Full-field strain of regenerated bone tissue in a femoral fracture model

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Summary

The mechanical behaviour of regenerated bone tissue during fracture healing is key in determining its ability to withstand physiological loads. However, the strain distribution in the newly formed tissue and how this influences the way a fracture heals is still unclear. X-ray Computed Tomography (XCT) has been extensively used to assess the progress of mineralised tissues in regeneration and when combined with in situ mechanics and digital volume correlation (DVC) has been proven a powerful tool to understand the mechanical behaviour and full-field three-dimensional (3D) strain distribution in bone. The purpose of this study is therefore to use in situ XCT mechanics and DVC to investigate the strain distribution and load-bearing capacity in a regenerating fracture in the diaphyseal bone, using a rodent femoral fracture model stabilised by external fixation. Rat femurs with 1 mm and 2 mm osteotomy gaps were tested under in situ XCT stepwise compression in the apparent elastic region. High strain was present in the newly formed bone ($\varepsilon_{p1}$ and $\varepsilon_{p3}$ reaching 29 000 $\mu$ε and $\sim$43 000 $\mu$ε, respectively), with a wide variation and inhomogeneity of the 3D strain distribution in the regenerating tissues of the fracture gap, which is directly related to the presence of unmineralised tissue observed in histological images. The outcomes of this study will contribute in understanding natural regenerative ability of bone and its mechanical behaviour under loading.

Introduction

Bone fracture healing is a complex process that has been extensively investigated (McKibbin, 1978; Perren, 1979; Zhao et al., 2009; Claes et al., 2012; Loi et al., 2016) and it is influenced by both the local biological and mechanical environment (Harwood et al., 2010). Optimal fracture healing requires mechanical stability in close proximity of the fracture ends, since large movements can negatively affect this process. However, some micromotion is necessary as completely rigid fixation has shown only partial fracture healing (Perren, 1979; Harwood et al., 2010). In cases where the two fracture ends are not in contact, secondary healing occurs. This can be described by three basic steps: inflammation, cellular proliferation and differentiation and remodelling (Marsell & Einhorn, 2011). These phases occur in different locations and at different rates. In the second step, intramembranous woven bone is deposited in the void between the ends, and it is later replaced in the remodelling process by more mature lamellar bone (Perren, 1979). Intramembranous and endochondral ossification can occur simultaneously leading to formation of both soft and hard callus (uncalcified and calcified/woven bone, respectively). It has been shown that hard callus starts forming in the inner layer of periosteum at a certain distance from the fracture site progressing towards the gap predominantly at the periphery (Claes et al., 2012; Loi et al., 2016), whereas soft callus is forming centrally (Harwood et al., 2010). The combination of these two actions increases the fracture stiffness and stability. Any defects that cannot heal spontaneously without intervention are considered critical-sized defects. It was recognised that defects without complete healing after 52 weeks would remain as such. In humans, nonhealed defects are those induced by trauma or ablative oncological surgery (Ochandiano Caicoya, 2007). A standardised method to stabilise a critical-sized defect is the use of external fixators (Lippert & Hirsch, 1974; Kaplan et al., 1985; Claes et al., 2002; Seide et al., 2004). They ensure alignment of the bone fragments and allow control over the degree of interfragmentary movement, which develops under external loading and muscle activity.

Histology has been widely used to assess bone healing at the microscale throughout the entire process (Manjubala et al., 2009; Mora-Macias et al., 2017) to identify the type of defect (Epari et al., 2006; Zandi et al., 2019), to evaluate fixation efficiency (Claes et al., 2008; Meeson et al., 2019) and to
classify the mineralised and unmineralised tissue formation in the healing stages (Epari et al., 2006; Manjubala et al., 2009). The mineralisation of the fracture over time provides information on the effect of fixator stiffness (Zandi et al., 2019), on the use of biomaterials and pharmaceuticals to enhance fracture healing. This investigates only selected two-dimensional (2D) regions of the tissue. Macroscopically, the fracture healing process has been also analysed in a three-dimensional (3D) manner with the use of high-resolution X-ray computed tomography (XCT) (Gabet et al., 2004; Shefelbine et al., 2005; Isaksson et al., 2009; Morgan et al., 2009; Kustro et al., 2018). Specifically, high-resolution XCT imaging allows details on the patterns of bone tissue remodelling as well as information on the time-dependent changes of bone structure during healing. However, both 2D and 3D imaging evaluations were only able so far to quantify the morphology of bone regeneration, without any specific information on its quality in terms of mechanical properties.

Digital volume correlation (DVC) is gaining increased popularity in the experimental computation of 3D full-field strain in bone. Typically, DVC correlates high-resolution XCT images obtained in situ to measure the full-field displacement and derived strain at the tissue (Gillard et al., 2014; Peña-Fernández et al., 2018) and bone apparent levels (Christen et al., 2012; Tozza et al., 2016; Dall’Ara et al., 2017). DVC has been used on long bones to better understand the strain distribution of in vivo experiments on mice tibia (Giorgi & Dall’Ara, 2018), where the tibiae were tested within an in vivo XCT system and DVC performed on these tomograms to identify the precision error of local strains. Additionally, DVC was employed to compute the full-field strain at tissue level in bone specimens where the newly formed bone was favoured by the action of resorbable biomaterials (Peña Fernández et al., 2019; Peña-Fernández et al., 2020). Digital image correlation (DIC) has also been applied to identify the 2D strains on the surface of intact whole bone (Szefek et al., 2010). To such extent, Yavari et al. (2013) used DIC to examine 3D surface strains in the linear elastic region of rat femora during compression testing until failure, in order to elaborate strain-based fracture criteria (Yavari et al., 2013).

To the best of the authors’ knowledge, the investigation of internal strain distribution in the regenerated bone of naturally healed fracture models using DVC has not been previously investigated. Therefore, the aim of this study is to assess the load-bearing capacity and the 3D full-field strain distribution in the regenerated diaphyseal bone tissue using DVC. Furthermore, histology is used to assess the regeneration status of both calcified and uncalcified tissues in the region.

Materials and methods

Materials

Seven Wistar rat female femurs with osteotomy in the diaphysis of 1 and 2 mm gap (S1-S4 and S5-S6, respectively) were used in this study. The gap was stabilised with a Stanmore Micro-External-Fixator (SMExF) (Meeson et al., 2019). The animals were sacrificed 5 weeks postoperative and the femurs were extracted and fixed in formalin solution (10% buffered formaldehyde). Both ends were embedded in Poly(methyl methacrylate) (PMMA) and Acetyl endcaps (Keaveny et al., 2004). The average height of the femurs was 38 ± 2.3 mm; consequently, the tissue in between the endcaps was 20 ± 2.3 mm. The fixator was removed prior to the in situ mechanical testing.
Fig. 2. XCT images of rat femurs with 1 mm osteotomy gap (red arrow) under a $7 \pm 2.7$ N preload. The red square is outlining the ROIs of S2 (A), S3 (B) and S4 (C) cropped during the image postprocessing.

**High-resolution XCT imaging**

High-resolution XCT was performed using a Versa 510 system (Carl Zeiss Microscopy, USA). The system operated at 110 kV/9 W and 1601 projections were collected on air using a $0.4 \times$ objective with exposure time of 3 seconds over $360^\circ$, resulting in a $12 \mu m$ voxel size. The resulting field of view (FOV) included the osteotomy gap and the 2 closest pin holes ($12 \times 12$ mm).

**In situ XCT mechanics**

In order to plan the in situ XCT experiments with incremental increases of compressive load, a preliminary test was conducted in order to document damage induced by fixator removal as well as defining the apparent elastic region extension to be used as a reference for the step-wise XCT mechanics. One femur with a 1 mm osteotomy gap (S1) was XCT imaged before and after removal of the fixator, then uniaxially compressed in situ with a loading device (CT5000, Deben, UK) until failure and finally XCT imaged postfailure. It was shown that removal of the fixator did not cause any visible damage to this specimen (Fig. 1A) and failure occurred at $\sim 1.2$ mm displacement (Figs. 1B, C).

For the in situ step-wise compression testing the bone was kept hydrated throughout the process by wrapping the tissue with a gauze immersed in saline solution and then in a parafilm to avoid leakage. The distal end of the femur was fixed to the bottom plate. An initial preload of $7 \pm 2.7$ N was applied and two consecutive tomograms were acquired to calculate DVC strain uncertainties (zero-strain test; Dall’Ara et al., 2017). The specimens were then step-wise compressed to 0.5 mm and 1 mm displacements, as dictated by the preliminary experiment, within their apparent elastic region. Following the application of each displacement step, the specimens were allowed to relax for 15 min, to compensate for stress relaxation, before acquiring XCT images (Madi et al., 2013).

**Image postprocessing**

The 3D datasets obtained from the tomograms after reconstruction ($1004 \times 1024$ pixels, 32-bit) were converted to 8-bit and rigidly registered using FIJI (ImageJ, USA). Parallelepipeds were cropped in the centre of the dataset ensuring that the osteotomy and one of the pin holes were in the region of interest (ROI) (Figs. 3 and 4). Nonbone voxels in the images were given a zero-intensity value in the grey-scale by applying a mask to each dataset (Peña-Fernández et al., 2018). The mask was created by running a purifying cycle on the binary image of each dataset with BoneJ plug-in. An arithmetic and logical operation was executed between the original dataset and the purified images to remove the noise using the same software (Doube et al., 2010).
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Digital volume correlation

A local approach DVC analysis was performed for the cropped volumes (Figs. 2 and 3) using DaVis 10.0.3 (LaVision, UK). A multistep processing scheme (128 to 48 voxels/subvolume) was used to compute the strain, followed by a vector postprocessing, where the correlation coefficient was thresholded to 0.8. The first two consecutive datasets where the preload was applied were used for the calculation of the strain error uncertainty (< 300 µε in all cases) (Palanca et al., 2015; Tozzi et al., 2016; Palanca et al., 2017). The 1st and 3rd principal (εp1 and εp3) strains were then computed.

Histological analysis

After in situ testing the specimens were decalcified in formic acid, dehydrated in increasing concentration of ethanol (50%, 75%, 85%, 95%, 100%) and cleared in xylene (C8H10). Then, they were infiltrated with paraffin wax for 30 h positioned in the same plane with the pin holes oriented vertically and embedded in wax. Sections of 5 µm were cut from the middle of each specimen using Leica RM2235 rotary microtome (Leica Biosystems, UK) (Meeson et al., 2019) and mounted on glass slides. The paraffin wax was removed and sections stained using Haematoxylin & Eosin (H&E) (Junqueira & Carneiro, 2005; Ross & Wojciech, 2006). The osteotomy gap region was located under 10x magnification lens and the tissue was analysed at 50x magnification using Olympus BX40 (Leica Biosystems, UK). The soft (i.e. cartilaginous matrix, skin, muscles, fibrous) and hard (i.e. cortical, woven bone) tissue were identified in the periosteal, intracortical and intramedullary zones.

Results

The XCT images showed differences in the morphology of the regenerated mineralised tissue. Only S1 showed complete healing after 5 weeks (Fig. 1A). In S2, S3, S4 (1 mm gap, Figs. 2A–C) and S5 (2 mm gap, Fig. 3A) partial healing was shown, whereas S6 (Fig. 3B) formed a nonunion. The strain induced on the regenerated tissue throughout the compression steps was assessed using DVC. In S2-S5 (Figs. 4A–C and 5A) an overall buckling of the structure was observed. The 1st principal strain (εp1) showed a wide variation of the strain concentration. S2 experienced higher strain level in the borders of the specimen where there was lack of woven bone (maximum ≈ 29 000 µε) (Figs. 4I, IIA). Conversely, in S3 and S4 strain concentration was present in the woven bone (exceeding 20 000 µε) (Figs. 4I, IIB, C). Due to their morphology, the femurs with a 2 mm osteotomy gap showed different strain distributions. S5 exhibited an increase in the strain inferiorly, not exceeding 15 000 µε.
Fig. 4. DVC analysis of 1 mm osteotomy gap, showing $\varepsilon_{p1}$ and $\varepsilon_{p3}$ of cross sections (A–A) in the ROIs of S2 (A), S3 (B) and S4 (C) in the first and second compression steps (I and II for $\varepsilon_{p1}$; III and IV for $\varepsilon_{p3}$, respectively).
At $\Delta L = 1.0$ mm, $\varepsilon_{p1}$ in S3 resulted in similar trends as before; however, the strain induced in the woven bone reached a value of $-5000 \mu e$ (Fig. 4IVB). The strain distribution in S4 was similar throughout its volume ranging between $-1000 \mu e$ and $-4000 \mu e$ after the first compression step (Fig. 4IVIC). The hard callus peripherally of the periosteum sustained the load, reaching $-43000 \mu e$ locally in the second compression step (Fig. 4IVC). S3 and S4 displayed lower strain levels compared to S2 in the first compression step (Fig. 4I, III). Specifically, lower strain was distributed when woven bone was present in either side of the gap, maintaining a stable mechanical environment. The callus formation in S4 supported the compressive load at $\Delta L = 1.0$ mm without signs of buckling and concentration of compressive strain experienced on both sides of the fracture (Fig. 4IVC). In S5 (Figs. 5III, IVA), the strain was concentrated in the middle of the woven bone locally in the first compression step, spreading laterally in the regenerated tissue on the second step. The behaviour of S6 (Figs. 5III, IVB) is different compared to S5; since the compression induced a sliding-like effect on the two parts of the fracture. The strain in the woven bone laterally showed an increase in magnitude of $10000 \mu e$ going from $\approx -20000 \mu e$ to $\approx -30000 \mu e$ (Figs. 5III, IVB).

Discussion

In this study XCT images showed that how fracture healing status differed in all six specimens, having complete (S1, Fig. 1) and partial (S2–S4, Fig. 2 and S5, Fig. 3A) healing, as well as nonunion (S6, Fig. 3B). In general, tissue formation patterns throughout the healing process are highly depended on the duration, gap size and quality of the fixation (Gómez-Benito et al., 2005; Vetter et al., 2010; Meeson et al., 2019; Betts et al., 2020). The consequent variation in tissue formation affected the strain distribution under the two displacement conditions. It has been shown that the intermittent tissue calcification during endochondral ossification and continuing growth of callus volume, increased the fracture stiffness and stability (Harwood et al., 2010). Here, not only there were differences in (Figs. 5I, IIA). The results for S6 were different from the rest, since the XCT images were unable to resolve callus bridging the osteotomy region. For this specimen the DVC analysis showed higher strain levels on the surface of the proximal fracture end (maximum $\approx 18000 \mu e$) (Figs. 5I, IIB). As for $\varepsilon_{p3}$, in S2 (Figs. 4III, IVA) compressive strains were accumulated laterally ($\approx -30000 \mu e$) in the first compression step; prompting contact between the two fracture ends at the second compression step, where the volume of the tissue under compression increased, not exceeding $-32000 \mu e$. The load in S3 was primarily maintained by the woven bone ($\approx -20000 \mu e$) at $\Delta L = 0.5$ mm and inducing compressive strains locally in regions with less hard tissue formation ($\approx -28000 \mu e$) (Fig. 4IIIIB).
strain between the 1 and 2 mm groups, but also high variability in the strain distribution within a single fracture and this explains how tissue formation in the fracture gap was not uniform. This was associated with variation of the tissue strain in the osteotomy gap. In the 1 mm osteotomy gap specimens (Fig. 4) at $\Delta L = 0.5$ mm, S2 exhibited high strain laterally. Compressive strain ($\approx -30\,000$ $\mu\varepsilon$) appeared in the area with visually higher levels of hard callus formation. Interestingly from the histology images, it was seen that area under tension is filled with cartilage matrix and mineralised chondrocytes (Fig. 6). This was promoted by an unstable in vivo mechanical environment, where the woven bone sustained the compression causing the cartilage matrix to tension ($\varepsilon_{p1}$ exceeding 20 000 $\mu\varepsilon$ locally; Figs. 4I, II and 5I, II).

The difference in the mechanical behaviour of S3 and S4 to S2 is better understood from the histology images. S3 illustrates an even distribution of soft and hard callus formation in the osteotomy gap (Fig. 6), with soft callus formation and advanced endochondral ossification where the cartilage matrix mineralised into woven bone. The combination of the two provided the mechanical support in the overall structure under loading (Jørgensen, 1972; Loi et al., 2016). However, the fact that endochondral ossification was more dominant on one side instigated a buckling effect. In the case of S4 the XCT images showed high levels of woven bone formation extraand intracortically and it could be assumed that bone was at the beginning of the remodelling stage in the fracture healing (Fig. 2) (Isaksson et al., 2009). Only a small region presented no hard callus formation. The histology images of that area showed presence of fibrous tissue along with osteoblasts in close proximity. S5 showed increased levels of compressive strain localised at the intracortical callus in the first compression step, which extended towards the rest of the callus as well as the cortex of the fracture ends in the second step. In this case of 2 mm osteotomy gap, union has formed, providing interfragmentary mechanical stability (Loi et al., 2016). The histological analysis showed the presence of cartilage matrix in that area that helped load transfer in these regions (Ghiasi et al., 2017). Although the peak value of strain is decreased at $\Delta L = 1.0$ mm, the volume under higher compressive strain levels increased, thus maintaining the higher load. The last specimen S6 was a clear case of nonunion. The load was conveyed between the two fracture ends through contact and sustained by the skin and muscles surrounding the bone. This assumption is made through observation of the specimen status post-XCT imaging and histological analysis (Figs. 3B and 7). By contact, the fracture ends of the specimen appeared to be more flexible than the rest and visually there was no tearing of the skin surrounding the bone. From the histological images, endochondral ossification was detected; however, there was no bridging and both ends were surrounded by cartilage matrix. The sliding-like effect of the compressive load deformed the interfragmentary mineralised tissue reaching $\approx -30\,000$ $\mu\varepsilon$ locally (Fig. 5IV B).
Controls were not available for comparison of the mechanics in intact and whole bone during fracture healing. However, the strain induced via compression on healthy young and mature rat femurs has been previously measured using DIC on the surface on the diaphysis (Yavari et al., 2013). The full-field measurements before failure displayed high levels of strain in the diaphysis reaching \( \approx -11\,000 \mu \varepsilon \) in the younger specimens and \( \approx -17\,000 \mu \varepsilon \) in the mature ones. Furthermore, full-field strain distribution using DVC in long bones under physiological loading conditions (not exceeding 13 N of total load) exhibited higher strain in the proximal and distal end of the bone (Giorgi & Dall’Ara, 2018). Specifically, the highest and lowest \( \varepsilon_{p3} \) values in the mid-shaft region in all the specimens reached \(-17\,000 \pm 2\,390\) and \(-7\,504 \pm 4\,347 \mu \varepsilon \). In the current study, strains obtained for the fracture site at the first compression step in S4 were one order of magnitude lower than the ones presented in both these studies (Yavari et al., 2013; Giorgi & Dall’Ara, 2018). Overall, the specimens with 1 mm osteotomy gap, only locally in the extracortical woven bone exhibited higher values of strain (\( \approx -30\,000 \mu \varepsilon \)) than that mentioned in Giorgi and Dall’Ara (2018) (Giorgi & Dall’Ara, 2018). The full-field strain measurements in the second group displayed higher compressive values (\( \approx -43\,000 \mu \varepsilon \)), which can be attributed to the larger osteotomy gap and therefore axial volume of woven bone. 3D full-field strain concentration in regenerated tissue has been examined in cylindrical trabecular bone specimens that included osteoregenerative grafts (Peña Fernández et al., 2019; Peña-Fernández et al., 2020). DVC analysis was performed in regions which included the interface between the native bone tissue and grafts. In the case of this study there are no biomaterials introduced to the structure; however, a correlation can be made in the strain values of regenerated tissue between the two studies. The specimen with the largest amount of new bone formation displaced higher compressive strain levels in the regenerated tissue compared to bone graft reaching a maximum of \(-15\,000 \mu \varepsilon \) (Peña Fernández et al., 2019). Similarly, in Peña-Fernández et al. (2020) the DVC analysis showed that the newly formed bone experienced the highest strain values in the specimen of \( \approx -10\,000 \mu \varepsilon \).
The mechanical behaviour of the soft tissue (i.e. cartilage matrix, muscles and skin) in the fracture site cannot be easily quantified by XCT-based evaluations, due to their low absorption. The cartilage matrix, where endochondral ossification occurred, should have experienced similar deformation as the woven bone around it. However, in areas of intramembranous ossification the strain in the adjacent soft tissues was unknown. Characterisation of the mechanical properties of the callus formed via intramembranous ossification is vital in the understanding of factors determining healing process and mechanical stability (Shefelbine et al., 2005). This could be potentially achieved by using in situ mechanics with an optimised combination of tissue staining and XCT phase-contrast, which would provide DVC with sufficient pattern to enable correlation in soft-hard tissue interfaces (Clark et al., 2020; Tozzi et al., 2020).

This study has a number of limitations. A limited number of specimens for both 1 and 2 mm osteotomy gaps were available and the ex vivo analysis is representative of a single time point during the healing processes. The main interest of this study was in understanding the load transferred through the osteotomy gap and the associated strain levels; for that reason, the bone was loaded after removal of the external fixator. In future, the strain pattern in the osteotomy gap with the fixator still in position can be investigated, as this will give a real value of the strain in the tissues during the healing process. Nevertheless, the differences observed in the local strain distribution for both 1 and 2 mm osteotomy gaps were related to the level of bone formation in vivo. It could be argued that mechanics of the tissue may be altered by the fixation process, but it has been shown that fixation should not alter the apparent elastic properties of bone (Wieding et al., 2015). In any case, the methodology reported in this study provide a valuable guideline for XCT-based analysis of regenerated fractures and can be used to inform/validate computational models predicting bone regeneration mechanism.

Conclusion

This study presented an approach to evaluate the 3D full-field strain distribution using DVC, within the regenerated bone tissue of two different osteotomy gaps in the diaphysis of rat femurs, induced by in situ XCT step-wise compression loading in the apparent elastic region. The regenerated mineralised tissue was evaluated via high-resolution XCT imaging and the soft callus formation was examined through histology. All specimens were at a different stage of healing leading to variability in the strain values under loading. The histological analysis showed endochondral ossification in all the specimens, except one where a nonunion had occurred. Overall, it was observed that in the specimens with partial regeneration, the newly formed bone experienced high strain ($\varepsilon_{p1}$ between $18\ 000\ \mu \varepsilon$ and $29\ 000\ \mu \varepsilon$; $\varepsilon_{p3}$ between $-30\ 000\ \mu \varepsilon$ and $-43\ 000\ \mu \varepsilon$). In the case of nonunion the strain reached $-30\ 000\ \mu \varepsilon$ ($\varepsilon_{p3}$) and the cartilaginous and soft tissues in the region of the osteotomy gap sustained strain reaching $18\ 000\ \mu \varepsilon$ ($\varepsilon_{p1}$).

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