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Cellularized versus decellularized scaffolds for bone regeneration

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Abstract

An optimal scaffold based strategy for in vivo repair of large bone defects and its associated problems is presented in this work. Three polymeric scaffolds produced by using an extrusion-based additive manufacturing system were examined in a rat critical bone defect model: scaffolds without cells, with undifferentiated Adipose-derived mesenchymal stem cells (ADSCs) and differentiated ADSCs (osteoblasts). Scaffolds with undifferentiated cells seem to be the best strategy as they exhibited around 22% more bone formation than natural bone healing, and around 15% more than the two other cases. Authors observed that scaffolds enabled cell migration and tissue formation. Results suggest that undifferentiated ADSCs strongly contribute to new bone formation with no rejection if scaffolds are used to support cell migration, proliferation and differentiation. Our long-term goal is to engineer high-quality cell seeded-scaffolds (autograft and allograft) for bone regeneration, mainly in elderly patients.

Keywords: Adipose-derived mesenchymal stem cells, Biomaterials, Bone regeneration, Polymers, Scaffold.

1. INTRODUCTION

Bone is able to heal and remodel without leaving any scar in cases of very limited damage or fracture. However, in pathological fractures, traumatic bone loss or primary tumour resection, when the defect exceeds a critical size, the bone is no longer able to heal itself [1,2]. In these cases, the clinical approach is the use of bone grafts, defined as an implanted material that promotes healing on its own or in combination with other materials, through osteogenesis, osteoinduction and osteoconduction [3]. Bone grafts can be divided into autografts, allografts and xenografts [1-6]. Autografts, harvested from one site and implanted into another site within the same individual, are osteogenic, osteoinductive and osteoconductive, and do not present risk of disease transmission or immune system rejection [2]. It is considered to be the most effective approach for bone regeneration, however it presents some major drawbacks such as site morbidity and pain, prolonged hospitalization, increased risk of deep infection and haematoma [2]. Allografts are harvested from one individual and implanted into another one of the same species. Its limitations are associated with rejection, transmission of diseases and infection. Additionally, the healing rate is generally lower than autografts [2]. Xenografts are harvested from one individual and transplanted into another one of a different species. They produce poor clinical outcomes and present high risk of disease transmission [3]. However, mesenchymal stem cells xenografts using different

biomaterials could be an alternative method for defect repair, especially for aged patients [7,8]. Thus, biofabrication, defined as the combined use of additive manufacturing techniques, biocompatible and biodegradable materials, cells, growth factors, etc. for the fabrication of scaffolds, is becoming a promising alternative for grafting [9]. Several studies reported the use of polymeric (poly(caprolactone), poly(lactic-go-glycolic acid), etc.), ceramic (hydroxyapatite, bioglass, etc.) and polymer-ceramic scaffolds with different porosities and architectures [10-12]. However, none of these research works were able to identify the best route for bone regeneration. Chuang and co-workers investigated the in vivo osteogenic potential of Human Mesenchymal Stem Cells/Poly Lactide Co-Glycolic Acid (PLGA) constructs for bone regeneration in a rat critical-sized calvarial defect model. Scaffolds were produced by dissolving PLGA in a medium containing chloroform and sodium chloride. After the vaporization of chloroform, the scaffolds were immersed in double-distilled water to dissolve the sodium chloride [13]. A similar study was performed by Yoon and co-workers [14]. In these cases, the techniques used to produce the scaffolds do not allow to control pore shape, pore size and pore interconnectivity of the scaffolds, critical parameters to design optimised constructs. To circumvent these limitations, Rai and co-workers [15] investigated the use of a filament-based extrusion additive manufacturing system to produce polycaprolactone (PCL)/tricalcium phosphate scaffolds seeded with Human Mesechymal Stem Cells (hMSCs). However, none of these research studies were able to identify the best strategy for bone regeneration.

In previous studies, we extensively investigated the design and fabrication of PCL scaffolds for tissue engineering [16-18]. The effect of processing conditions (temperature, deposition velocity, screw-rotational velocity and slice thickness) on the morphological and mechanical properties of extruded scaffolds was investigated and optimal processing conditions determine [16]. Experimental results reveal that deposition velocity and screw-rotational velocity have the highest influence in terms of porosity and mechanical properties. The *in vitro* biological behaviour was also assessed using scaffolds with different architectures (0º/90º, 0º/60º/120º and 0º/45º/90º/135º) seeded with hMSCs [17,18]. After 21 days of static culture viability/proliferation appeared to be strongly influenced by the pore size and pore shape. Results also showed that large quadrangular pores enhance hMSC viability and proliferation and cell morphology was not affected by pore topology. This research work investigates the use of polymeric scaffolds with controlled architecture and three biofabrication routes for bone regeneration: scaffolds without cells, scaffolds seeded with differentiated cells and scaffolds seeded with undifferentiated cells.

2. MATERIALS AND METHODS

Scaffold fabrication: PCL (Mw 50,000 Da, CAPA 6500, Perstorp Caprolactones, Cheshire, UK) scaffolds were produced through a screw-assisted additive biomanufacturing system named 3D Discovery (RegenHU, Switerzland). 3D cylindrical scaffolds were designed with dimensions of 30 mm (diameter) x 5 mm (height), a constant filament distance of 650 µm and a lay- down pattern of 0º/90º. The operating conditions are indicated in Table 1. After the fabrication, produced scaffolds were cut using trephine drill according to the dimensions of the created bone defect (5mm (diameter) x 2mm (height)), and they were surface treated in 5M NaOH for 3h to enhance their hydrophilicity. After rinsing with phosphate buffered saline (PBS, pH 7.4), the scaffolds were sterilized in 70% ethanol for 24 h and 12h under UV light. The scaffolds were then rinsed again with PBS, air-dried and soaked for at least 3 h in basal media prior to cell seeding.

Table 1

Processing conditions used to produce the PCL scaffolds

Processing Temperature	Air Pressure	Screw Rotational Velocity	Deposition Velocity	Nozzle Tip Size
90 ºC	6 bar	22 rpm	20 mm/sec	300 µm

Cell source and culture: ADSCs were isolated from liposuction of patients from Clinical Hospital of Ribeirão Preto Medical School (Brazil), after obtaining informed consent from all patients and approval from the hospital ethical committee (number 2722/2014). The cell isolation method is based on extracellular matrix (ECM) digestion in 0.075% collagenase (SIGMA) during 30 minutes, at 37°C, according to a method previously reported [19]. ADSCs were cultured and expanded at 37°C, under 5% of CO_2, using basic culture medium (α-MEM, 10% fetal bovine serum, 1% antibiotic-antimyotic, 1% L-Glutamine 200mM; Invitrogen, Brazil). The medium was changed twice a week. After reaching 80-90% confluence, cells were detached by the use of 0.25% trypsin-EDTA solution (from Invitrogen) and re-suspended in 10 mL of culture medium. Only passages 3 up to 5 were considered in this present work.

Cell seeding: scaffolds were seeded with $5x10^4$ cells in 50µL of α-MEM medium. The cell-seeded scaffolds were incubated at standard conditions during 2h allowing cells to diffuse and adhere to the scaffold, before the addition of 1 mL of fresh basic or osteo-differentiating culture medium, as previously

reported [20]. All cell-seeded scaffolds were maintained in a humidified incubator, at 37°C and 5% CO_2, with medium changes every 3 days for 21 days prior implantation.

Calvarial defects: Defects were induced in male Wistar rats (weight of 250-300 g), according to the ethical guidelines of the Brazilian College of Animal Experimentation, approved by the Ethical Committee on Animal Experimentation from Ribeirão Preto Medical School, University of São Paulo (number 024/2015-1). The animals were anesthetized by intraperitoneal administration of a mixture of ketamine hydrochloride (80 mg/Kg) and xylazine hydrochloride (15 mg/Kg). The hair over the calvarium was shaved and, after asepsis with 70% ethanol, a circular bone defect (5mm in diameter) was created on the right side of the calvarial bone using trephine drill at 1200 rpm with constant PBS irrigation. According to previous studies, 5 mm calvarial defect is considered a critical-sized, non-healing defect along the length of the study [21,22].

Sixteen animals were divided into four testing groups as follows: NBR group or control – New Bone Regeneration without scaffold; SCA group - Scaffold without cells; SUC group - Scaffolds with human ADSCs; SDC group - Scaffolds with human ADSCs differentiated to osteoblast *in vitro*. After the scaffold implantation, wounds were sutured with nylon 5-0 sutures (Johnson&Johnson, Brazil). During the immediate postoperative period, one dose of a systemic antibiotic (1mg/kg; Pentabiotic, FortDodge®) was administered. Postoperative pain was also controlled by oral administration of Dipyrone (1 drop/kg; Biovet, Brazil), three times a day for 2 days. The animals were euthanized after 8 weeks with excessive anesthetic dose, and all calvarial bones were collected for further analysis.

Histology: The calvarial bones biopsies were fixed in 3.7% buffered formaldehyde solution (pH 7.4) for 48 hours, and demineralized with 10% ethylenediaminetetraacetic acid for 30 days [23]. After this period, biopsies were dehydrated and embedded in paraffin, and cross-sections of 5.0 μm thick were cut with a micrometer, mounted on glass slides, dewaxed, rehydrated and stained with hematoxylin and eosin. Biopsies were analyzed using a LEICA DM 4000BVR microscope equipped with a LEICA camera (Leica Microsystems, Germany) and images were captured at 50x and 200x magnification. Colour Deconvolution ImageJ software was used to evaluate the percentage of new bone and connective tissue formation on 50x magnification images. The morphometric analysis, corresponding to red color, was measured as the percentage of the total number of pixels in each original image, as described elsewhere [24].

3. RESULTS AND DISCUSSION

The scaffold implanted in the calvarial defect is indicated in Fig. 1.

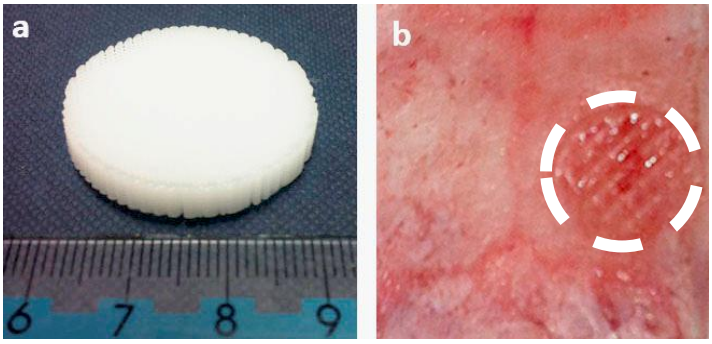

Fig. 1. PCL scaffold. a) Scaffold after printing and b) scaffold implanted in the calvarial defect after 8 post-operative weeks.

Fig. 2 shows histological images at 50x and 200x magnifications considering new bone and connective tissue formation after 8 post-operative weeks for all four groups. No inflammatory effects were observed in all groups neither before the sacrifice of the animals nor in histological images. In the control group (NBR group) case, where scaffolds were not used to fill the bone defect, the central part of the defect healed through the formation of a thin connective tissue layer. In this case, only a small portion of new bone is observed, mostly close to the edges of the defect. In the other three cases, where PCL scaffolds were used to fill the bone defect, it is possible to observe the presence of new bone and more connective tissue between the defect edges. For the SCA group, it is clear the presence of areas of new bone at the defect edges and also “islands” of bone spread along de defect embedded in a connective tissue, which seems to be inside the scaffold pores. Similarly, it is also possible to observe identical “islands” of new bone embedded in connective tissue in the SDC testing group. In the case of the SUC group, most of the new tissue corresponds to areas of new bone close to the edge, and also towards and along the center of the defect.

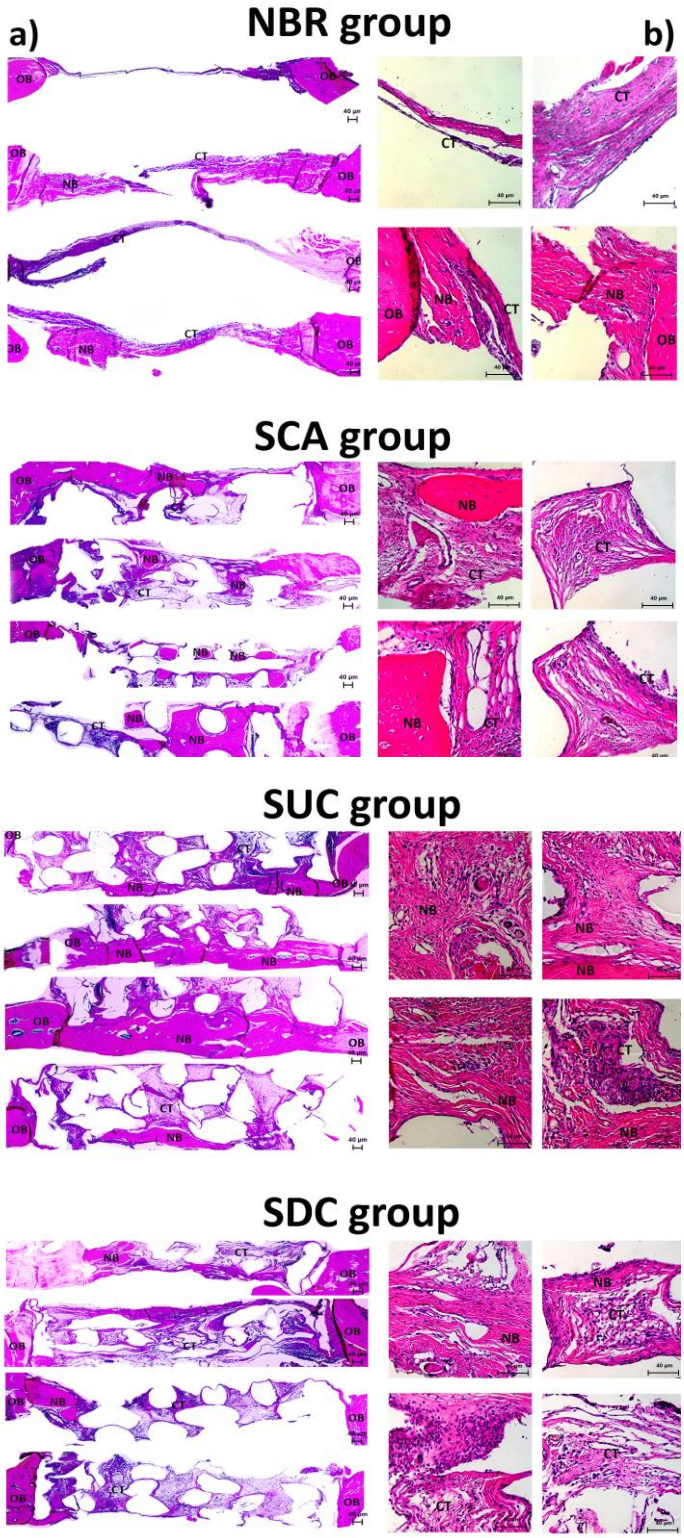

Fig. 2. Photomicrography of the defect area for all groups after 8 weeks, stained with hematoxylin and eosin. a) 50x magnification showing the entire bone defect; b) 200x magnification showing closer captions of the new tissue. In these images, it is possible to observe the original bone (OB), new bone (NB) and connective tissue (CT).

Fig. 3 shows the percentage of new bone and connective tissue formation in all considered cases.

As expected, all three groups that received scaffold in the bone defect had higher tissue connective

formation ($p<0,05$) compared to NBR group as the PCL scaffold enables cell influx and tissue formation. The bone formation observed in SUC group is significantly higher compared to NBR, SCA and SDC groups (22%; 14% and 15% higher, respectively) and higher than its tissue connective formation ($p<0,05$). Based on these findings, it seems that human ADSCs participate in the new bone formation process, probably due their regenerative and immunosuppressive properties. Results strongly suggest that PCL scaffolds seeded with ADSCs present high osteoinduction properties. It is also possible that growth factors released by human ADSCs stimulate animal cells to produce new bone. This effect was not observed in the SDC group, where ADSCs were previously *in vitro* differentiated to osteoblasts. However, contrary to other studies using bone marrow stem cells, where transplanted cell-scaffold implants were rejected one week after implantation [13], the obtained results seem to confirm that ADSCs xenotransplanted are able to evade the immune surveillance and contribute to healing. This can also be related to the fact that ADSCs express less human leukocyte antigen (HLA-ABC) than bone marrow stem cells, indicating they may have more potential in transplantation [25].

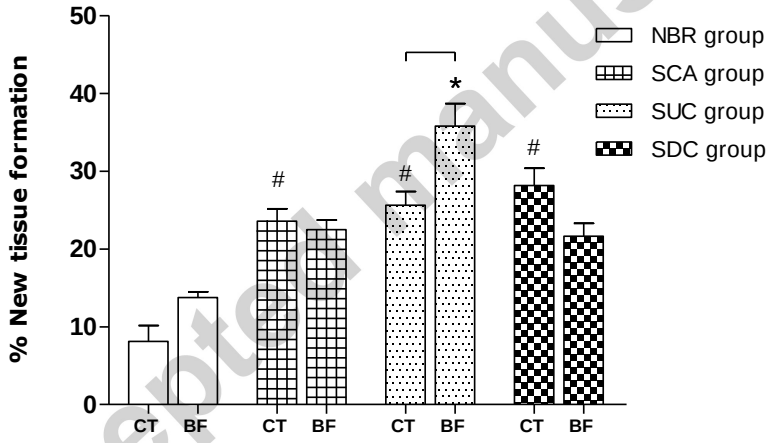

Fig. 3. Percentage of new bone formation (BF) and connective tissue (CT) quantified by ImageJ software at 50x magnification (hematoxylin and eosin staining). * Statistical evidence ($p<0.05$) was analyzed by one-way-ANOVA show difference to all other groups regarding to BF ; # Statistical evidence ($p<0.05$) regarding to CT from SCA, SUC and SDC groups compared to NBR group.

4. CONCLUSION

The design of an ideal scaffold-based strategy for *in vivo* bone regeneration is critical to repair large bone defects and treat bone problems in aged patients, who may already have compromised cellular function. This work investigated the regenerative performance of PCL scaffolds seeded with ADSCs, showing a better performance compared to PCL scaffolds without cells or seeded with differentiated cells. Results suggest that undifferentiated cells, even in a xenotransplantation case, strongly contribute to new bone

formation. It was also possible to observe that xenotransplanted cells are able to evade the immune surveillance, not only immediately after implantation but also for a longer period. ADSCs in a xenograft model were also considered in attempting to extrapolate the construct behavior for future autograft and allograft treatments.

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Highlights

- Scaffolds enabled cell influx and differentiation
- Scaffolds seeded with undifferentiated ADSCs induce higher bone regeneration *in vivo*
- The use of cellularized scaffolds seeded with undifferentiated ADSCs seem to be the best strategy for *in vivo* bone regeneration