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**Evaluation of the ability of collagen-glycosaminoglycan scaffolds with or without mesenchymal stem cells to heal bone defects in Wistar rats**

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**Running Title:** Collagen scaffolds promote bone regeneration

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**Key words:** Collagen-glycosaminoglycan scaffold, Mesenchymal stem cells, Osteogenesis, Histology.

## **Abstract**

**Purpose** The aim of this experiment was to examine the capacity of collagen-glycosaminoglycan scaffolds, with or without mesenchymal stem cells, to satisfactorily repair a 5-mm rat calvarial defect.

**Methods** 55 Wistar rats were used in the study. The defects were either left empty to serve as controls ( $n = 7$ ), filled with cell-free scaffolds ( $n = 11$ ), cell-seeded scaffolds that were pre-cultured in standard culture medium ( $n = 13$ ), cell-seeded scaffolds that were pre-cultured in osteoinductive factor-supplemented medium ( $n = 12$ ) or particulate autogenous bone ( $n = 12$ ). The animals were sacrificed at 12 weeks after surgery and specimens were prepared for histomorphometric analysis. The linear bone healing and the bone area within the defect were measured.

**Results** Comparable results were obtained using cell-free collagen-glycosaminoglycan scaffolds and autogenous bone both in terms of linear bone healing ( $P < 0.986$ ) and area of new bone ( $P < 0.846$ ). While the test groups showed significantly more bone formation compared to the empty defect control group, the linear bone healing and area of new bone within the defect were significantly lower in the cell-seeded scaffolds than in the cell-free scaffolds. The results have demonstrated that a cell-free collagen-glycosaminoglycan scaffold is capable of repairing a 5-mm rat calvarial defect as effectively as autogenous bone and that seeding the scaffold with pre-cultured mesenchymal stem cells prior to implantation offered no beneficial effect and resulted in incomplete healing of the defect.

**Conclusions** The results thus suggest that the scaffold has immense potential for tissue repair showing favourable osteoconductive properties, biocompatibility and degradability.

## Introduction

Reconstruction of bony defects using autogenous bone grafting is widely considered the gold standard by which all techniques of osseous reconstruction of the cranio-maxillofacial skeleton must be judged. However, both practitioners and patients have perceived this reconstructive method as being rather invasive, requiring bone removal from non-oral sites which may be associated with donor site morbidity. In order to eliminate the need to create a second surgical donor sites, recent studies have defined the field of scaffold-guided bone tissue engineering to be a promising strategy for the treatment of many skeletal defects [1]. Regenerative scaffolds allow for the repopulation and differentiation of various cell types and are the focus of many current investigations.

There are two major approaches to tissue engineering for bone regeneration. One tissue engineering approach entails cell-free scaffolds in which the scaffold is used to act as a template for new tissue in-growth and to enhance the maturation and function of tissue that has been directed to grow into the scaffold. Cell-based tissue engineering approaches, on the other hand, incorporate seeding of a scaffold with cells that are delivered to and retained within the recipient site. In some cases, the cell/scaffold construct is pre-cultured *in vitro* to generate the required tissue and later transplanted to the recipient site [2]. Several studies have shown that the implantation of certain cell/scaffold combination can lead to better results in bone regeneration than the implantation of scaffolds alone [3].

A bone tissue engineering approach utilizing murine bone marrow-derived mesenchymal stem cells (MSCs) and collagen–glycosaminoglycan (CG) scaffolds has been investigated in our laboratory [4-6]. These studies have shown the ability of the CG scaffold to support the osteogenic differentiation of MSCs *in vitro*. The results demonstrated that MSCs can undergo osteogenesis followed by matrix deposition and mineralisation when grown on CG scaffolds and stimulated with osteoinductive factors (dexamethasone, ascorbic acid, and  $\beta$ -glycerophosphate), as evaluated by the temporal induction of the bone-specific proteins (collagen I and osteocalcin) and by evident mineral deposition when stained with alizarin red and von Kossa staining.

After *in vitro* proof-of-concept, we are interested in the current study in studying the behaviour of this tissue-engineered mineralized construct *in vivo*.

Although a large number of materials have been developed for bone tissue engineering [7-9], only a few are currently being tested in critical size defects [10]. The aim of this study was to examine the capacity of CG scaffolds with or without MSCs to satisfactorily repair a critical sized defect in a rodent calvarium. The healing response of CG scaffolds is compared to that achieved by the “gold standard” autogenous bone graft. We are also interested in observing the difference between using osteoinductive factor-stimulated MSCs and non-stimulated MSCs.

## **Material and methods**

### **Scaffold fabrication**

The CG scaffold was fabricated using a lyophilization (freeze-drying) method as detailed previously by O'Brien et al. [11]. Briefly, collagen type I from bovine tendon (Collagen Matrix, NJ, USA) was mixed with shark chondroitin 6-sulfate in acetic acid. The slurry was then freeze-dried at -40°C. This forces the co-precipitate into the spaces between the growing ice crystals to form a continuous, interpenetrating network of ice and the co-precipitate. Sublimation of the ice crystals under a vacuum leads to the formation of a highly porous sponge with an average pore size of 96 µm [12-13]. To stiffen and sterilize the collagen network, the scaffold was crosslinked using dehydrothermal processing at 105°C.

### **Cell culture and scaffold seeding**

Under Irish Government License, MSCs were isolated from the bone marrow of adult male Wistar rats (250–350 g) as detailed in Farrell et al. [4]. MSCs were expanded for 3 weeks in a pre-warmed Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, UK) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin/streptomycin, 2 mM Glutamax, 1 mM of L-glutamine, and 1% nonessential amino acids (all from GIBCO, supplied by Biosciences, Dublin, Ireland). Medium was replaced every 3–4 days.

Previous work in our laboratory has demonstrated the presence of undifferentiated MSCs when bone marrow cells were isolated and cultured according to the above protocol [4-5]. Cells analyzed displayed positive endoglin immunoreactivity. Endoglin (CD105) is a cell surface marker of MSCs which has been used to distinguish between haematopoietic stem cells and MSCs [14]. Furthermore, Fluorescence-activated cell sorter (FACS) analysis verified that the cell population expressed high levels of the putative MSC surface marker CD90 [15], in agreement with previous published data on MSC cell surface markers [16]. The purity of the MSC population used in these experiments was concluded to be comparable with that used by others [17].

To seed the CG scaffolds, cells were rinsed with phosphate-buffered saline, detached with trypsin-ethylenediaminetetraacetic acid (EDTA) solution and centrifuged at 650g for 5 min at 20°C. The resulting pellet was re-suspended in a 2-ml pre-warmed culture medium, and a single-cell suspension was obtained by aspirating through a 20-gauge needle. The cells were counted and diluted to achieve a cell density of  $1 \times 10^6$  cells/ml. Discs of CG scaffolds (16-mm in diameter) were seeded with 300  $\mu$ l of cell suspension and incubated for 30 min at 37°C. The scaffolds were then overturned onto agarose-coated wells, and a further 300  $\mu$ l of cell suspension was placed onto the scaffold. After 30 min, 2 ml of culture medium was added to each well and the scaffolds were submerged. To induce osteogenic differentiation, scaffolds were placed in osteoinductive factor-supplemented medium containing 0.68 nM of dexamethasone, 10 mM of  $\beta$ -glycerophosphate, and 50  $\mu$ M of ascorbic acid (Sigma-Aldrich, UK). Non-seeded scaffolds to be used as controls were maintained in standard culture medium. Half of the respective media were replaced every 3 days. Scaffolds were maintained for 4 weeks in a humidified atmosphere of 95% air/5% CO<sub>2</sub> at 37°C.

### **Surgical Procedure**

In this study, 55 adult male Wistar rats (250–350 g) were used. The care and use of animals were in accordance with the ethical principles for animal research established by the Department of Health in Ireland. Anaesthesia was achieved with a mixture of 75mg/kg of xylazine hydrochloride (Rompun®, Bayer Healthcare, Germany) and 10 mg/kg of Ketamine hydrochloride (Vetalar™, Pharmacia Animal Health Ltd, UK) at a

dose of 0.2 ml/100 g body weight given intraperitoneally. The skin of the head was shaved and disinfected with 10% aqueous povidone-iodine solution (Seton, Healthcare Group Plc, England). To expose the calvarial bone a 1.5-cm vertical skin incision was made, and periosteum was carefully reflected. A standardized, circular, and transosseous defect, measuring 5-mm in diameter, was created on the parietal calvarial bone using a saline-cooled trephine drill at low rotational speed. The circular bone plug was gently removed, and care was exercised to avoid injury to the dura matter. To prepare the autogenous bone graft, the trephined calvarium was manually ground with a # 11 blade and collected in a sterile glass dappen dish. The harvesting of particulate autogenous bone resulted in predominantly cortical bone chips of various shapes and sizes.

The defects were rinsed with normal saline and filled with graft material (Figure 1) in each experimental group as follows:

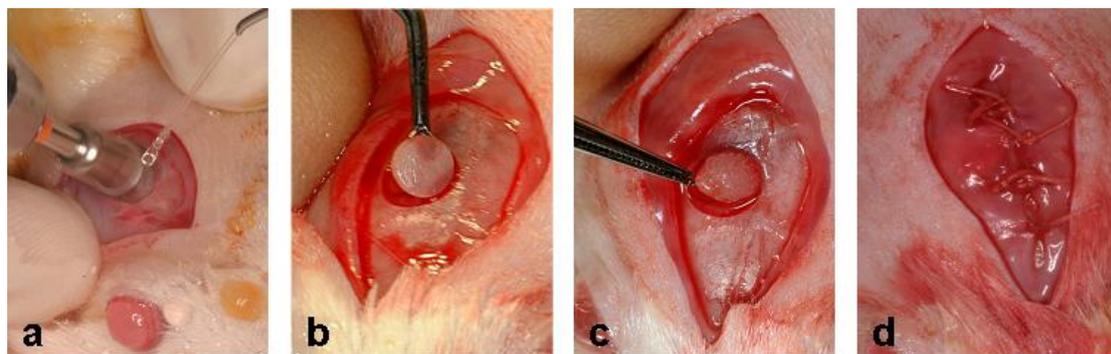
**Group 1** ( $n = 7$ ) - The defects were left empty to serve as a control.

**Group 2** ( $n = 11$ ) - Cell-free scaffolds

**Group 3** ( $n = 13$ ) - Cell-seeded scaffolds maintained in standard DMEM.

**Group 4** ( $n = 12$ ) - Cell-seeded scaffolds maintained in osteoinductive factor-supplemented DMEM.

**Group 5** ( $n = 12$ ) - Particulate autogenous bone.



**Figure 1.** Surgical procedure: (a) Preparing the surgical defect. (b) 5-mm critical sized defect in the rat calvarium. (c) Placement of CG scaffold. (d) Closure of the overlying periosteum

The overlaying periosteum and skin flaps were then closed using absorbable sutures (Ethicon™, NJ, USA). For the duration of the recovery period, animals were housed in the Bioresources Unit, Trinity College, Ireland under veterinary supervision.

### **Histological Preparation**

Animals were sacrificed by CO<sub>2</sub> asphyxiation at 12 weeks after surgery. Block sections, including the surgical sites, were removed and fixed in 10% neutral buffered formalin. Samples were decalcified in EDTA and embedded in paraffin. Before the embedding procedure, an incision was made exactly through the middle of the bone defects to ensure that the microtome sections were made in the area of interest. Coronal sections of 5-µm thickness were cut through the centre of the defect and stained with haematoxylin and eosin (H & E).

From each animal, three representative H & E-stained sections were randomly selected for histomorphometric analysis. Computer-assisted measurements were obtained using image analysis software (Analysis<sup>®</sup> Soft Imaging System GmbH, Germany). Sections were analyzed under light microscopy (x10 magnification). All measurements were made by an examiner who was blinded with regard to treatment.

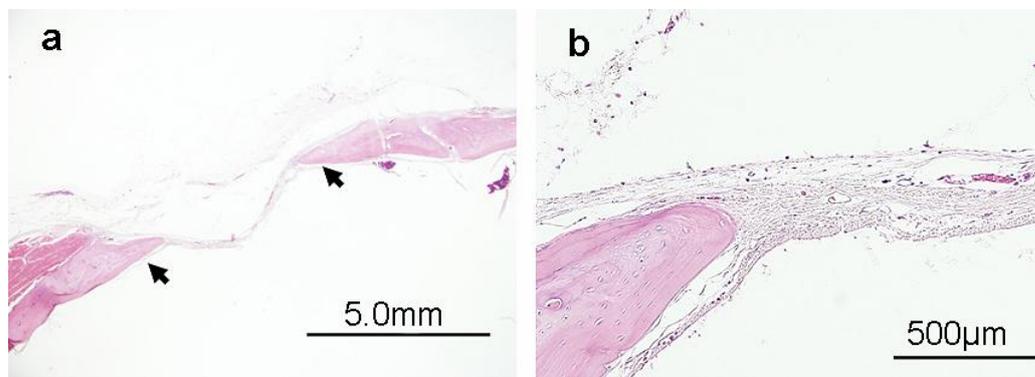
### **Results**

All animals survived the surgical procedures and were available for evaluation. Wound healing progressed without any signs of infection, material exposure or other complications during the healing period and at the time of retrieval. Weight gain was normal in all animals.

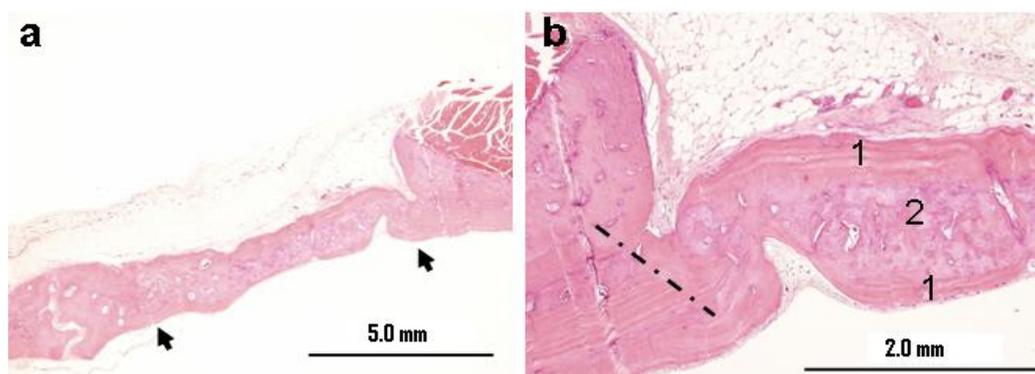
#### **Qualitative histological analysis**

**Group 1:** The empty defect group demonstrated only a small amount of new bone which was confined to areas at the edge of the defect with no new bone formation in the other defect areas. Bone at the margins of the defect showed a mixture of lamellar and woven bone indicating persistent new bone formation. In most of the specimens, the central part of the defect healed with a thin connective tissue as shown in Figure 2. There was no sign of inflammatory infiltrates throughout the surgical defect.

**Group 2:** The implantation of the cell-free scaffold resulted in extensive new bone formation apparent in all of the defects. Microscopically no residues of the CG scaffold were found after 12 weeks. At the defect margins, the newly formed bone appeared well integrated with the host bone. A thickening of bone at the bony margin of the defect was also observed. The outer surface of the new bone that faced the scalp and the inner surface that faced the dura mater demonstrated a mature lamellar bone, while the middle part of the new bone demonstrated a woven bone pattern with increased haematopoietic stromal areas (Figure 3).



**Figure 2.** Representative micrograph of the empty defect group showing a thin layer of connective tissue occupying the defect. H & E stained. Arrow = defect margin. (a) Original magnification x1.25. (b) Original magnification x10

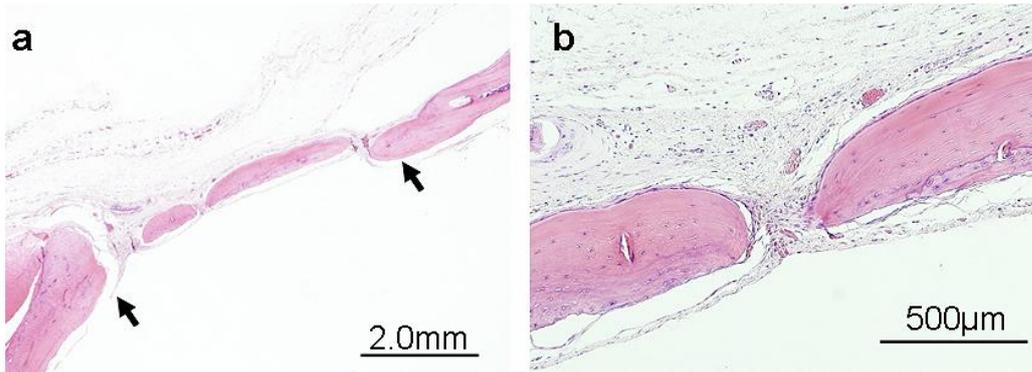


**Figure 3.** (a) Micrograph showing marked bone formation within defects treated with cell-free scaffolds. Arrows indicating the edges of host bone. Original magnification x1.25. (b) Micrograph showing good integration and bone thickening at the interface between host and new bone. (1) Lamellar bone. (2) Woven bone. H & E stained. Original magnification x4.

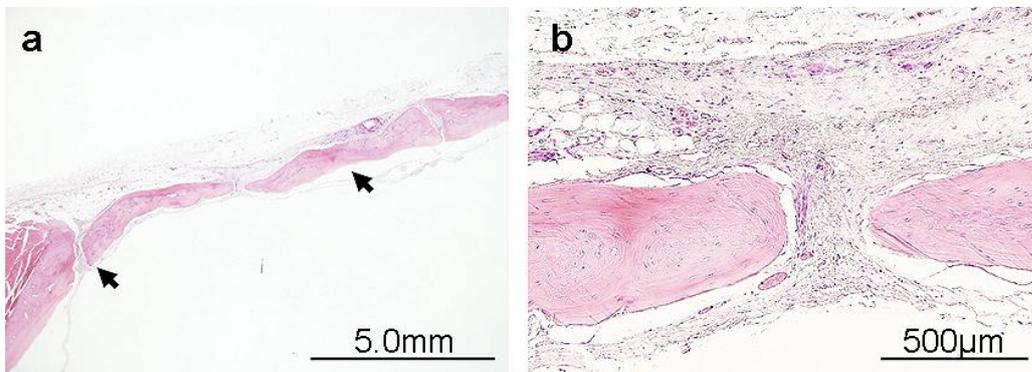
**Group 3:** The overall extent of bone regeneration in this group (cell-seeded scaffolds that were cultured in standard medium) was limited. Areas of new bone were evident at the margins and towards the centre of the defects. In most of the examined sections, there was no solid osseous bridging of the defect, but islands of bone were distributed in the former defect area and embedded in a fibroblast-rich connective tissue that was organized and dense. However, new bone formation, originated from the margins and/or as bone isles throughout the defect, was greater than in the empty defect group. The newly formed bone in the centre of the defect appeared lamellar in nature with mature osteocytes (figure 4).

**Group 4:** The defects which were grafted with MSC-seeded scaffolds that were cultured in the presence of osteoinductive factors showed similar healing pattern to those cultured in standard culture medium (figure 5). The extent of bone regeneration in this group was less than the new bone seen with non-seeded scaffolds.

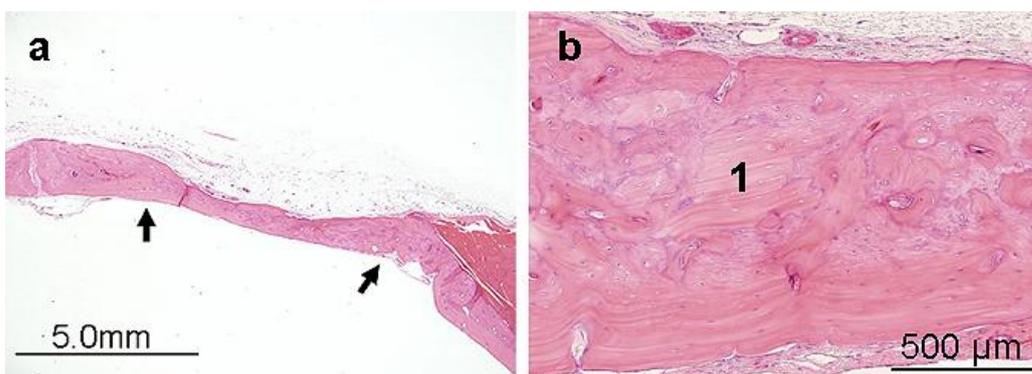
**Group 5:** In animals that received particulate autogenous bone, the defect was completely filled by new bone. The grafted bone particles did not exhibit signs of significant degradation and remnants of grafted chips were evenly distributed within the defect area. Similar to the defect margins, the graft surfaces were lined to a large extent by layers of new bone that bridged the gaps between the particles and connected them to the defect walls. The newly formed bone appeared less mature than the implanted bone particles, as demonstrated by the disorganization of its bone lamellae and osteocytes. Most defects were completely filled with bone which exhibited a cortical structure close to the surface, and was rather cancellous towards the centre of the defect with wide intra-trabecular spaces, collagen fibres and blood vessels. Close to the newly formed bone tissue margins and the graft fragments, there were numerous osteoblasts characterizing areas with intense bone apposition (Figure 6).



**Figure 4.** H & E stained representative micrographs of the cell-seeded scaffolds (group 3). (a) Defect healing by islands of new bone embedded in connective tissue. Original magnification x2. (b) Mature lamellar bone. Original magnification x10.



**Figure 5.** H & E stained representative micrographs of the cell-seeded scaffolds (group 4). (a) Defect healing by islands of new bone embedded in connective tissue. Original magnification x2. (b) Mature lamellar bone. Original magnification x10.



**Figure 6.** (a) Micrograph exhibiting complete bone bridging in a defect grafted with particulate autogenous bone. (H & E stained. Original magnification x1.25). (b) A close-up view of the newly formed bone, exhibiting graft particles (1) surrounded by less mature new bone (H & E stained, original magnification x10).

## **Histomorphometric and statistical analyses**

Histomorphometric parameters were defined as follows:

- Percentage of linear bone healing in the defect.
- Area of new bone in the defect.

### ***(A) Measurement of percentage of linear bone healing in the defect.***

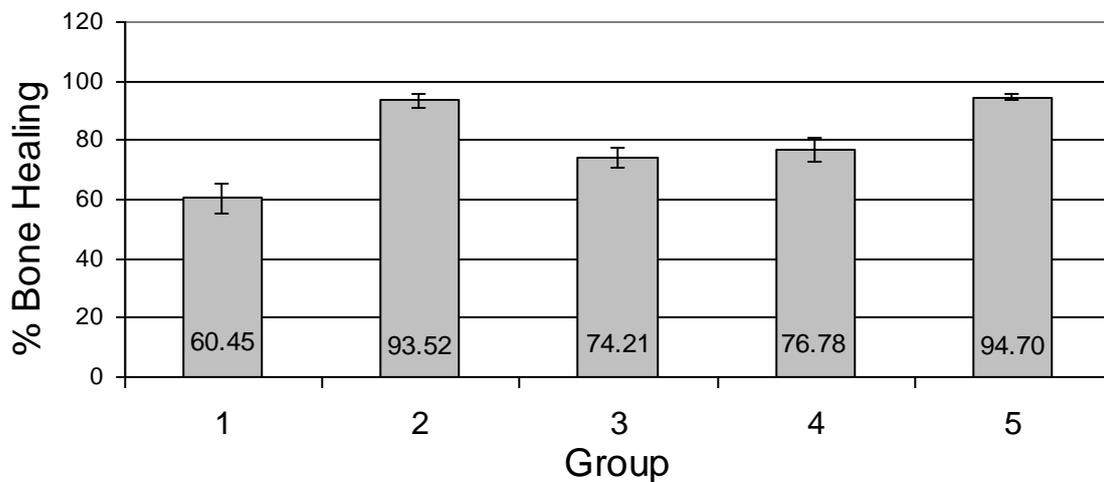
The percentage of new bone in the defect was evaluated to determine which treatment was able to facilitate bony bridging of the defect. Similar levels of linear bone healing were found in surgical defects that received cell-free collagen-GAG scaffolds ( $93.52 \pm 2.42$ ) when compared to particulate autogenous bone ( $94.70 \pm 1.24$ ). On the other hand, the cell-seeded scaffolds in group 3 and group 4 resulted in a mean percentage of bone healing of ( $74.21 \pm 3.59$ ) and ( $76.78 \pm 4.10$ ) respectively. Defects which were left empty showed the least mean percentage of bone healing of ( $65.45 \pm 5.21$ ). Figure 7 illustrates the mean percentage of bone healing amongst the study groups by the end of 12 weeks *in vivo*.

Analysis of variance (ANOVA) showed no significant difference between the bone healing in the cell-free scaffold (group 2) and the autogenous bone (group 5) ( $P < 0.986$ ). However, the bone healing in the cell-free scaffold group was found to be significantly greater than the bone healing achieved by the rest of the study groups ( $P < 0.0001$ ). The test showed no difference between the bone healing generated by the cell-seeded scaffolds that were cultured in standard culture medium (group 3) and the bone healing by the cell-seeded scaffold that were cultured in presence of osteoinductive factors (group 4) ( $P < 0.774$ ).

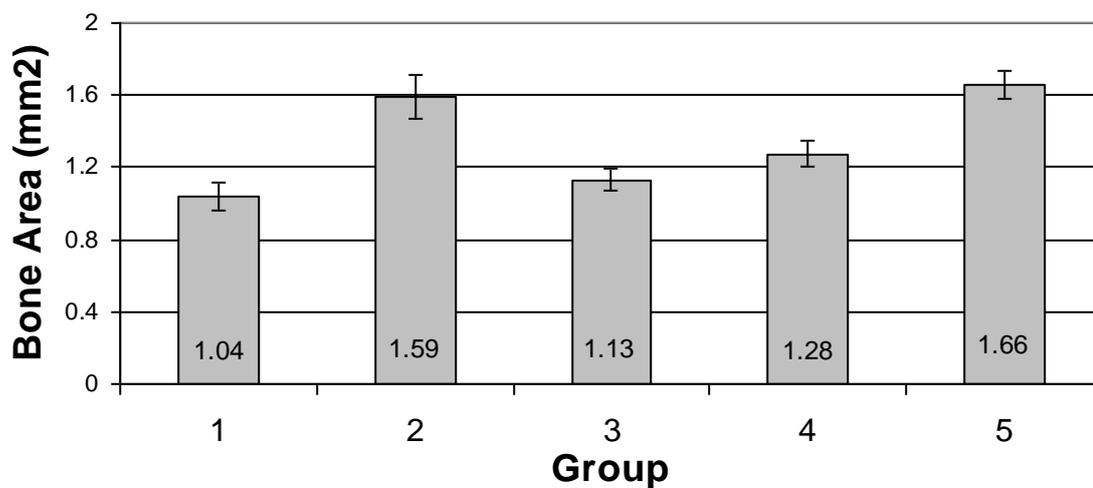
### ***(B) Area measurement of newly formed bone within the defect.***

The average bone area within the defect per group after 12 weeks *in vivo* is shown in figure 8. Defects which were left empty showed the least mean value of bone area ( $1.04 \pm 0.079$ ). On the other hand, a higher mean value of bone area was seen in the defects that were grafted with cell-free collagen-GAG scaffold ( $1.59 \pm 0.12$ ). This was similar to the mean bone area measured in autogenous bone group ( $1.66 \pm 0.08$ ). The cell-seeded scaffolds in group 3 and group 4 resulted in a mean value of bone area of ( $1.13 \pm 0.07$ ) and ( $1.28 \pm 0.07$ ) respectively.

Statistical analysis of the mean bone area in the cell-free scaffold (group 2) and the autogenous bone (group 5) showed similar effects ( $P < 0.846$ ). The bone area in the cell-free scaffold group, however, was found to be significantly greater than the bone area achieved by the rest of the study groups ( $P < 0.0001$ ). The test showed that the bone area generated by the cell-seeded scaffolds that were cultured in standard culture medium (group 3) and the bone area by the cell-seeded scaffold that were cultured in presence of osteoinductive factors (group 4) did not significantly differ ( $P < 0.115$ ).



**Figure 7.** Percentage linear bone healing within the defect (Mean ± SEM)



**Figure 8.** Bone area within the defect (Mean ± SEM)

## Discussion

The aim of this investigation was to assess the ability of MSC-seeded CG scaffolds that were pre-cultured *in vitro* for 4 weeks to regenerate bone tissue in a 5-mm rat calvarial defect. We also compared the healing response of the CG scaffolds to autogenous bone. Following a healing period of 12 weeks, comparable results of complete bone healing were obtained using cell-free CG scaffolds and autogenous bone. While all the test groups showed significantly more bone formation compared to the empty defect control group, the percentage of bone healing and bone area within the defect were significantly lower in the cell-seeded scaffolds than in the cell-free scaffolds.

We assessed bone healing 12 weeks after surgery, considering that bone healing might plateau between 4 to 12 weeks after surgery. Additional healing over an 8-week time frame was described by Gasain et al. [18], and for that reason we sacrificed all animals at 12 weeks after surgery considering the potential healing between 8-week and 12-week time frame. No additional healing is expected after the 12-week time point as previously showed by Nguyen et al. [19]. We defined long-term evaluation as the longest period of postoperative care during which bone formation had stopped and no additional healing would be found. Thus, a 12-week time point was the optimal time to evaluate the clinical consequences and side effects of cell-free and cell-seeded CG scaffolds.

In the control group, where the defects were left empty, the central part of the defect healed with a thin connective tissue. This demonstrated that 5-mm calvarial defect was of a critical size (non healing within the length of the study). This is in line with the results of several previous studies [20-23]. The bone healing in the test groups was found to be significantly higher than the bone healing achieved by the empty defect group ( $P < 0.0001$ ).

The cell-free scaffolds facilitated bony healing of the defect similar to autogenous bone and significantly more new bone formation than all the other groups, both in terms of bone area and bony bridging of the defect. In a previously published work we demonstrated the tissue compatibility and the ability of cell-free CG to provide a

scaffold which allows infiltration of host cells [24]. Its full mineralization after 12 weeks of implantation is a further proof of its osteoconductive properties. The newly formed bone appeared well integrated with the host bone with a bony thickening at the interface. The scaffold appeared completely degraded and replaced by new bone. This is an extremely important property of scaffolds used for bone regeneration. It has been reported in previous studies that type I collagen had been resorbed and substituted by mature bone 9 weeks after implantation [25]. The authors of that study concluded that this histological sequence was ideal for induction of osteogenesis.

The CG scaffold might have played a role in the early cell proliferation, differentiation and subsequent secretion of extracellular matrix. Petrovic et al. [26] studied the suitability of different alloplastic and xenogenic biomaterials as scaffolds for *ex vivo* osteoblast cultivation. The authors concluded that a 3-dimensional collagen type-I scaffold can provide a more favorable environment for the attachment, proliferation, and differentiation of *in vitro* osteoblast-like cells, at least until the initial stage of differentiation, than non collagenous biomaterials. Mizuno et al. [27] demonstrated that bone marrow stromal cells expressed osteoblastic phenotypes (high alkaline phosphatase activity and synthesis of osteocalcin), when they were maintained on type I collagen matrix gel *in vitro*, and formed bone in the subcutaneous region of nude mice *in vivo*. According to our knowledge, however, we are the first to report on the use of CG scaffold for bone regeneration in an *in vivo* setting.

The use of autogenous bone graft resulted in extensive new bone formation within the defect. The deposition of woven bone was not only observed on the exposed defect walls, but also extensively on the graft surfaces. This confirmed the excellent osteoconductive properties of autograft. Studies have shown that autogenous bone graft gives the best post bone grafting results [28]. In a study on miniature pigs, Buser et al. [29] compared autogenous bone to DFDBA, tricalcium phosphate, and coral-derived hydroxyapatite. After 4 weeks of healing, they found that autologous bone graft had the best bone regenerative properties during the initial healing period with 39% of newly formed bone inside the membrane-covered defect.

The cell-seeded scaffolds showed greater new bone formation than the empty defect group but significantly less than the cell-free scaffold. Islands of newly formed bone immersed in loose fibrous connective tissue were distributed throughout the defect but complete bony bridging was not seen in most of the animals. Interestingly, comparable levels of new bone were produced by MSC-seeded scaffolds that were pre-cultured in standard culture medium and those pre-cultured in the presence of osteoinductive factors.

The findings of our previously published *in vivo* experiment may provide an explanation to the lack of continuous bony bridging of the defects grafted with MSC-seeded scaffolds [24]. In particular, inflammation induced initially after scaffold implantation has been linked with unsuccessful engraftment of tissue-engineering constructs [30-31]. A foreign body reaction to the implanted MSC-seeded scaffold may have resulted in premature scaffold degradation [32]. If degradation is too rapid, osteoblasts will be deprived of a surface on which to migrate and secrete bone matrix, the result being fibrous repair rather than osseous regeneration. To ensure optimal healing and incorporation results, the augmentation material must not generate a specific immune response of the host cells, with initiation of chemotaxis, activation and proliferation [33].

Cell-mediated contraction of CG scaffolds is another possible explanation for the interrupted pattern of bone formation in MSC-seeded scaffolds. Because of the contraction of cell-seeded scaffolds, changes in the size and microstructure of the implants take place frequently. Cell-mediated contraction can collapse the pores of the scaffold, thereby possibly affecting cell migration and proliferation and the flow of nutrients, and can distort the shape of the construct [34-35]. A change in size will cause difficulty in fitting a specific implant site, or cause separation from the surrounding tissue host [36-37]. Furthermore, *in vivo* deformation of the scaffold could result in a loss of contact between the implanted device and the host tissue, thereby decreasing the chances for successful integration of the repair tissue.

A limitation of the present study is that the MSCs used were not labelled and thus could not be distinguished from the infiltrating host-derived cells. As such, the possibility that the newly formed bone in the cell-seeded scaffolds was laid by the

infiltrating host-derived cells cannot be ruled out. Accordingly, because new bone formed in the cell-free grafted defects, it seems that the host cells which populated the cell-free scaffold were able to initiate new bone filling of the defect without the aid of implanted MSCs. These findings are in conflict with other studies, in which it has been reported that implants with differentiated osteoblasts and developed matrix are not only involved in direct bone formation, but also appear to be osteoinductive, inducing differentiation of host mesenchymal cells into osteoprogenitor cells during repair of the critical-size bone defects [38-40].

It has been reported previously that both delivery and migration of osteoprogenitor cells into bone defect areas establish the most definitive reconstruction of bone defects [41-42]. Potentially, these cells secrete specific extracellular matrix components [43], which could induce proliferation and differentiation of mesenchymal cells into osteoblasts and result in the formation of new bone [44]. The above-described findings implicate an active role for implanted osteoblasts in bone repair; however, in our study, cell-free CG scaffolds resulted in mineralization not only on collagen fibrils, but also within newly formed extracellular matrix. Such mineralization was significantly decreased in CG scaffolds seeded with MSCs.

## **Conclusions**

We have demonstrated that grafting a 5-mm rat calvarial defect with cell-free CG scaffold results in a complete bone healing comparable to that achieved by autogenous bone. The scaffold showed favorable osteoconductive properties, biocompatibility and degradability. In addition, we found that using pre-cultured MSC-seeded CG scaffolds resulted in incomplete healing of the defect. Irrespective of these findings there is a need to determine the precise role of implanted vital cells in the mineralization process and what role, if any, the implanted cells play in the induction of mineralization *in vivo*.

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