osteogenic marker genes and have a higher ALP activity. Moreover, the dynamic expression of the genes in osteogenic assays are comparable to the alternatives, BMAC and RIA. This indicates that the BoneFlo is a promising surgical tool to acquire bone grafts. Extension of the sample size is needed to determine the inter-personal variability.

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