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Research Article

Improved Bone Formation by Differentiated Mesenchymal Stem Cells and Endothelial Progenitor Cells Seeded on High Concentrated Bioglass-Polylactic Acid Composite in Calvarial Rat Bone Defect

Karam Eldesoqi[1,2], Dirk Henrich[1]*, Abeer M El-Kady[2], Bothaina M Abd El-Hady[2], Karima M Sweify[3], Borna Relja[1], Christoph Nau[1], Ingo Marzi[1] and Caroline Seebach[1]*

[1]Department of Trauma Surgery, Johann-Wolfgang-Goethe University Hospital, Frankfurt, Germany

[2]Department of Biomaterial, National Research Centre, Al-Bohous St. Dokki, Cairo, Egypt

[3]Department of Zoology, Women's College for Arts, Science and Education, Ain Shams University, Cairo, Egypt

Abstract

Objective: New developed composite biomaterials with a Bioglass (BG) and Polylactic Acid (PLA) component are promising candidates for the treatment of bone defects. There is evidence that adding Mesenchymal Stem Cells (MSC) and Endothelial Progenitor Cells (EPC) significantly improve new bone formation. Thus, cell adhesion, cell viability and bone formation of these composites, when seeded with undifferentiated or differentiated progenitor cells, respectively were tested.

Materials and methods: We investigated newly developed composite material consisting of Polylactic Acid (PLA), PLA and 20% Bioglass (PLA+BG 20%) or PLA and 40% Bioglass (PLA+BG 40%). These materials were seeded with either undifferentiated MSC / EPC or differentiated MSC / EPC and tested for cell adhesion and cell viability *in vitro*. Moreover, these composites were evaluated for bone formation *in vivo*. A Critical Size Defect (CSD) was made in each calvarium of 76 rats and composites were implanted. Animals were sacrificed after 14 weeks. Formation of new bone was evaluated by histomorphometry.

Results: Cell adhesion and cell viability *in vitro* is not significantly influenced by our tested composites, but differentiated MSC/EPC seeded onto PLA+BG40 improve significantly bone formation in a calvarial rat bone defect *in vivo* and represent a novel cell-based therapy for bone regeneration.

Keywords: Bioglass, Bone tissue engineering, EPC, MSC, PLA

Introduction

Over 2 million bone graft materials are used every year worldwide, thus bone is second only to blood on the list of transplanted materials. With increasing demand and known limitations with traditional bone graft materials, new approaches are developed to provide alternatives for bone regeneration [1-3].

Bone tissue engineering tries to mimic the physiologic situation [4]. The addition of osteogenic and angiogenic cells to a synthetic biomaterial increases their local density and rely on locally secreted growth and differentiation factors to induce bone formation. The biomaterials should present good biocompatibility for cell adhesion and cell viability [5,6] as well as controlled degradation kinetics to match the ratio of replacement by new tissue. Also, the biomaterials should provide an initial biomechanical support until cells generate the extracellular matrix [7,8].

Bioactive Glasses (BG) are a subset of inorganic bioactive materials, which are capable of reacting with physiological fluids to form tenacious bonds to bone through the formation of bone-like hydroxylapatite layers and the biological interaction of collagen with the material surface [9,10]. It has been found that reactions on BG surfaces lead to the release of critical concentrations of soluble Silicon (Si) and Calcium (Ca) ions, which induce favourable intracellular and extracellular responses leading to rapid bone formation [11]. Although BG has traditionally been employed for its osteoconductive and osteostimulative properties, BG also exhibit proangiogenic potential *in vitro* and *in vivo*. Soluble dissolution products of BG up-regulate the production of numerous angiogenic factors by stimulated cells providing a potentially promising strategy to enhance early vascularisation and resultant bone formation [12-15].

However, BG, compared to cortical and cancellous bones, usually present low mechanical properties, especially in porous forms [16,17]. This disadvantage significantly limits the use of these materials in a very broad range of applications. Fortunately, one solution came from mimicking nature, which provides the inspiration to design materials with optimal organized structures under dynamically changing conditions. Many of these structures are composed of an intrinsically complex matrix based on organic and inorganic components which produce a natural hybrid material, usually referred to as composites. By combining two or more materials in a predesigned manner, a biomaterial can be created with properties that are not possible to be attained when considering each of the individual components separately [18].

***Corresponding author:** Caroline Seebach, Department of Trauma Surgery, Johann-Wolfgang-Goethe University Hospital, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany, Tel: +49 6963017110; Fax: +49 6963017108; E-mail: caroline.seebach@kgu.de

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Synthetic polymers (e.g., Poly-Lactide Acid (PLA)) have numerous advantages, such as excellent processing characteristics, which can ensure the off-the-shelf availability as well as being biocompatible and biodegradable at rates that can be tailored for the intended application [19,20]. Additionally, synthetic polymers possess predictable and reproducible mechanical and physical properties (e.g., tensile strength, elastic modulus, and degradation rate) and can be manufactured with great precision [21].

Thus, PLA/BG composite biomaterials present an ideal clinical solution to the limitations of traditional bone graft. But the optimal composition of PLA/BG composite for cell adhesion, cell viability and bone tissue engineering is still unknown.

Therefore, we prepared and investigated three different compositions of PLA/BG composite: Polymer (PLA), PLA+BG 20% and PLA+BG 40% (the glass content is 0, 20 and 40 wt% respectively) for bone tissue engineering *in vitro* and *in vivo*.

Moreover, recent investigations of our laboratory have focused on implantation of undifferentiated MSC and EPC seeded onto Tricalciumphosphate (TCP) which demonstrated enhanced bone regeneration and improved vascularization of critical size bone defects [22-24]. In these previous studies EPC demonstrated real angiogenic contribution. In this context it is unknown if differentiation of MSC/EPC can enhance bone formation. Thus, we hypothesized that the localized delivery of differentiated MSC/EPC onto PLA/BG composite enhance bone formation and promote bone healing in a critical-sized calvarial bone defect in rats.

The specific aims of this study were twofold:

1. To compare the osteogenic potential of various concentration of bioglass in the composite: PLA; PLA+BG 20%, PLA+BG 40%
2. To determine the osteogenic potential of undifferentiated MSC/EPC versus differentiated MSC / EPC.

Materials and Methods

Ethic statements

All animal experiments were performed in accordance with the institutional animal care and oversight committee (Project No. F3/22; Regierungspräsidium Darmstadt, Germany). All efforts were made to minimize suffering.

Characteristics of composite biomaterials: PLA, PLA+BG 20 and PLA+BG 40

Depending on the rate of burning, we created a new particle size of Bioglass (BG). The composite biomaterial consists of a PLA-component supplemented with increasing amounts of BG. Tetraethyl orthosilicate (TEOS, ≥ 99%) and Nitric acid 65% were supplied by Merck Chemicals KGaA, Darmstadt, Germany. Calcium nitrate [Ca $(NO_3)_2$-$4H_2O$, ≥ 99%], Poly (L-lactide) and Chloroform ($CHCl_3$, ≥ 99.4%) were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. All chemicals were reagent grade and were used as received without further purification. For the synthesis of BG CaO-SiO_2 (SiO_2 80mol-%-CaO 20mol-%), low viscosity gel was obtained by mixing 31 mL of Tetraethyl Orthosilicate (TEOS) and 8.6g of $Ca(NO_3)_2.4H_2O$ in a solution of 5.5mL of HNO_3 2M, used as catalyst, in 31.5 mL of H_2O. The initial pH of hydrolysis was 0.5. The BG was cast at room temperature in Teflon container (Thermo Scientific Nalgene, Germany) until the gel was formed. Aging was performed at 60°C for 3 days. Drying was carried out at 120°C. The glass was collected in laboratory porcelain crucible (Haldenwanger GmbH, Waldkraiburg, Germany) after that was burned in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany) at rate 3°C/1 minute till 700°C, then 700°C for 3 hours. The glass particles were grinded in small porcelain mortal (Haldenwanger GmbH, Waldkraiburg, Germany) to form glass powder. Finally, Bioglass particles sizes were sieved to be in the range of 106-125µm by Test sieves (Retsch GmbH, Haan, Germany).

Composite biomaterials were prepared by mixing polymer [poly(L-lactide) (PLA)] and Bioglass (BG) with 10 ml chloroform as follows: PLA, PLA/BG 20% (PLA+BG 20%) and PLA/BG 40% (PLA+BG 40%) biomaterial. The bioglass content was 0, 20 and 40 % by weight. These biomaterials will be referred to as PLA, PLA+BG 20% and PLA+BG 40%. Disc shaped specimens with a diameter of 5 mm and a thickness of 1 mm were cut and stored at room temperature under sterile conditions until use.

Cell isolation and preparation of rat Endothelial Progenitor Cells (EPC) from rat spleen

Rat spleens (donor rats; n=7) were cut in tiny pieces and gently rubbed. Viscous solution were resuspended in Phosphate Buffered Saline (PBS) and filtered through 100mm, 70mm and 40mm mesh (BD-Bioscience, Heidelberg, Germany). Subsequently, the cell suspension was layered on a Ficoll density gradient (1.077g/mL; Biochrom, Berlin, Germany) and density gradient centrifugation (30min, 900g). Cells were washed twice with cold PBS (10min, 900g), and 4×106 cells were cultivated on a fibronectin-coated (10mg/mL; Sigma, Deisenhofen, Germany) 24-well culture dish in 1mL of endothelial basal medium (Cambrex, Verviers, Belgium) supplemented with endothelial growth medium (Cambrex) at 37.8°C. After 48h, nonadherent and weakly adherent cells were removed, the medium was changed. The cells were cultivated for an additional 72h. A parallel preparation was performed to evaluate the percentage of endothelial cell-like differentiated cells. EPC were identified using the method previously described [37]. Briefly, cells were incubated for 1h with 2.4mg=mL DiLDL (Cell-Systems, St. Katharinen, Germany) in EBM supplemented with 20% FCS. Cells were fixed with 2% paraformaldehyde for 10min, and after washing with PBSþ=þ, FITC-labeled Ulex europaeus agglutinin-1 [10 mg=mL] (lectin; Sigma) was incubated for 1h. Cells presenting double-positive fluorescence were considered to be EPC. Only preparations with a percentage of endothelial-like differentiated cells greater than 80% were used. For the experiments the cells were detached by incubation (10min) with accutase (PAA-laboratories, Linz, Austria), washed once with MesenCultþ Supplements (Cell-Systems), and subsequently adjusted to a density of 2.5x10^5 cells in 100mL.

Cell isolation of rat Mesenchymal Stem Cells (MSC) from rat femur

Mesenchymal stem cells were isolated from rat femurs (donor rats; n=3). Bone marrow aspirate was washed once using PBS. The pellet was resuspended in PBS and layered on a Ficoll density gradient (d = 1,077g/mL, Biochrom, Berlin, Germany).

After centrifugation (30min, 1100g) the cells in the interphase were collected and washed twice using PBS (10min, 900g) containing 2% Fetal Bovine Serum (FBS). The cells were resuspended in 3 ml DMEM/F-12 and Supplements (gibco® by life technologies, Germany) and were counted.

Differentiation of rat Mesenchymal Stem Cells (MSC)

MSC were incubated with osteogenic differentiation medium: DMEM/F-12 (gibco® by life technologies, Germany) medium, Supplements, dexamethason [1µM], ascorbic acid [50µg/ml] and β-glycerol phosphate [10 000µM] (Stem Cell Technologies, Germany) for 3 weeks. Extracellular calcium deposition was evaluated by van Kossa staining (Figure 1A and B).

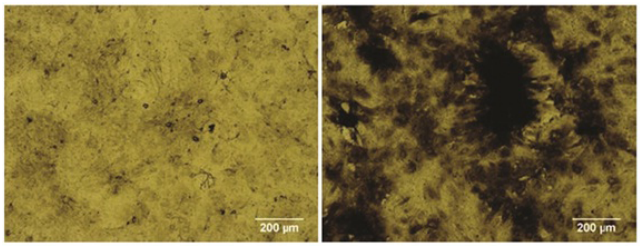

Figure 1A: Images of von Kossa staining of undifferentiated MSC and differentiated MSC.

Increased calcium deposition of differentiated MSC (right image) versus undifferentiated MSC (left image) obtained from the rat femur. Original magnification 50×, space bar indicates 200µm.

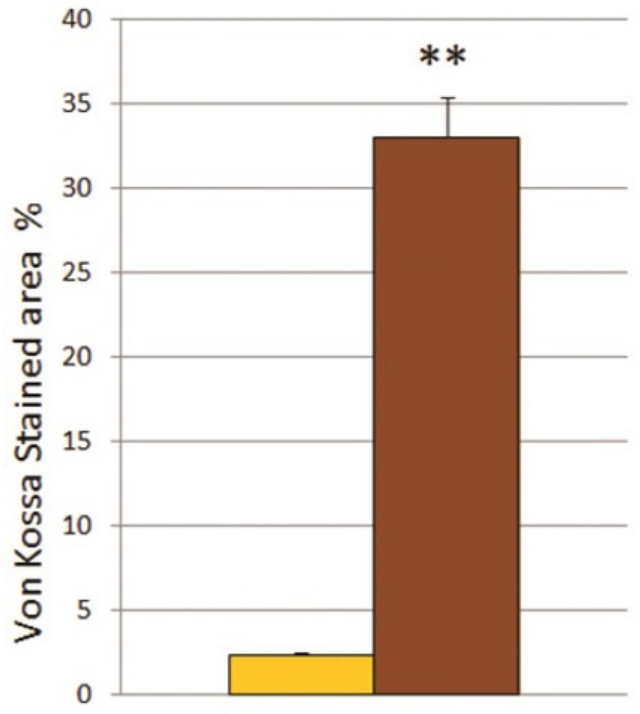

Figure 1B: Calcium deposition of undifferentiated MSC or differentiated MSC.

Analysis of von Kossa stained area [%] of undifferentiated MSC (yellow column) versus differentiated MSC (brown column).

** The mean difference is significant at the 0.01 level.

Cell seeding onto composite biomaterials

10µl medium containing 2.5x105 undifferentiated MSC and 2.5x105 EPC were dropped over one disk of biomaterial (PLA vs PLA+BG20 vs PLA+BG40), then cells seeded onto composite were incubated for one hour in CO_2 incubator at 37°C. This cell seeding procedure was also performed with differentiated MSC/EPC.

Scanning Electron Microscopy (SEM) of MSC and EPC onto composite biomaterials

Qualitative analysis of the morphology of adherent MSC and EPC were assessed by Scanning Electron Microscopy (SEM). The seeded biomaterials were fixed with glutardialdehyde for 30min and subsequently dehydrated by incubation of each 15min in a 4-step ethanol gradient. Then they were incubated overnight in 1,1,1,3,3,3-hexamethyldisilazane (Merck-Schuchardt, Hohenbrunn, Germany) and drained. Afterwards the samples were sputtered with gold (3_60s, Agar Sputter Coater, Agar Scientific Ltd., UK) using a Hitachi FE-SEM S4500 (Hitachi, Düsseldorf, Germany) with a voltage of 5kV. The images (Figure 2) were digitally recorded using the Digital Image Processing System 2.6 (Point Electronic, Halle, Germany).

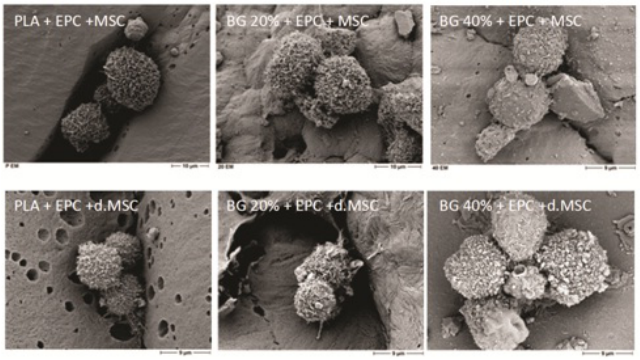

Figure 2: Cell adhesion and phenotype of MSC/EPC on the composite biomaterial.

Surface characteristics and direct proof of undifferentiated MSC+EPC (upper panel) and differentiated MSC+EPC (lower panel) on various biomaterials: PLA (first column); PLA+BG 20% (second column) or PLA+BG 40% (third column), respectively by SEM. The scale bar indicates 9µm and 10µm, respectively

Cell viability of MSC and EPC after seeding onto composite biomaterials

Cells (undifferentiated MSC/EPC and differentiated MSC/EPC) were detached by 10 minutes incubation with accutase (PAA-lab oratories), then washed (5minutes, 300g), resuspended in DMEM supplemented with 10% fetal calf serum (FCS; PAA Laboratories) and adjusted to a density of 500 x105 cells in 1ml medium.

In each well from 24 well plate 10µl medium which contains either 2.5x105 undifferentiated MSC and 2.5x105 EPC or 2.5x105 differentiated MSC and 2.5x105 EPC, respectively were dropped over the biomaterial and incubated in CO_2 incubator at 37°C for 1hour.

In order to stain MSC and EPC nuclei, cells were fixed with 3% paraformaldehyde for 10 minutes and after washing with PBS++(with calcium and magnesium), 1µL 40,6-Diamidino-2-Phenylindole (DAPI; Sigma-Aldrich, Deisenhofen, Germany; final concentration 1µg=mL) were added to each well followed by further incubation for 15 minutes at 37°C.

In order to detect EPC, cells were incubated for 1h with 2.9µg/mL 1,1=-dioctadecyl-3,3,3=,3=-tetramethylindo-ca bocyanine-labeled acetylated low density lipoprotein (DiLDL; Cell-Systems, St. Katharinen, Germany) in EBM supplemented with 20% FCS.

After three washes with PBS++, the biomaterials were subjected to fluorescence microscopy (Axio Observer; Zeiss, Göttingen, Germany)

in order to view DAPI-stained MSC as well as DiLDL/DAPI-stained EPC (Figure 3).

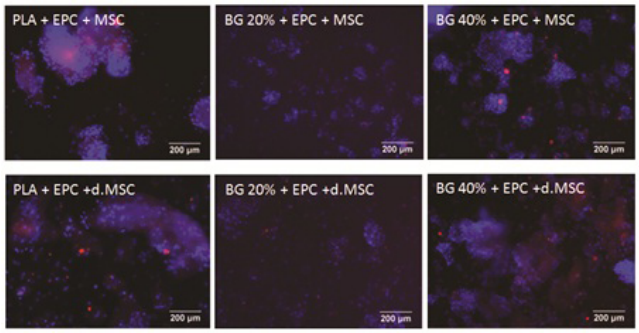

Figure 3: Fluorescence microscopy of co-cultured MSC and EPC onto composite biomaterials.

Fluorescence microscopy of co-cultured MSC and EPC adhering to three different biomaterials: PLA (first column), PLA +BG20% (second column), PLA+ -BG40% (third column). At day 1, staining of EPC (DiLDL, red) and cell nuclei of MSC and EPC (DAPI, blue), respectively, were performed. Endothelial-like differentiated cells were stained with both an orange-red and blue fluorescence, whereas cells without endothelial-like differentiation appear only blue, DAPI stained nucleus. The EPC uptake of DiLDL indicating endothelial cell differentiation.

The scale bar indicates 200µm.

Animals and cell transplantation

76 sixteen-week-old male albino (Sprague Dawely Strains) rats (Charles River, Germany) weighting approximately 350-450g were housed, four animals per cage under standardized conditions: 15-21°C, air flow, 12h light cycle, rat food and water ad libitum. The rats were randomly allocated to the experimental groups (Table 1).

Group	Biomaterial	Cells	Animals (n)
1	bone	--	6
2	Empty	--	6
3	PLA	--	6
4	BG 20	--	5
5	BG 40	--	6
6	PLA	EPC + MSC	8
7	PLA+ BG20%	EPC + MSC	8
8	PLA+ BG40%	EPC + MSC	7
9	PLA	EPC + d.MSC	8
10	PLA+ BG20%	EPC + d.MSC	8
11	PLA+ BG40%	EPC + d.MSC	8

Table 1: Group setup and number of animals per group.

PLA: Polymer (poly (L-lactide)); PLA+Bioglass 20% (PLA+BG20): composite of 80% PLA and 20% Bioglass; PLA+ Bioglass 40% (PLA+BG40): composite of 60% PLA and 40% Bioglass; EPC: Endothelial Progenitor Cells; MSC: Mesenchymal Stem Cells; dMSC: osteogenic differentiated MSC

A general anesthesia with a mixture of Ketavet and Rompun was given intraperitoneally. All efforts were made to minimize suffering. In order to create a CSD in skull, the head was shaved and cleaned with antiseptic fluid. A midlongitudinal incision was made on the dorsal surface of the cranium under aseptic conditions, and care was taken to ensure that the periosteum was completely cleared from the surface of the cranium by scraping. The skull cortex was drilled (X CUBE V2.0 drill, Avtec Dental, USA) using a 6 mm Trephine burr (VWR International GmbH, Darmstadt, Germany), so that a critical calvarial bone defect of 6 mm was created. The full thickness of the cranial bone was removed.

According to the experimental groups (Table 1) the composite implants were immediately placed in the defects. Some defects were left unfilled to confirm that the defect was critical sized. The inczision was closed with a continuous suture (4-0 nylon, Ethicon, Somerville, NJ). Animals had free access to food and water and were monitored daily in the postoperative period for any complications or abnormal behaviour.

The animals were sacrificed with an overdose of pentobarbital (150mg/kg intraperitoneal) and weighed after 14 weeks. The skull bone was dissected free and removed. Bones were wrapped in gauze moistened with physiologic PBS-solution and stored at -80°C until preparation for histomorphometrical examination.

Histomorphometry of bone formation

Skull bones were decalcified over 7 days in a 10% Tris buffered EDTA-solution under continuous stirring and embedded in paraffin in an established procedure of our lab. Sections (5µm) of the decalcified specimens parallel to the long axis of the head were stained with Hematoxylin and Eosin (H&E). All slides were analyzed using light microscopy (Axioobserver Z1, Zeiss, Göttingen, Germany) in combination with a computer-supported imaging picture analysis system (Axiovision 4.7; Zeiss, Göttingen, Germany). Histomorphometric assessments were performed by Image J to evaluate the new bone formation stained areas. Bone formation was counted in 6 non overlapping images/slide/animal surrounding the defect area and the mean value was calculated. These mean values were subsequently used for statistical analysis, which were examined in random order and blinded to the group setup.

Statistics

Results are presented as mean ± SD. Statistically significant differences were determined using F test (one way ANOVA and Post hoc Tests by SPSS program), statistical significance was achieved with $p<0.05$.

Results

Characterization of MSC and EPC by FACS analysis showed a typical pattern of MSC (CD34-, CD45-, CD71+, CD73+, CD90+, CD105+) and EPC (CD31, vWF, VEGFR-2) surface markers. Osteogenic differentiation in MSC was observed using von Kossa staining, while the induction of endothelial differentiation in EPC was evidenced by DilDL uptake and binding of UEA-1-lectin.

Differentiation of rat Mesenchymal Stem Cells (MSC)

Using von Kossa staining [area%] differentiated MSC (33.05 ± 2.33) demonstrated a significant higher calcium deposition in comparison to undifferentiated MSC (2.33 ± 0.12; Figure 1A and B).

Adhesion of cells onto composite biomaterials

At 2hours after cell seeding onto various biomaterials no significant differences in cell adhesion of undifferentiated MSC+EPC (Figure 2, upper panel) or differentiated MSC+EPC, respectively (Figure 2, lower panel) were observed. Neither various biomaterials (PLA, BG20%; BG40%) influenced cell adhesion *in vitro* (Figure 2).

MSC and EPC viability

Viability of undifferentiated MSC+EPC and differentiated MSC+EPC, respectively was investigated on all tested biomaterials (PLA; PLA+BG20%; PLA+BG40%). We could not detect any difference in cell viability on day 1 according neither differentiation of cells nor biomaterials (Figure 3).

Histology of bone formation

All specimens were included in this study at 14 weeks after surgery. No infection or delayed wound healing were observed.

When CSD remained empty, newly formed bone surrounded by an osteoid matrix rich in osteoblasts were only close to the borders of the surgical defect observed (Figure 4). The connective tissue in the central part of the defect was thinner than the original calvarium. It was well vascularized and rich in fibroplasts with oriented collagen fibers. Thus, in the histomorphometrical analysis (Figure 4) bone formed area [%] were evaluated in the empty defect (10.5 ± 4.2) of the skull bone. Cell based therapy with seeding of undifferentiated MSC+EPC to our three tested composites, only PLA+BG40% demonstrated a significant increase of bone formation (39.5 ± 12.1) compared to empty defect. PLA+MSC+EPC (24.6 ± 10.3) and PLA+BG20%+MSC+EPC (30.0 ± 8.1) showed bone formation with tendency to rise.

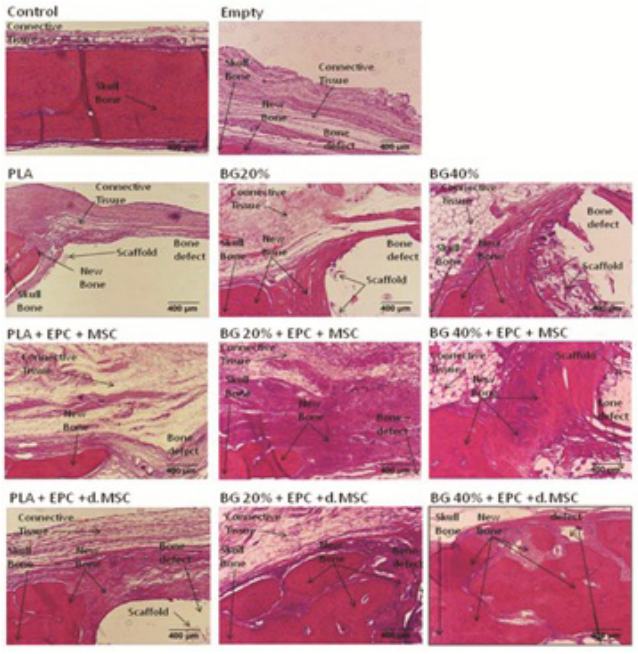

Figure 4: PLA+BG40%+dMSC/EPC increase bone formation in CSD after implantation.

Representative images of a histological sections (H & E staining) of the Critical Size Defect (CSD) in calvarial rat bone were demonstrated. Due to the decalcification process, the BG was almost completely dissolved and left a gap corresponding to the BG area. At the border skull bone was detectable.

The scale bar indicates 400µm.

Interestingly, when differentiated MSC+EPC were used for cell seeding to our three tested composites, all three experimental groups demonstrated significantly higher bone formation compared to control. The significant highest degree of new bone formation was observed in animals that received PLA+BG40% seeded with differentiated MSCs and EPC (49.2 ± 11.1). Well-developed newly formed bone was observed at the borders of the surgical defect and it extended toward the center of the defect in this group, thus a bony bridging over skull defects almost overall were done. Also high values were detectable when PLA+BG20% were seeded with differentiated MSC and EPC were implanted (42.0 ± 13.7). Less bone formation was observed in animals that received PLA with differentiated MSC and EPC (34.4 ± 11.6, figure 5).

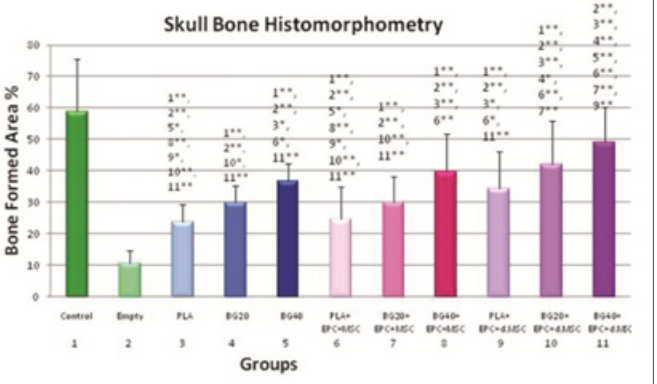

Figure 5: Quantitative analysis of Skull Bone Histomorphometry.

Bone formation area [%] in CSD increased significantly in animals when PLA+BG40 was used as biomaterials compared to PLA groups. Moreover significant bone formation area was evaluated in animals treated with PLA+BG40%+differentiated MSC/EPC, compared to PLA+BG40% alone.

* indicates significant level (0.05)

** indicates high significant level (0.01) compared to group number x

Discussion

In this study, we established a new developed bioglass-PLA-composite. Depending on the rate of burning, we created a new particle size of bioglass (106-125µm) for cell based therapy of a calvarial critical size defect in rats. Here we could demonstrate that PLA+BG40% seeded with EPC in coculture with differentiated MSC significantly improve bone formation in CSD.

Optimal biomaterials for bone tissue engineering should be biocompatible, biodegradable, possess an ideal porosity for cell attachment and cell integration, respectively as well as useful biomechanical stability. Single component materials do not meet all these requirements, thus composite biomaterials are needed.

PLA is highly biocompatible with a better thermal procedure, compared to other biopolymers. The main limitations of PLA are poor toughness, slow degradation and hydrophobic properties, which results in low cell affinity [25]. Pure bioglass is hard and brittle but offers a surface suitable for cell attachment. It is highly biodegradable and influences the local environment by releasing bioactive ions such as ionic calcium [26], which may lead to improved cellular responses at the implantation site [27].

Disadvantages of BG like lack of porosity occurred because it crystallizes during sintering. Recently, this has been overcome by understanding how the glass composition can be tailored to prevent crystallization [28]. Procedure developments have now allowed the production of bioactive glass polymer hybrids (composite of PLA and BG, e.g., PLA+BG40%) for bone regeneration which share load with bone and are not brittle under cyclic loads [26,29-32]. In several studies, bioactive glasses are reported to be able to induce the up-regulation of genes in bone cells and their effect in enhancing bone formation [33]. Due to their dissolution products bioactive glasses

stimulating osteoprogenitor cells at the genetic level and bond with bone more rapidly than other bioceramics [11,34,35]. Moreover, early vascularization is a prerequisite for successful bone healing and Endothelial Progenitor Cells (EPC), seeded on appropriate biomaterials, can improve vascularization. In our former study, PLA+BG40% released the most calcium, and improved endothelial differentiation and vitality. This indicated that Ca^{2+} release improved EPC differentiation and enhanced early vascularization in critical size bone defects [36].

Interestingly in our present study, various BG content in PLA/BG composite (PLA; PLA+BG20%; PLA+BG40%) did not effect cell adhesion and cell viability *in vitro* when MSC and EPC were seeded on these biomaterials.

But, high concentrated PLA+BG40% demonstrate its osteogenic potential for bone formation. Moreover, pre-seeding this composite biomaterial (PLA+BG40%) with tissue-specific cells (MSC/EPC) prior to implant, especially when MSC are pre-differentiated, enhance bone formation significantly *in vivo* at 14 weeks compared to bioglass/PLA alone (cell free). This can be due both to the osteogenic and the vascular differentiation potential of MSC and EPC. In fact, cell-based therapy of MSC and EPC has been previously reported in literature in different studies [22,23,37-40] indicating a potential to provide vascularization for constructs used in bone regeneration. Our findings, that pre-differentiated MSC/EPC showed higher bone formation by trend, confirm these cell-based strategies.

These findings are in a line to Yu et al., [41]. It is one method which is being examined to improve bone tissue regeneration. According to safety of a bioglass-polylactic acid composite scaffold seeded with progenitor cells in a rat skull critical-size bone defect we observed in a previous study no side effects or complications [42].

One limitation of this study is that we observed at a very early time point for cell adhesion and viability, but according to our previous study it is possible to detect differences [5]. Also 2 hours after cell seeding is in this experimental setting more practical.

Cell transplantation onto an optimal biomaterial is a promising alternative to the "gold standard" of autologous bone grafting to stimulate bone repair even in this presented severely compromised model of bone healing. As known a skull defect model without bone marrow inside is a severely compromised model because recruitment of progenitor cells is more difficult.

Therefore, our data support the hypothesis that this new created bioglass/PLA composite is a useful biomaterial, which improve bone formation at a critical-sized bone defect.

This work provides important insights into the interaction between cell-based therapy (EPC/MSC) and the currently available PLA/Bioglass composites. This information can be valuable for choosing which substitute to use clinically and, more importantly, for further development of these and new materials.

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