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Development of a biomimetic collagen-hydroxyapatite scaffold for bone tissue engineering using a SBF immersion technique

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Abstract

The objective of this study was to develop a biomimetic, highly porous collagen-hydroxyapatite composite scaffold for bone tissue engineering, combining the biological performance and the high porosity of a collagen scaffold with the high mechanical stiffness of a hydroxyapatite scaffold.

Pure collagen scaffolds were produced in a lyophilisation process as previously described. The collagen scaffolds were immersed in simulated body fluid (SBF) to provide a biomimetic coating. Pure collagen scaffolds served as a control. The mechanical, material, and structural properties of the scaffolds were analysed and the biological performance of the scaffolds was evaluated by monitoring the cellular metabolic activity and cell number at 1, 2 and 7 days post seeding.

The SBF- treated scaffolds showed a significantly increased stiffness compared to the pure collagen group (4-fold increase). While the porosity of the scaffolds was reduced, the scaffolds still retained a highly interconnected structure with a porosity as high as 95%. FT-IR indicated that the SBF coating exhibited similar characterisitcs to pure hydroxyapatite while the excellent biological performance of the collagen scaffolds was maintained in the collagen-hydroxyapatite scaffolds as demonstrated from cellular metabolic activity and total cell number.

This investigation has successfully developed a biomimetic collagen-hydroxyapatite composite scaffold. An increase in the mechanical properties combined with an excellent biological performance *in vitro* was observed, indicating the high potential of the scaffold for bone tissue engineering.

Keywords:

Bone repair

Bone tissue engineering

Scaffold

SBF

Hydroxyapatite coating

Introduction

The annual worldwide market for bone grafts or bone graft substitutes is approaching $US 1 billion, with up to 4 million bone replacement procedures. This makes bone second only to blood transfusions on the list of transplanted materials (American Association of Orthopaedic Surgeons Report 2002). The most common graft is an autograft, whereby bone is taken from the patient‘s own body and re-implanted. However, there is a limited amount of bone which can be removed from a particular donor site and additional invasive surgery is necessary which can be associated with donor site morbidity. An alternative treatment is the use of an allograft whereby bone is removed from an organ donor. The drawbacks with this approach include a risk of infectious disease being transmitted and a shortage of available donors. Therefore, bone graft research has begun to focus on bone tissue engineering (TE) and novel bone graft substitutes.

Collagen makes up to 89% of the organic matrix and 32% of the volumetric composition of bone [1]. Collagen scaffolds produced according to a standardised protocol [2,3] show an excellent biological performance due to their high porosity and permeability. Previous investigations in our laboratory indicated that scaffolds require a high porosity and surface area [2], combined with a good permeability and pore interconnectivity [4], for cell migration and nutrient perfusion during the cell culturing process. The main disadvantage of collagen as a

scaffold material for bone tissue engineering is that it has relatively poor mechanical properties.

In bone tissue engineering, much interest has focused on using synthetic polymers or pure ceramic materials for scaffold fabrication. These materials tend to have better mechanical properties than collagen scaffolds; however, other disadvantages appear while using these scaffolds. Many synthetic polymers demonstrate hydrophobic behaviour. Hydrophobic surfaces show a lower proliferative and a higher apoptotic rate for osteogenic cells [5-7]. In addition, polymeric biomaterials have bioinert surfaces that lack bioactive functions for bone formation [7]. Upon implantation, fibrous tissue often encapsulates these bone substitutes. Recent investigations have focused on chemically coating various scaffold types with calcium-phosphate (CP) or hydroxyapatite (HA) using different immersing techniques to improve their material properties [8-12]. Bone-like apatite layers on the surface of bone grafts improve their ability to create a bond with the living host bone and enhance osteoconductivity [7]. In particular, simulated body fluid (SBF), an acellular solution developed in 1990 by Kokubo et al. [13] with an ion concentration similar to that of human extracellular fluids has been used to precipitate HA or CP on scaffolds [7,11,14]. SBF is a metastable solution containing calcium and phosphate ions supersaturated with respect to the apatite [13] and can be used to coat various materials with apatite under biomimetic conditions. Different SBF treatments have been investigated to form CP layers to improve

surfaces for better bioactivity and osteointegration on titanium alloys [15,16], polymers [9,10,17] and composite biomaterials [7].

Scaffolds for tissue engineering require a highly porous structure and an excellent biocompatibility as commonly exhibited by collagen scaffolds. However, scaffolds for bone tissue engineering require good mechanical properties to facilitate load-bearing after implantation, a characteristic common to ceramic scaffolds but not found in biological scaffolds. The goal of this study was therefore, to develop a novel collagen-hydroxyapatite composite scaffold which combined the advantageous properties of both materials by using a SBF coating treatment on a highly porous collagen scaffold. The specific goals of the study were to develop a collagen-hydroxyapatite composite scaffold using a SBF treatment to (i) improve the mechanical properties of the composite scaffolds relative to pure collagen scaffolds and (ii) to maintain the high porosity and the high biological performance of the pure collagen scaffolds in the new variants.

Methods

Fabrication of scaffolds

Pure collagen scaffolds were fabricated using a standardised protocol [2-4]. Briefly, a collagen suspension was produced from microfibrillar type I collagen, isolated from bovine tendon (Integra Life Sciences, Inc., Plainsboro, NJ, USA) and 0.05 M acetic acid by mixing at 15,000 rpm in an overhead blender (IKA Works, Inc., Wilmington, NC, USA). The slurry was poured into a stainless steel tray which was placed into a freeze-dryer (VirTis Co., Gardiner, NY, USA). The temperature was then lowered at a constant cooling rate of 1º C/min to the final temperature. A final freeze-drying temperature of -40ºC was used to produce scaffolds with a mean pore size of approximately 95 μm [3]. The shelf and chamber temperature were then held constant at the final temperature for 60 minutes to complete the freezing process. The shelf temperature was then ramped up to 0ºC for 160 minutes. The ice phase was then sublimated under a vacuum of approximately 200 mTorr at 0ºC for 17 hours to produce the porous collagen scaffold. Samples with a diameter of 9.5 mm and a height of 4 mm were cut out for further experiments using a punch.

Simulated body fluid (SBF) was prepared by dissolving reagent-grade chemicals NaCl, $MgCl_2$, $CaCl_2$, Na_2HPO_4 and $NaHCO_3$ (Sigma Aldrich, Germany) into demineralized water according to Kokubo et al [7,13]. A SBF solution with a

concentration of five times the ion concentration of human plasma was made up to restore a pH value of 7.4 [7,13]. Pure collagen scaffolds (n=6) were then immersed into the solution. Each scaffold was immersed in 3ml of the SBF solution for 4 days with daily replenishment of the solution. During this time period calcium and phosphate should deposit on the collagen scaffolds to form a HA layer on the collagen struts. This treatment was performed at 37°C in an incubator.

Characterisation of scaffolds

Fourier transform infrared (FT-IR) spectra measurements were performed to investigate the material properties of the scaffolds. In order to evaluate the typical collagen and HA characteristics, FT-IR spectra were made in extinction using a controlled potassium bromide (KBr) embedding technique in the range from 400 to 4000 cm^{-1} at a resolution of 4 cm^{-1} (Bruker Tensor 27, Nicolet Instruments, USA). 300 mg of dried KBr powder and approximately 1 mg of pulverized collagen-hydroxyapatite samples were mixed and ground using an agate mortar and pestle.

Mechanical characterisation of the scaffolds (n=6) was performed on a uniaxial testing system (Zwick Z005 with a 5 N load cell). Compression tests were performed at room temperature in phosphate buffered saline (PBS). The slope of

the plot of the stress strain curve was determined between strains of 2 and 5%, resulting in a Young's Modulus value for each scaffold.

The scaffolds were weighed before and after SBF treatment using a digital scale (Mettler Toledo, PB 153-S, Switzerland; accuracy of 0.1 mg). The individual dimensions of the scaffolds were measured using a digital camera and the image editing software ImageJ. The weight of the composite scaffolds was then compared to the weight of the pure collagen scaffolds to find the percentage of collagen and hydroxxyapatite in the scaffolds (Equation 1 and 2). The density was calculated using the weight and volume of each individual scaffold (equation 3).

$$\%_{collagen} = \left(\frac{weight_{collagen}}{weight_{scaffold}} \right) \quad (1)$$

$$\%_{HA} = \left(\frac{weight_{HA}}{weight_{scaffold}} \right) \quad (2)$$

$$\rho_{scaffold} = \left(\frac{weight_{scaffold}}{volume_{scaffold}} \right) \quad (3)$$

The collagen/HA ratio was calculated using the percentage of collagen and HA the scaffold and the densities of pure collagen (1.343 g/cm^2) [18] and the density of HA (3.14 g/cm^2) (Equation 4).

$$ratio_{coll/HA} = \frac{\%_{collagen} * \rho_{coll}}{\%_{HA} * \rho_{HA}} \quad (4)$$

The relative density of the scaffolds was then calculated using the collagen/ HA ratio of each scaffold (Equation 5). The individual porosities of the scaffolds were calculated using equation 6 [19].

$$\rho_{relative} = \left(\frac{\rho_{scaffold}}{\rho_{coll/HA}} \right) \quad (5)$$

$$porosity = 1 - \rho_{relative} \quad (6)$$

Biological performance of scaffolds

12.7 mm diameter scaffold samples of pure collagen (control group) and the SBF treated collagen scaffolds were seeded with 1.5 million MC3T3-E1 mouse pre-osteoblast cells. 100 µL of cell suspension was seeded drop-wise onto each scaffold side using an established technique [3]. The scaffolds were kept in alpha minimum essential medium supplemented with 2% penicillin/streptomycin, 1% L-glutamine and 10% foetal bovine serum in an incubator at 37℃ and 5% CO2 for

up to 7 days. Metabolic activity and cell number were monitored at 1, 2 and 7 days post-seeding.

Metabolic activity was measured using a non-destructive colourimetric assay (alamarBlue®). The scaffolds were incubated with a 10% solution of alamarBlue® for 2 hours on an orbital shaker in an incubator. Three 100 µL samples of the medium/ alamarBlue® solution were taken from each scaffold and analysed regarding the absorbance of the supernatant at 540nm and 620nm was on a spectrometer (Titertek, Germany). The percentage reduction of the blue dye to the reduced pink form which represents the metabolic activity of the cells is obtained using:

$$\%reduction = (A_{540} - (A_{620} R_0)) \times 100 \quad (7)$$

$$R_0 = \frac{(A_{blue} - A_{medium})_{540}}{(A_{blue} - A_{medium})_{620}} \quad (8)$$

where A_{540} is the absorbance of the sample at 540nm, A_{620} is the absorbance at 620nm. A_{blue} is the absorbance of a control sample of 10% solution of alamarBlue®, not incubated with a cell-seeded scaffold and A_{medium} is the absorbance of a sample of culture medium.

Cell number was quantified using a DNA assay (Hoechst 33258). This is a fluorescent dye that bonds to double stranded DNA. Scaffolds were digested in papain (Sigma-Aldrich, Germany) in a water bath at 60℃ over 6 hours. 30 µL of

the digested scaffolds were mixed with 600 µL of a working dye solution made up of Tris, Na_2EDTA, NaCl, distilled water and the Hoechst 33258 dye solution (Sigma-Aldrich, Germany). The fluorescence of the samples was measured at 460nm after excitation at 355nm in a multilabel counter (Wallac Victor2™ 1420, Perkin Elmer Life Sciences, Finland). Fluorescence readings were compared to a standard curve to give cell number.

Statistical analysis

Statistical analysis was performed in SPSS using a 2-tailed student-T test for 2 variables or a one-way ANOVA with the Tukey PostHoc test for more than 2 variables. The level for significance was set as $p < 0.05$.

Results

Figure 1 shows the effect of SBF treatment using FT-IR. The spectra of the SBF treated scaffolds were analysed and compared to the spectra of pure untreated collagen scaffolds and HA powder (Plasma Biotal Limited, North Derbyshire UK). HA powder shows distinctive peaks in the region of 500 - 600 cm^{-1} and at 1080 cm^{-1}. Pure collagen scaffolds show peaks at 1600 cm^{-1} and 3400 cm^{-1}. The SBF treated scaffolds possess the typical HA (500 - 600 cm^{-1} and 1080 cm^{-1}) and collagen peaks (1600 cm^{-1} and 3400 cm^{-1}) indicating the presence of a collagen and hydroxyapatite phase in the scaffolds.

Figure 2 shows the effect of SBF treatment on the mechanical properties of the scaffolds, with pure collagen scaffolds serving as control. The compressive moduli were determined from stress-strain curves. Pure collagen scaffolds demonstrated a mean compressive modulus of 0.23 kPa. The treated scaffolds demonstrated a mean modulus of 0.9 kPa which was significantly higher ($p < 0.005$) than the control group.

Figure 3 shows the effect of SBF treatment on the porosity of the scaffolds. The SBF treated scaffolds show a significantly ($p<0.01$) decreased porosity compared to the pure collagen scaffolds. However, while pure collagen scaffolds have a porosity of 99.5%, the SBF treated scaffolds still show a porosity as high as 94.9%.

Figure 4 shows the effect of SBF treatment on the metabolic activity of cells seeded on the scaffolds at 1, 2 and 7 days post seeding. A non-significant increase in metabolic activity over cell culture time was seen from 1 to 7 days for both scaffold types. No statistical difference was found in metabolic activity between pure collagen scaffolds and SBF treated scaffolds.

Figure 5 shows the effect of SBF treatment on the cell number in the scaffolds at 1, 2 and 7 days post seeding. No statistical difference was found comparing the SBF- treated scaffolds with the pure collagen scaffolds, although the SBF treated scaffolds showed a non-significant increase after 7 days post seeding compared to the collagen scaffolds ($p = 0.112$). The pure collagen scaffolds showed similar cell numbers from 1 to 7 days post seeding. However the SBF treated scaffolds showed an increase from day 1 to day 7 post seeding ($p < 0.15$) demonstrating that the composite scaffolds support cell proliferation.

Discussion

Pure collagen and pure hydroxyapatite scaffolds have been commonly used in tissue engineering to date [3,20-25]. While collagen scaffolds show excellent biological performance [2-4,26], they do not possess adequate mechanical properties on their own [24] which limits their potential use for bone tissue engineering. On the other hand hydroxyapatite scaffolds show excellent mechanical properties [27]. However, many investigations have demonstrated that the brittle behaviour and low-degradibility limits the use of pure hydroxyapatite scaffolds in tissue engineering [20-22,28]. In this investigation a novel collagen-hydroxyapatite composite scaffold has been successfully developed by immersing a collagen scaffold in simulated body fluid. The new scaffolds indicate a 4-fold increase in stiffness compared to the control pure collagen scaffolds. Although the porosity was decreased, the porosity remains as high as 95% and the biological performance analysed by cell number and metabolic activity showed promising results *in vitro*.

Adequate mechanical properties are essential in bone TE, not only for implanting cell-scaffold constructs into load-bearing areas, but also for improving their handling attributes during surgery. In this investigation the mechanical properties of pure collagen scaffolds have been improved significantly. Despite this increased stiffness from 230 Pa to almost 900 Pa, the construct does not exhibit the mechanical properties necessary for implantation into load bearing defects without external fixation. However, the handling properties of the scaffolds have

been significantly improved which expands their use for surgical application. Furthermore, from a bone tissue engineering perspective, mineralisation of the scaffolds following extra cellular matrix deposition by osteoblasts would lead to a further increase in the mechanical properties. The novel collagen-hydroxyapatite scaffolds show sufficient mechanical properties for maxillofacial, cranial or other non-load bearing bone defects and the improved mechanical properties may facilitiate increased cellular penetration to the centre of the scaffolds by helping to maintain the interconnected pore structure of the scaffolds during hydration.

The permeability of scaffolds used for tissue engineering is very important as it controls the rate of cell migration, as well as the diffusion of nutrients and waste products in and out of the scaffold [29,30]. The permeability of scaffolds is related to porosity, pore size and the interconnectivity/distribution of the pores [31]. Lyophilised collagen scaffolds developed by Yannas *et al.* and optimised by O'Brien *et al.* show a homogenous pore structure and a mean pore size that can be controlled to vary between 70 μm to 150 μm [2,23]. In this investigation pure collagen scaffolds produced at a final freezing temperature of -40 ºC have been used to obtain a mean pore size of approximately 95 μm and a porosity of 99.5%. Similar scaffolds showed promising results in cell adhesion of MC3T3-E1 mouse clonal osteogenic cells [2] and permeability [4]. The SBF immersion treatment generates a HA layer of only a few micrometers thick which does not affect the interconnected pore structure or alter the mean pore size of the scaffolds [11].

In this investigation, the porosity of the SBF- treated scaffolds was seen to decrease compared to the original pure collagen scaffolds which have a porosity of 99.5%. However, the collagen-hydroxyapatite scaffolds retained a porosity of 95% after the immersion in SBF which is still a highly porous construct compared to many other scaffold types intended for use in bone tissue engineering which typically have porosities ranging from 40-70% [28,32,33]. In particular, in comparison with dense sintered ceramic scaffolds made from CP or HA, the high porosity collagen-hydroxyapatite composite scaffolds have an enormous potential for bone TE or bone repair.

FT-IR spectra of the SBF treated scaffolds show the presence of HA on the SBF treated scaffolds (Figure 3). Compared to the control group (pure collagen), distinctive peaks between the wavenumbers 560–660 cm^{-1} can be seen. These peaks exhibit similar characterisitcs to HA powder [34]. The peak at 600cm^{-1} represents calcium crystal formation [34]. A second peak at 1030cm^{-1} with a shoulder peak at 1045cm^{-1} also indicates a similarity to the HA powder peaks. This peak seen in the spectra of the treated scaffold represents orthophosphate (PO_4^{3-}) formation in the scaffold [34]. Both peaks show the presence of HA in the scaffolds after treatment with SBF. The shape of the peaks between 500cm^{-1} and 600cm^{-1} with some distinctive peaks on a broad curve indicates the presence of crystalline HA, and amorphous CP. The peaks at 1630cm^{-1} and 3410cm^{-1} with their similarities to the collagen scaffold spectra indicate the presence of collagen after the treatments.

The novel developed biomimetic collagen-hydroxyapatite scaffolds showed an excellent biocompatibility and bioactivity *in vitro* using assays to analyse the cell number (Hoechst 33258) and metabolic activity (alamarBlue®). An increase in mean cell number per scaffold was observed on the composite collagen-hydroxyapatite scaffolds after 7 days. No statistical difference in the biological performance could be seen between collagen-hydroxyapatite scaffolds and pure collagen scaffolds. Analysing the metabolic activity of the cells supported these findings, indicating an increase in metabolic activity at seven days compared to the first day. This trend was not significant due to high deviations but is nonetheless a very promising result as it suggest that the composite scaffolds support cell proliferation. Pure collagen scaffolds are known to exhibit excellent biological performance and to support cell adhesion, growth and proliferation [3,25,35]. Statistical analyses showed no significant differences between the pure collagen scaffolds and the collagen-hydroxyapatite scaffolds, indicating the same excellent biological performance as pure collagen scaffolds in the new constructs.

Taken together, these cell culture experiments indicate cellular proliferation on composite scaffolds over a 7 day period. This is the first step in the development of bone tissue: proliferation occurs for the first 10-12 days of culture, followed by ECM synthesis and maturation and finally mineralisation [36]. The composite scaffolds support this initial step which is promising. Longer culture periods using osteogenic medium are needed to ascertain whether or not the scaffolds support further development of bone tissue.

Conclusion

This investigation has successfully developed a biomimetic collagen-hydroxyapatite composite scaffold using a SBF immersing technique. This novel collagen-hydroxyapatite scaffold demonstrates increased stiffness compared to pure collagen scaffolds while still retaining a highly porous structure (95% pore volume). These results, combined with the promising biological performance of the scaffold demonstrate its potential for bone tissue engineering.

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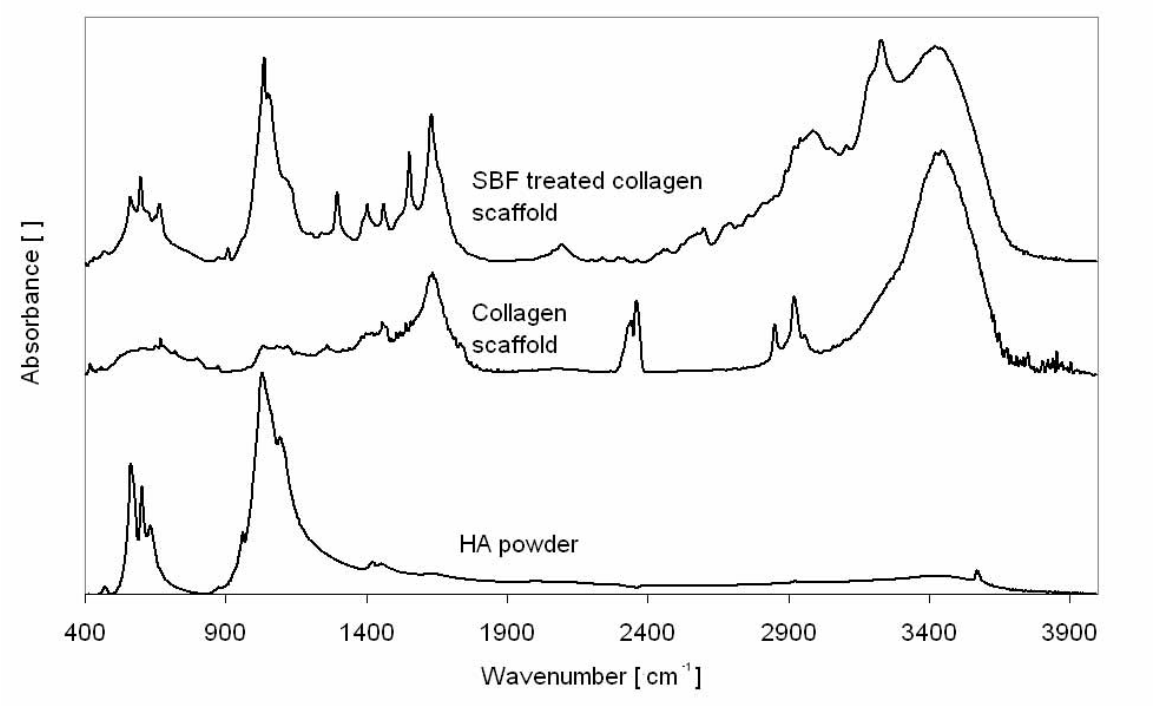

Figure 1: FT-IR Spectra of HA powder, pure collagen scaffolds and SBF treated collagen scaffolds (n=6).

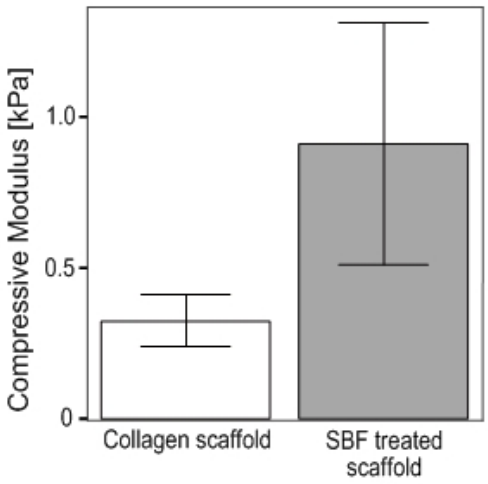

Figure 2 Compressive moduli of the collagen scaffold and the SBF treated scaffolds (n=9).

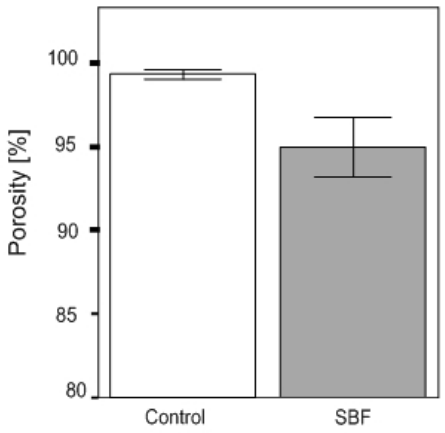

Figure 3: Porosity of the collagen scaffold and the SBF treated scaffolds (n=6).

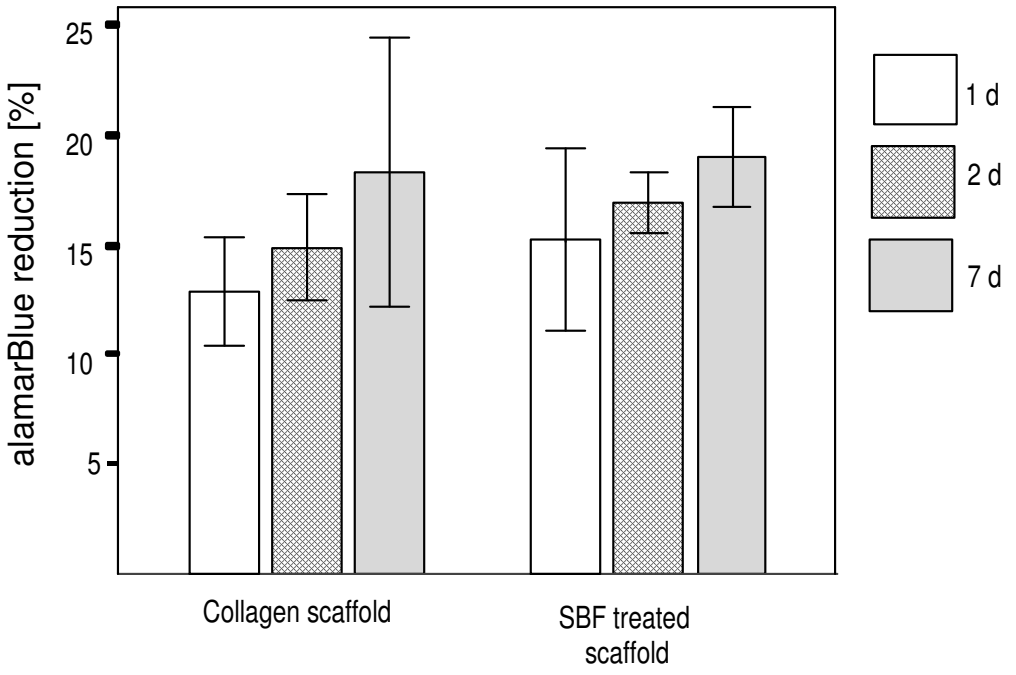

Figure 4: Metabolic activity of SBF treated collagen scaffolds and pure collagen scaffolds at 1, 2 and 7 days post seeding (n=9).

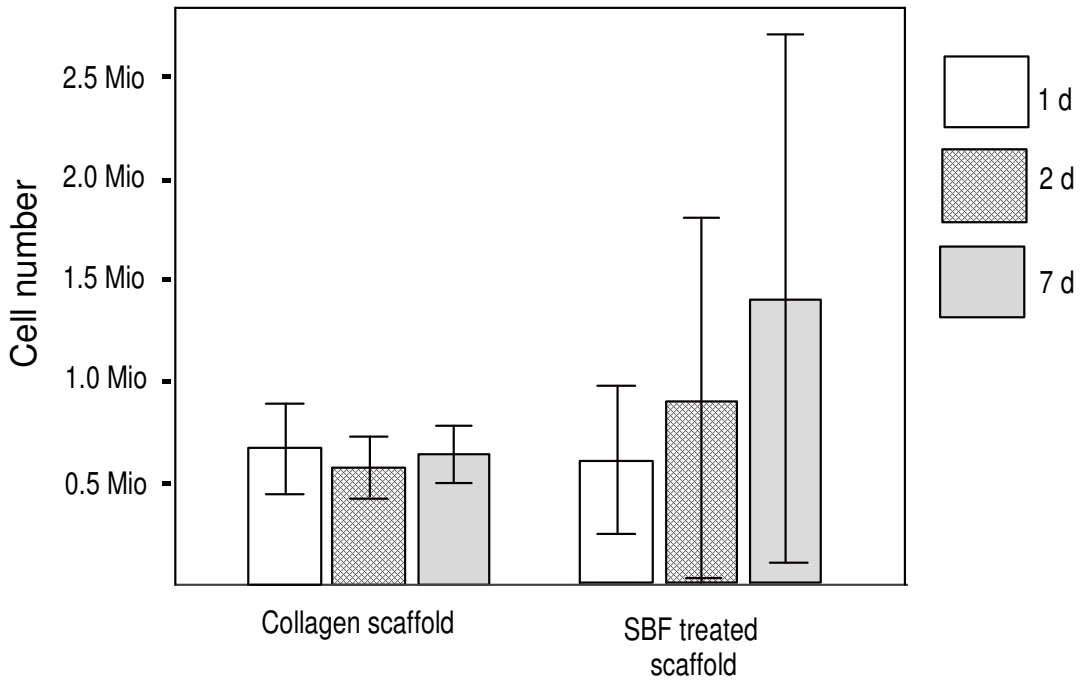

Figure 5: Cell number of SBF treated collagen scaffolds and pure collagen scaffolds at 1, 2 and 7 days post seeding (n=9).