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Mechanical characterization via nanoindentation of the woven bone developed during bone transport

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ABSTRACT

Nanoindentation has been used successfully in the determination of the mechanical properties of bone. Its application in fracture healing provides information on the evolution of material properties of the woven bone during regeneration process. However, this technique has not been applied in assessing the mechanical properties of woven bone during distraction osteogenesis. Therefore, the aim of this work is to evaluate the spatial and temporal variations of the elastic modulus of the woven bone generated during the bone transport process. Callus samples were harvested from intervened animals at different time points during the bone transport process (35, 50, 79, 98, 161 and 525 days after surgery) for nanoindentation measurements. Results clearly showed that the mean elastic modulus of the woven bone increased during the bone transport process reaching 77% of value for cortical bone after 525 days (from 7 GPa 35 days after surgery to 14 GPa 525 days after surgery approximately). Woven bone generated during bone transport seems to present similar evolution of elastic modulus with time as values reported for fracture healing. Furthermore, different spatial variations of elastic modulus within the callus were found for different stages of the process.

1. Introduction

Since its introduction by Ilizarov (1989a, 1989b), distraction osteogenesis has been studied by means of histology and radiography studies (Ilizarov, 1989a, 1989b; Aronson, 1994; Aronson et al., 1989, 1997; Forriol et al., 2010; Kojimoto et al., 1988; Okazaki et al., 1999; de Pablos and Canadell, 1990). These works evaluated the callus tissue types, the ossification modes and other parameters such as angiogenesis within the callus. It was demonstrated that the differentiation and the stiffening of the tissues within the distraction callus depend on the mechanical environment (Lanyon, 1987; Riddle and Donahue, 2009). Therefore, knowing the evolution of the callus mechanical properties and tissue types over time contributes to understand the mechanobiology of the new bone regeneration and optimizes the application of distraction osteogenesis in clinical practice by means of numerical models (Isaksson et al., 2007; Reina-Romo et al., 2009, 2010b, 2010a, 2011b, 2011a, 2012). Macroscopic mechanical properties of the distraction callus have been studied in the literature both *ex vivo* (Ohyama et al., 1994; Floerkemeier et al., 2010) and *in vivo* (Mora-Macías et al., 2015a, 2015b, 2015c; Aarnes et al., 2005; Dwyer et al., 1996; Brunner et al., 1994; Hyodo et al., 1996; Claes et al., 2000). *Ex vivo* studies (Ohyama et al., 1994; Floerkemeier et al., 2010) have provided values of the stiffness of the distraction callus at different time points during the process. *In vivo* studies also provided the evolution of the callus stiffness with time (Mora-Macías et al., 2015a, 2015b, 2015c; Aarnes et al., 2005; Dwyer et al., 1996) although measurements could only be performed before the distractor removal. In addition, these studies reported the increase of the force through the callus and the woven bone tissue volume with time during the consolidation phase. Some of the *in vivo* studies focused on other mechanical aspects such as the force relaxation within the callus tissue during the distraction phase (Brunner et al., 1994; Hyodo et al., 1996; Mora-Macías et al., 2016) or the influence of the interfragmentary displacement (Claes et al., 2000). Still all methods used in these works do not provide information on the evolution through the stages of the bone tissue regeneration of the material properties of the distraction callus at a microstructural level. In addition, they do not allow obtaining local variations of the mechanical properties within the callus tissue.

Nanoindentation measures the elastic modulus and contact hardness of materials point by point with a high spatial resolution (Oliver and Pharr, 1992). It has been applied successfully to assess the mechanical properties of biological tissues (Oyen, 2010), both soft (Grant et al.,

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2011; Tracqui et al., 2011; Yuya et al., 2010; Oyen, 2006) and hard (Amanata et al., 2008; Leong and Morgan, 2008, 2009; Lucchini et al., 2011; Manjubala et al., 2009; Rodriguez-Florez et al., 2013, 2015, 2014; Tai et al., 2006, 2007; Paietta et al., 2011; Bushby et al., 2011; Rho et al., 2002). These studies carried out in bone have provided information about the influence of nanoscale heterogeneity in bone strength (Tai et al., 2006, 2007), the bone permeability changes (Rodriguez-Florez et al., 2014) or the values of elastic modulus of the callus in bone healing (Amanata et al., 2008; Leong and Morgan, 2008, 2009; Manjubala et al., 2009). In the last case, Leong and Morgan (2008) measured the elastic modulus of the tissues within the callus during fracture healing. Although they obtained the elastic modulus for different tissue types, the study was limited to one time point during the bone healing process. Manjubala et al. (2009) reported, for the bone tissue of the callus during bone healing, the evolution with time of the elastic modulus mean values, the spatial variation with the distance from the cortex periosteum and the heterogeneity by means of elastic modulus maps, among others.

To the best of the author's knowledge, no analogous analysis of callus mechanical properties have been performed during distraction osteogenesis. Information about spatial and temporal variation of the local elastic modulus and hardness of the callus during distraction osteogenesis could contribute to clarify controversial or unknown aspects about distraction osteogenesis. For example, it is unclear to what extent the increase of the whole bone callus stiffness is due to the augmentation of callus volume or increases in the tissue stiffness (Mora-Macías et al., 2016, Mora-Macías et al., 2015b). On the other hand, in the particular case of bone transport, little is known about the mechanical parameter at the docking site callus since existing reports are, to the best of our knowledge, limited to analyzing the distraction callus (Brunner et al., 1994; Hyodo et al., 1996; Claes et al., 2000). However, docking site is known to be a frequent source of problems because of the difficulty of its consolidation (Marsh et al., 1997; Iacobellis et al., 2010; García et al., 2009). Therefore, the aim of this work is to assess the spatial and temporal variations of the elastic modulus of the woven bone tissue within the distraction and docking site calluses during bone transport experiments (Mora-Macías et al., 2015a, 2015b, 2015c) performed in sheep.

2. Material and methods

2.1. Sample preparation

The samples used in this study came from previous bone transport experiments carried out in sheep metatarsus (Mora-Macías et al., 2015a, 2015b, 2015c). The bone transport protocol consists of a latency period of 7 days, followed by a distraction phase in which a previous defect of 15 mm in length (distraction callus) is filled by displacing a bone transportable segment 1 mm/day during 15 days. After the distraction phase, the consolidation phase starts. During these experiments, animals were sacrificed at different time points: 17, 22, 29, 35, 37, 51, 79, 98, 161, 525 after surgery so that one animal could be used per time point. A quarter of the intervened bone (right metatarsus) cut in longitudinal direction was preserved for indentation experiments by freezing at −80 °C. Since the aim of this study was the characterization of the woven bone, only specimens in which most of the tissue within the callus had been ossified were used (35, 51, 79, 98, 161 and 525 days after surgery). For each animal, two woven bone samples were extracted from the two focuses of woven bone generated during bone transport: the distraction and the docking site calluses. In addition, a cortical bone sample from the proximal bone segment was also obtained for control measurements. The geometrical configuration and the dimensions of the samples used for nanoindentation, together with the location of the indentation sites, are shown in Fig. 1 for the three segments extracted from the quarter of the limb: cortical bone (a, d, g), distraction callus (b, e, h) and docking site (c, f, i).

For each piece, a 2 mm thick sheet in the surface parallel to the frontal plane was extracted and embedded in an Epofix® (Struers, California, US) resin cylinder (25 mm in diameter and 25 mm in height). The surface of the samples was polished with carbide papers (P600 to P4000) and diamond slurry (from 3 to 0.25 µm). Colloidal silica slurry (0.04 µm) was used for the final polishing step. The samples were cleaned ultrasonically with distilled water between each polishing step. Figs. 1d, e, f show pictures of the three types of samples for one of the animals (161 days after surgery) after the polishing process was completed.

2.2. Nanoindentation measurements

Indentation experiments were performed to determine the reduced elastic modulus (E_r) of the cortical and woven bones. Indentation test distribution and duration for the three types of samples of each sheep (cortical bone, distraction callus and docking site samples) are detailed in Figs. 1g, h, i and Table 1. A matrix of 16 × 16 indentations (750 × 750 µm) was performed in the cortical bone samples (A). In the callus samples two indentation lines were done in transverse and in longitudinal direction. Lines in longitudinal direction were positioned following the cortical bone orientation, approximately among 3 and 7 mm from the central axis of the limb (B1). Lines in transverse direction were positioned approximately between 2 and 4 mm from the proximal end of the callus (B2), which is about a quarter of its total length. In addition, three matrices of 16 × 16 indentations (750 × 750 µm) were performed: in the middle of the section (B4), a quarter from proximal end (B3) and a quarter from distal end (B5). In the docking site samples two indentation lines were done in transverse direction just between two bone fragments. (D1). Furthermore, a matrix of 16 × 16 indentations (750 × 750 µm) was performed just between the two cortical bone fragments in longitudinal direction (D2), in the exterior of the callus (some millimeters from cortical bone external diameter).

Indentations were performed using a Nanotest indenter (Nanotest, Micro Materials Ltd. Wrexham, UK) under ambient laboratory conditions using a Berkovich diamond indenter. The load was increased monotonically at a rate of 0.5 mNs^{-1} to a maximum load of 5 mN and held for 40 s before unloading at 0.5 mN s^{-1} rate. The load-depth data were processed using Oliver and Pharr method (Oliver and Pharr, 1992) to determine E_r. The accuracy of the nanoindentation equipment was evaluated by means of indentation in fused silica, whose mechanical properties are homogeneous and do not vary with time. Differences in these measurements were evaluated to be below ± 2 % for E_r. For the tests carried out, distance between indentations and between lines was chosen to be 50 µm. This distance corresponds to the minimum trabecula thickness of the new bone tissue of the callus, evaluated from histology images of additional samples from the same experiments (López-Pliego et al., 2016).

The time necessary to carry out the experiments is included in Table 1. Cortical bone sample (A) could be carried out in approximately 1 day, distraction callus samples (B1–B5) would need approximately 6 days and docking site samples 2 days. It has been checked that the duration of the experiments do not affect the mechanical properties measured (see supplementary material).

2.3. Data processing

Variation of the woven bone elastic modulus E_r with position along indentation lines were represented to analyze spatial variations. Besides, maps of E_r in matrix indentations were used to see and characterize the mean value and the heterogeneity of the tissues. The heterogeneity within each matrix was also evaluated by means of Whisker plots. Moreover, for each matrix of indentations the mean and the standard deviation of E_r at each time point in distraction and docking site calluses specimens were evaluated. This analysis neglected moduli where the indenter tip did not hit bone. The measurements carried out