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# Effect of multiple unconfined compression on cellular dense collagen scaffolds for bone tissue engineering

**Malak Bitar · Vehid Salih · Robert A. Brown · Showan N. Nazhat**

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**Abstract** Plastic compression of hydrated collagen gels rapidly produces biomimetic scaffolds of improved mechanical properties. These scaffolds can potentially be utilised as cell seeded systems for bone tissue engineering. This work investigated the influence of multiple unconfined compression on the biocompatibility and mechanical properties of such systems. Single and double compressed dense collagen matrices were produced and characterised for protein dry weight, morphology and mechanical strength. Compression related maintenance of the seeded HOS TE85 cell line viability in relation to the extent of compression was evaluated up to 10 days in culture using the TUNEL assay. Fluorescence Live/Dead assay was conducted to examine overall cell survival and morphology. Cell induced structural changes in the dense collagenous scaffolds were assessed by routine histology. The mechanical properties of the cellular scaffolds were also evaluated as a function of time in culture.

It is clear that a single plastic compression step produced dense collagenous scaffolds capable of maintaining considerable cell viability and function as signs of matrix remodeling, and maintenance of mechanical properties were evident. Such scaffolds should therefore be further developed as systems for bone tissue regeneration.

M. Bitar (✉) · V. Salih · S. N. Nazhat
Divsion of Biomaterials and Tissue Engineering, UCL Eastman Dental Institute, 256 Gray's Inn Road, London, WC1X 8LD, UK
e-mail: m.bitar@eastman.ucl.ac.uk

R. A. Brown
UCL Tissue Repair & Engineering Centre, Institute of Orthopaedics, Stanmore Campus, London, HA7 4LP, UK

S. N. Nazhat (✉)
Department of Mining, Metals and Materials Engineering, McGill University, 3610 University Street, Montreal, QC, H3A 2B2, Canada
e-mail: showan.nazhat@mcgill.ca

## 1 Introduction

It is estimated that each year, approximately 1 million patients in the United States undergo bone graft augmentation surgery to repair skeletal defects [1]. Autologous bone grafting is often associated with donor site pain and morbidity whilst, the use of allografts may introduce the risk of disease transmission and immunorejection [2–4]. Such disadvantages can be avoided by the use of biocompatible scaffold systems as cell delivery vehicles for tissue repair where the maturity and integration into the host may occur. When dealing with critical size defects, such cell-seeded systems can be configured to encompass *in vitro* morphogenesis of *de novo* tissue units. These units can later be implanted into the area of need [5–7].

Numerous forms of scaffold materials are currently under investigation among which, naturally derived polymers have been shown to possess potentially excellent characteristics as templates for tissue regeneration. These biopolymers, whether animal or plant in origin, have attracted much interest due to their low immunogenic potential, unlimited source and the capacity of interacting with the host tissue [8]. Collagen, fibronectin, chitosan, starch, hyaluronic acid, and poly (hydroxybutyrate) are examples of the many biopolymers currently in use. As the main extracellular matrix (ECM) protein in musculoskeletal tissues, collagen has been the scaffold material of choice.

Collagen directly supports integrin-mediated cell attachment and the subsequent expression, and maintenance, of the seeded cell differentiated state. Several studies have also concluded that collagen based matrices may exhibit

bioactive properties by promoting cell migration into the scaffold [9, 10]. Collagen matrices can also be remodelled by cell action resulting in greater functional integration into the host tissue. When derived from xenogenic sources (e.g. rat tail or bovine skin), collagen can be treated with various purification techniques thus minimizing potential immunogenicity [11].

Hydrated fibrillar collagen gels are of particular interest as scaffolds for tissue engineering and can be prepared from purified soluble collagen in acidic solution. Stable gel scaffolds can be produced by exposing the solution to neutralisation and temperature changes [12]. Owing to an extremely low protein to water ratio, these hydrated matrices have no inherent mechanical strength thus limiting their immediate *in vivo* application. This in turn has necessitated the use of various methods to enhance the mechanical properties and integrity of such substances. For example, hydrated collagen gels were subjected to chemical cross-linking to produce rigid structures [13]. Such modifications may render fibrillar collagen matrices non-susceptible to cell based remodelling thus compromising the biomimetic characteristics of the scaffold. Also, chemical cross-linking, may reduce the possibility of incorporating cells during scaffold preparation.

*In vitro* cell mediated collagen remodelling, followed by hydrated gel contraction, has also been shown to enhance both density and mechanical strength of the scaffold, therefore the potential for *in vivo* application [13, 14]. However, this process is time consuming and can only result in a limited increase in collagen density. On the other hand, the feasibility of a rapid delivery cell-seeded system with appropriate biological and mechanical properties, for the repair of tissue defects, remains critically time dependent.

Recently, Brown et al. [15] developed a novel method of rapidly producing dense collagen fibrogels through unconfined compression, which resulted in the exclusion of up to 98% of the scaffold water content. This plastic compression (PC) process produced tissue-like collagenous scaffolds with considerable mechanical strength supporting cell viability and proliferation of human dermal fibroblasts for 5 weeks in culture. PC processed collagen matrices may therefore be considered as potentially ideal scaffold systems for bone tissue engineering. Moreover, it has been shown that a further increase in mechanical properties could be produced by subjecting the materials to a secondary compression process [15, 16]. This controlled "double compression" of collagen matrices reduced the fluid content by a further 50–60%, and reproducibly increased the mechanical properties. Therefore, it appears that control of the scaffold mechanical properties can be achieved by the level of compaction and, accordingly, the extent to which interstitial fluid is expelled.

As fibrillar collagen content and tensile mechanical strength of PC scaffolds appear to be directly linked to the extent of compression, this work investigated the relationship between single and double compression processes and the seeded cell viability together with the potential for fine control of matrices.

## 2 Materials and methods

### 2.1 Production and characterisation of dense collagen matrices through multiple compression

To prepare acellular scaffolds 0.2 ml of X10 D-MEM (Dulbecco's Modified Eagle Medium) was added to 1.6 ml of sterile rat-tail type I collagen dissolved in acetic acid (2.2 mg/ml, First Link Ltd., West midlands, UK). Collagen solution was neutralised by sequentially introducing 3 $\mu$l aliquots of 5 M NaOH. Immediately thereafter, 0.2 ml of D-MEM at 37°C was added and the solution transferred into rectangular moulds (13 × 33 × 4 mm) and left to set at 37°C for 30 min. Dense collagen scaffolds of varying collagen concentrations were produced as previously described [15,16] by either single (SC) or double (DC) compression. Briefly, the resulting hydrated collagen gels were subjected to unconfined compressive load of 1.4 $kN{\cdot}m^{-2}$ (50 g/ 4.29 $mm^2$) for 5 min (SC) to produce collagen sheets which were subsequently rolled, along the short axis, to give concentrically multi-layered cylinder scaffolds of ∼2 mm diameter. Double compression was carried out by a further application of a 50 g weight for 5 min, normal to the long axis of the cylinder (stress equivalent to approximately 22.6 $kN{\cdot}m^{-2}$) resulting in a strap like structure.

Water and collagen content were measured for both SC and DC scaffolds through gravimetric weight measurements before and after a freeze drying process. Once compressed, samples were immediately immersed in liquid nitrogen for 1 min and subsequently freeze-dried (Heto Drywinner, Birkerod, Denmark) for 24 h; and finally placed in a desiccator for an additional 48 h. Weight measurements up to 5 decimal places were recorded using an analytical balance (BDH, UK) at every stage until an equilibrium weight was reached.

Scanning electron microscopy was carried out to determine relative scaffold morphology following compression. Freeze dried samples were sectioned along the long axis using no. 10 surgical scalpel, mounted and coated with gold-palladium using a Polaron E5 100 sputter coater (Quorum Technologies, East Sussex, UK). Images were obtained using a Stereoscan 90B electron microscope (Cambridge Instruments Ltd., Cambridge, UK).

### 2.2 Effect of multiple compression on cell viability and activity

The effect of SC and DC on cell viability within the dense collagen gels was assessed using human osteosarcoma (HOS

TE85, ATCC, Manassas (VA), USA) cell line cultured for up to 10 days. These cells were selected for their osteoblastic characteristics. All cultured cells were maintained in a humidified atmosphere, in 5% $CO_2$ and at 37°C. The standard growth medium used was prepared using D-MEM supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (all from Invitrogen, Paisley, UK). Cells were propagated in 10 ml of medium using 80 $cm^2$ cell culture flasks (BD Biosciences, Oxford, UK) with gas-permeable caps. SC and DC cellular scaffolds were prepared as above however, with HOS cell addition at a pre-compression density of $3 \times 10^5$ cells/ml.

Initially, cell viability was assessed after 10 days in culture using the LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen, Paisley, UK). Samples were incubated for 45 min in D-MEM supplemented with 1:1000 concentration of Calcein AM and Ethidium homodimer-1 (Invitrogen Ltd., Paisley, UK) to stain live and dead cells respectively. Samples were fixed in 99% ice cold methanol for 10 min, washed x3 in PBS (Phosphate buffered saline) and mounted between two glass cover slips in citiflour AF2 mounting medium (Citifluor Ltd., London, UK) for examination under the Leica DM-IRB flourescence inverted microscope (Leica Microsystems UK Ltd., Bucks, UK). For each area of interest, 10 sequential images were obtained in the vertical (Z) plane covering 50 $\mu$m of the samples' depth and later superimposed to produce composite images.

Following the initial assessment of cell viability, signs of the seeded cell death were assessed for up to 10 days in culture using the TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) reactivity assay. At day 1, 5 and 10 in culture, samples were fixed in 10% formol saline and auto-embedded in paraffin wax by an overnight automated process using a Citadel 1000 tissue processor (Thermo Electron Corporation, Cheshire, UK). Samples were subsequently sectioned at a depth of 100 $\mu$m to produce 5 $\mu$m sections. Sections were later mounted on poly-l-lysine coated slides, rehydrated and nuclear DNA fragments were labelled using TUNEL-based DAB-conjugated TACS-XL *In Situ* Apoptosis Detection Kit (R&D Systems Europe Ltd., Oxon, UK). A minimum of $1 \times 10^3$ cells were counted in adjacent X20 objective light microscope fields using an eyepiece micrometer (Type H12 SQUARE 10MM/20D). The number of cells with TUNEL reactivity (positive brown DAB stain) was established to obtain a TUNEL index. Methyl green dye was used to counter stain all nuclei.

To assess cell induced structural changes in the seeded collagenous scaffolds, samples were histologically fixed and embedded in paraffin as described above. Sections were Hematoxylin/Eosin stained for light microscopy. Scaffolds seeded with dead cells were used as control to assess any changes in the scaffold morphology as a result of cell action. These control cells were treated prior to seeding in 4% glutaraldehyde solution for 10 min.

### 2.3 Investigations into the mechanical properties of scaffolds as function of time in culture

Tensile testing was carried out to investigate the mechanical properties of cellular and acellular single and double compression produced scaffolds after 1, 5 and 10 days in culture. Samples were clamped at each end using 2 mm wide stainless steal mesh strips, mounted on a dynamic mechanical analyser (DMA-7e, Perkin-Elmer, Bucks, UK) and subjected to quasi-static uniaxial tension at the rate of 200 mN $min^{-1}$. Data was collected using the Pyris software (version 5.02, Perkin-Elmer, Bucks, UK) where the breaking force values were obtained as an indication of strength.

## 3 Results

### 3.1 Acellular scaffold characterization

As seen from Table 1, weight loss for both SC and DC scaffolds following water exclusion suggested that despite the compression process, water content within the scaffold remains significantly higher than that of protein. It is clear following double compression that the scaffolds accommodated higher protein concentration ($23.1 \pm 3.1\%$) than SC scaffolds ($12.6 \pm 2.0\%$). Also the multi-layered structure of the scaffold was apparent when samples were examined using SEM. As expected, clear differences in layer cross-section measurements were observed between SC (Fig. 1(A)) and DC (Fig. 1(B)) processed scaffolds.

### 3.2 Cell viability assessment

Fluorescence microscopy revealed a dense and predominantly viable cell population in the SC processed scaffolds at day 10 in culture (Fig. 2(A) and (B)). Live cells (green cytoplasm) exhibited a well spread morphology throughout the examined areas whereas relatively few dead cells, labelled with red nuclear stain, were observed. In DC scaffolds on the

**Table 1** Weight measurements to determine protein concentration in SC and DC processed scaffolds ($n = 3$)

| Stage of compression | Weight loss (wt%) | Measured collagen concentration (wt%) |
|---|---|---|
| Single compression | ~ 98% from original neutralized weight | $12.6 \pm 2$ |
| Double compression | ~ 50% from single compression | $23.1 \pm 3.1$ |

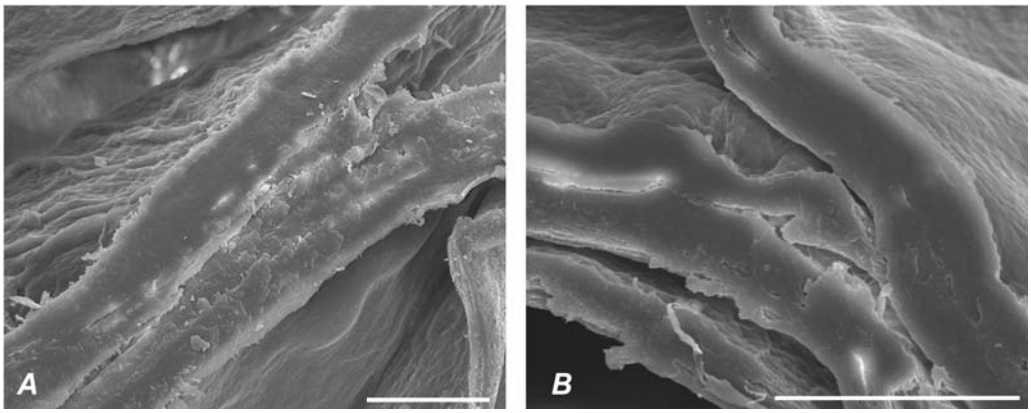


**Fig. 1** Scanning electron microscope images of sectioned freeze dried SC (A) and DC (B) scaffolds showing the multi-layered roll structure. Scale bars = 30 $\mu$m

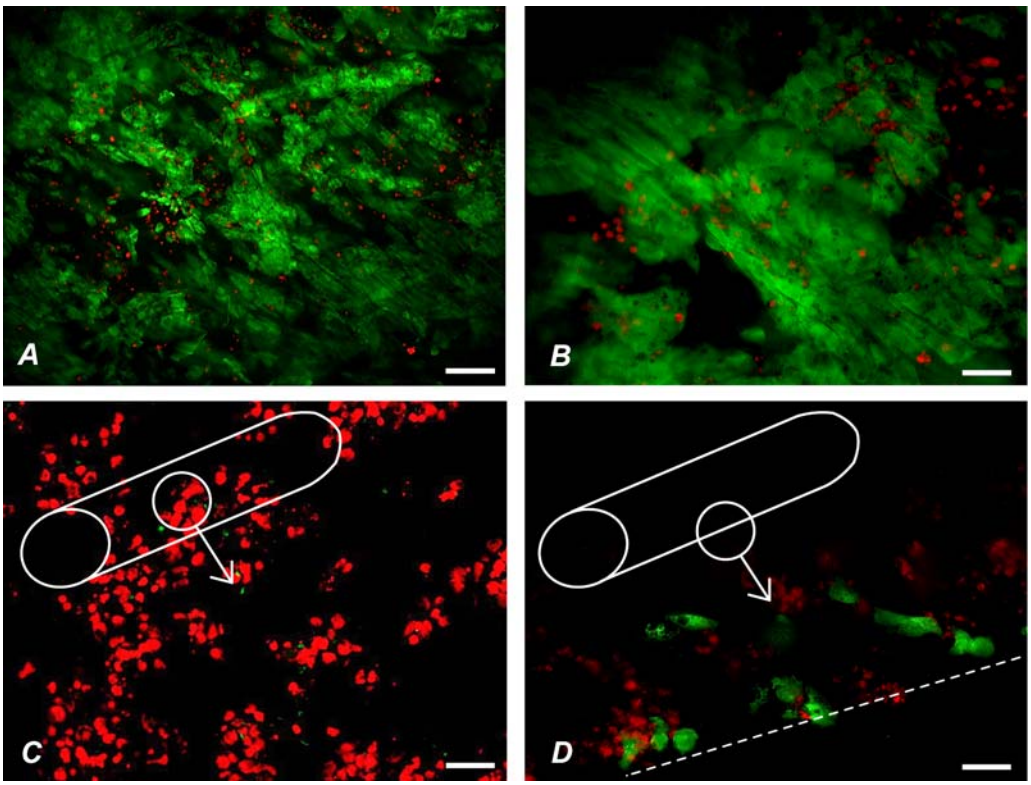


**Fig. 2** Fluorescence microscope images of HOS cells seeded in collagen scaffolds at day 10 in culture. A and B, SC scaffolds. C and D, DC scaffolds. Samples were stained for Calcein AM (green cells) and Ethidium homodimer-1 (red nuclei). Scaffold edge is demarcated by the dashed white line. Scale bars: A = 50 $\mu$m, B, C and D = 100 $\mu$m

other hand, clear signs of cell death were observed as the presence of red stained nuclei was predominant throughout the entire structure (Fig. 2(C)). However, few live Calcein AM positive cells were detected. The presence of this limited cell viability was confined to the external edges of the scaffold (50–100 $\mu$m, Fig. 2(C)) and therefore, to the superficial layers of the collagen roll.

Accordingly, the TUNEL reactivity assay was carried out on histology sections obtained at 100 $\mu$M from the scaffold surface. As seen from Fig. 3, a number of seeded cells in both SC (Fig. 3(A)) and DC (Fig. 3(C)) scaffolds exhibited signs of nuclear DNA degradation by day 1 in culture. By day 10, the cells seeded within SC processed scaffolds remained predominantly viable (Fig. 3(B)) however, all the observed nuclei in the DC processed scaffold exhibited TUNEL reactivity (Fig. 3(D)). Despite evidence of cell death (12.6% of the total cell count, Fig. 4) at day 1 in culture, no significant increase in the number of cells exhibiting TUNEL positivity occurred at later time points in the SC scaffolds. In contrast, within the DC processed scaffolds there was a

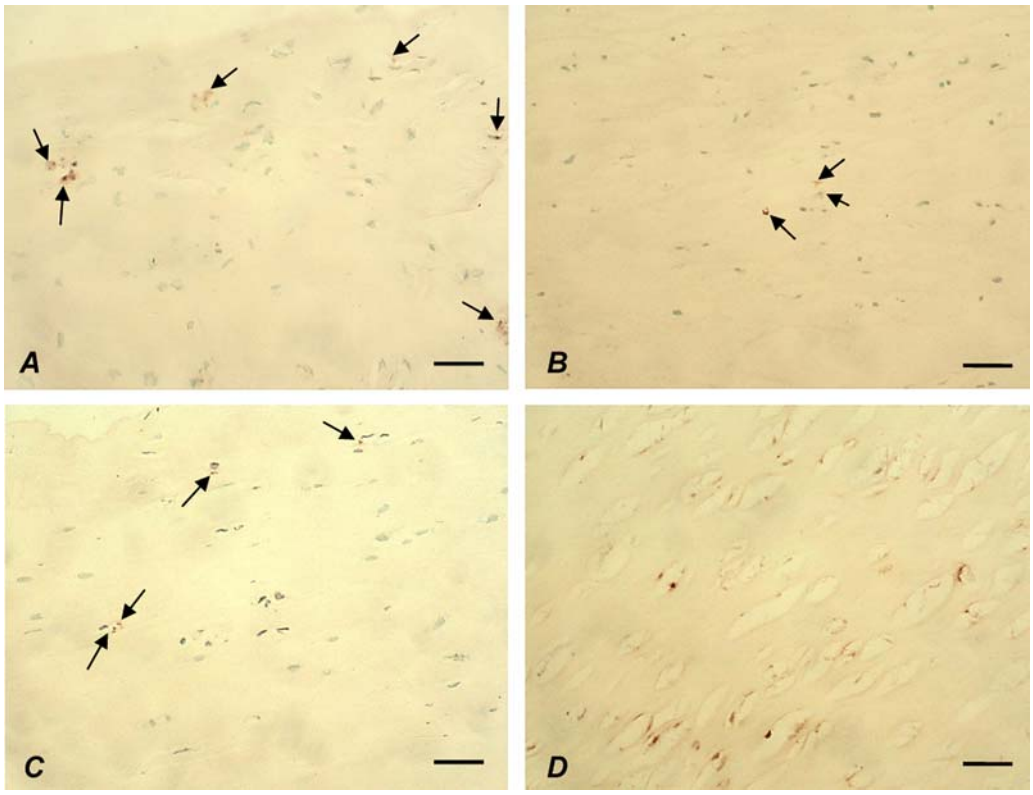


**Fig. 3** Light microscope images of paraffin embedded histology sections showing TUNEL reactivity positive nuclei (DAB positive brown nuclei, black arrows). A and B, SC scaffolds at days 1 and 10 in culture respectively. C and D, DC scaffolds at days 1 and 10 in culture respectively. Counterstain (green nuclei) was performed using Methyl Green dye. No arrows illustrated in Figure D as all nuclei exhibited DAB positivity. Scale bars = 50 $\mu$m

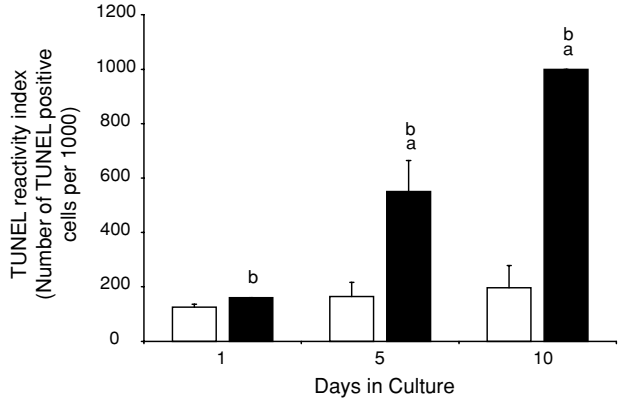


**Fig. 4** TUNEL reactivity index for HOS cells seeded in SC (white bars) and DC (black bars) scaffolds at days 1, 5 and 10 in culture. For each sample, $10^3$ nuclei were counted in X20 objective adjacent light microscopic fields using an eyepiece micrometer (Type H12 SQUARE 10MM/20D). *a* indicates significant differences in value for each compression method as a function of time. *b* indicates significant differences in value between SC and DC at single time points. ($n = 3$, error bars + SD)

significant increase in TUNEL positive cell numbers reaching $\approx 55\%$ at day 5, and 100% by day 10. Numbers of TUNEL positive cells were also significantly higher in DC scaffolds when compared to those obtained for the SC scaffolds at all time points ($p < 0.005$). It is worth noticing nonetheless, that TUNEL positive cell number in DC scaffolds was comparatively low (average of 16%) at day 1 in culture thus the effect of multiple compression on cell viability could be considered minimal.

### 3.3 Cellular scaffold characterisation

Following H&E staining, light microscope examination revealed HOS cells to be embedded in a dense collagenous matrix at day 1 time point for both, SC and DC scaffolds (Fig. 5(A) and (D)) where the multi-layered nature of the collagen scaffold was indistinguishable. By day 5, and for both SC and DC compressed scaffolds (Fig. 5(B) and (E)), a network-like structure appeared to have formed around the seeded cells. The occurrence of this lamellae reorganisation of collagen fibrils was also detected by day 10 in culture (Fig. 5(C) and (F)) and is consistent with previous findings [15]. This lamellae formation was not observed when dead cells were deliberately seeded with the scaffolds (Fig. 5(G) and (H)).

Tensile mechanical analysis data (Fig. 6) showed that at day 1 for cellular and acellular scaffolds, the DC processed scaffolds exhibited significantly ($p < 0.005$) greater break

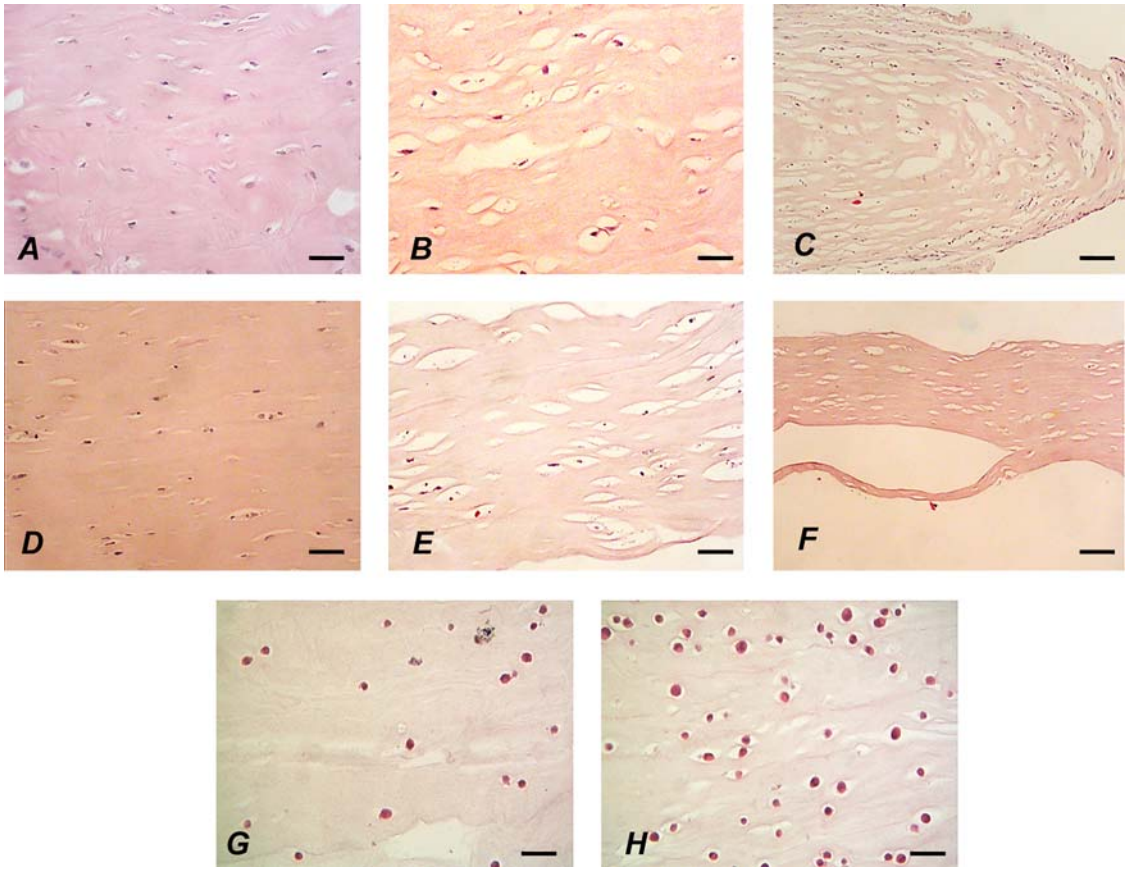


**Fig. 5** Light microscope images of paraffin embedded H&E stained histology sections. HOS seeded SC scaffolds at days 1 (A), 5 (B) and 10 (*C, Low magnification*). HOS seeded DC scaffolds at days 1 (D), 5 (E) and 10 (*F, Low magnification*). *G* and *H*, dead cell SC processed scaffolds at day 10. Scale bars A, B, D, E, G and H = 50 $\mu$m, C and F= 100 $\mu$m

force values than SC scaffolds. By day 5 in culture, no significant differences in this value were apparent among all samples. Similarly, and by day 10, break force values were similar for all the samples investigated however, a significant drop in mechanical strength for both SC and DC cellular scaffolds occurred. It can be concluded therefore, that HOS cells did not significantly increase matrix strength during remodelling.

## 4 Discussion

Bone is an extremely dynamic structure continuously undergoing remodelling and is thus maintained in a state of functional homeostasis. Despite being mainly constituted by an inorganic mineral, hydroxyapatite (up to 70% weight of the mature cortical bone), bone structural cohesion and architecture are vitally supported by a highly organised collagenous matrix [17, 18]. During *in vivo* bone remodelling, and throughout the formation phase, a pre-mineralised osteoid is deposited by osteoblasts comprising primarily type I collagen (95%). The use of collagenous matrices therefore to either conduct, or induce, *de novo* bone formation is of extreme significance particularly if the mechanical properties of such matrices can be enhanced via the application of plastic compression.

Dilute acidic collagen solutions can be converted into a stable hydrated gel through pH modification whereby hydrophobic interactions become predominant leading to the aggregation of collagen fibrils [12, 19]. This process is based on exploiting the capacity of collagen molecules to self-assemble under certain conditions in a process strongly resembling *in vivo* fibrillogenesis [20, 21]. These hyperhydrated gels can be compressed to produce structurally stable, high density collagenous scaffolds. The extent and duration of the compression process influenced the final protein concentration. DC scaffolds were almost twice as dense as the SC scaffolds (23 versus 12wt% collagen respectively), which significantly increases the mechanical properties of the scaffold [16]. It is important however, to define and control the balance between increasing collagen density and maintaining the appropriate environment for cell survival and function.

The TUNEL assay was used to establish, and quantify, signs of cell death at a 100 $\mu$m depth from the scaffold surface. The TUNEL assay was developed to detect specific signs of nuclear DNA degradation as an indication of apoptosis (programmed cell death, PCD). The onset of PCD,

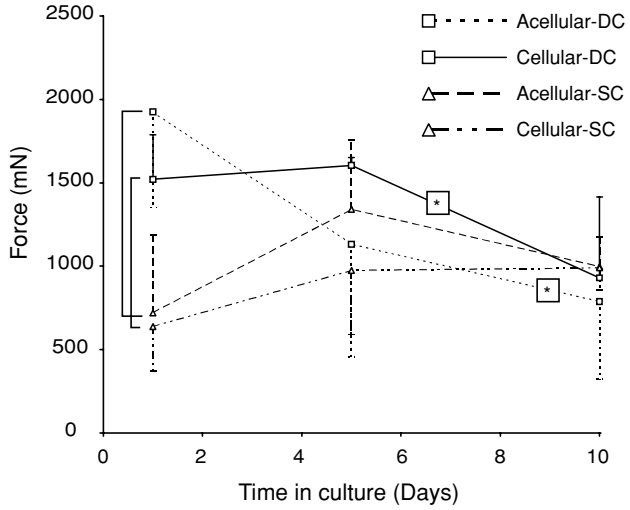


**Fig. 6** Break force values for SC and DC processed scaffolds. Significant differences in break forces between different samples and at each time point are connected using square brackets. Significant differences in each sample break force as a function of time in culture are indicated by (*). ($n = 3$, error bars − or + SD)

at early stages, is followed by chromatin condensation and nuclear DNA "laddering" (cleavage) into large fragments by endonucleases. TUNEL reactivity is induced by labelling "nick" DNA fragments (single stranded) using the tdt enzyme (terminal deoxynucleotidyl). It has been shown however, that cells undergoing necrosis exhibit the same signs in terms of DNA cleavage as apoptotic cells [23]. This has put into question the specificity of TUNEL alone as a marker technique for PCD [24–26]. Indeed, TUNEL reactivity can be used as labelling method for cell necrosis [27]. Accordingly, it was clear that at a certain depth cell survival in DC processed scaffolds became limited as a function of time in culture as, and by day 10, no signs of cell viability were detected. This may be attributed to the lack of nutrient and oxygen diffusion into the scaffold due to DC related increase in collagen density and more significantly, the increase in seeded cell density. Compression related increase in cell density and oxygen diffusion into these scaffolds is currently under investigation (Brown, Cheema, Alp and McRobert In preparation).

The use of fluorescence microscopy highlighted the above findings where viable cells were detected in DC processed scaffolds were only limited to the superficial layers. SC processed scaffolds on the other hand maintained a viable population up to day 10 in culture with no significant increase in cell death as a function of time. In fact, cell viability remained above 80% for the seeded population at all the investigated time points. Furthermore, at day 10, examination under the fluorescence microscope revealed dense viable cell populations across the SC scaffold where cells exhibited a well spread morphology indicative of maintenance of cell function. It is well recognised that following the attachment of anchorage-dependent cells, reorganization of actin microfilaments initiates various intracellular signalling mechanisms influencing major cellular events such as proliferation, differentiation, protein synthesis and survival. A well spread morphology is therefore vital for cell survival and expression of the differentiated function [28].

Following histological evaluation, it was evident that a degree of cell-scaffold interaction took place, and was clearly related to the presence of a viable cell population where a network-like structure appeared to have formed around the seeded cells. This strongly suggested an active role of the seeded HOS cells in conducting a certain degree of matrix remodelling, which could also be influenced by fluid infiltration into the scaffolds followed by a swelling effect. The extent and exact nature of such remodelling however, must be further investigated and confirmed as the maturity of the collagenous matrix in terms of collagen fibrils alignment, orientation and cross-linking is important for subsequent mineralization [29].

This study used the break force value as an indicator of tensile mechanical strength when comparing SC and DC processed scaffolds. At day 1 tensile tests showed significant increase in the break force when comparing DC versus SC, which is clearly related to the increase protein concentration and the physical interaction between the nano-sized fibrils as well as the micro-layering of the spirals. Following compression, and as collagen is the load bearing component, the tensile mechanical properties of collagen gels are dominated by the nature, orientation and physical interaction of the constituting protein fibrils. Also the level of frictional forces required to break DC as apposed to SC may be higher. However, this higher break force value obtained for DC processed scaffolds underwent a gradual decrease, as a function of time, to a level matching that of the SC scaffolds by day 10 in culture. This may be related to post-compression relaxation in the physical entanglement within the fibrillar collagenous network followed by a significant decrease in the level of the intra-fibrillar frictional forces. The mechanical behaviour of cellular scaffolds showed that after 10 days of culture, HOS cells did not significantly increase matrix strength during remodelling.

## 5 Conclusions

Double multiple compression of hydrated collagenous scaffolds may initially result in enhanced mechanical properties nonetheless, the DC process exerted a negative impact on the seeded cell survival. In contrast, SC processed scaffolds supported cell viability with a strong indication of the cell-collagenous matrix interaction. SC processed collagenous scaffolds should therefore be further investigated as cell-seeded matrices for bone regeneration.

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