Comparison of the volumetric composition of lamellar bone and the woven bone of calluses

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Abstract

Woven tissue is mainly present in the bone callus, formed very rapidly either after a fracture or in distraction processes. This high formation speed is probably responsible for its disorganized microstructure and this, in turn, for its low stiffness. Nonetheless, the singular volumetric composition of this tissue may also play a key role in its mechanical properties. The volumetric composition of woven tissue extracted from bone transport callus of sheep was investigated and compared with that of the lamellar tissue extracted from the cortical shell of the same bone. Significant differences were found in the mineral and water content, but they can be due to the different age of both tissues, which affects the mineral/water ratio. However, the content in organic phase remains more or less constant through the mineralization process and resulted a good variable to measure the different composition of both tissues, being that content significantly higher in woven tissue. This may be linked to the abnormally high concentration of osteocytes in this tissue, which is likely a consequence of the more abundant presence of osteoblasts secreting osteoid and burying other osteoblasts, which then differentiate into osteocytes. This would explain the high formation rate of woven tissue, useful to recover the short term stability of the bone. Nonetheless, the more abundant presence of organic phase prevents the woven tissue from reaching a stiffness similar to that of lamellar tissue in the long term, when it is fully mineralized.

Keywords

Woven bone, Bone transport, Callus, Mineral content, Volumetric composition

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Introduction

Woven bone is a tissue produced during skeletal development; in the callus, which appears during fracture healing, distraction osteogenesis or bone transport; in Paget's disease or in response to the administration of anabolic drugs in high doses[1–3]. It is formed very rapidly and this is likely the reason for being highly disorganized[4].

Woven tissue is less stiff than lamellar tissue[5–7]. This has been traditionally attributed to its disorganized arrangement, with the collagen fibers more or less randomly oriented. This contrasts with the hierarchical organization of lamellar tissue in which different substructures such as fibrils, fibers and lamellae can be distinguished, but overall with a well defined pattern of fibers orientation[8]. García-Rodríguez and Martínez-Reina[9] have recently proposed a multiscale micromechanical model concluding that the disorganized microstructure of woven tissue may be one of the reasons why its stiffness is lower than that of lamellar tissue.

Another reason for the low stiffness of woven bone may be its composition, compared in this paper with that of lamellar bone. Bone tissue, in general, has an organic phase, predominantly Type I collagen, and an inorganic phase primarily composed of crystals of non-stoichiometric hydroxyapatite. Hydroxyapatite crystals have hexagonal symmetry in its stoichiometric form, with well defined lattice parameters that may be altered by impurities[10]. Therefore, these impurities may distort the shape of crystals, so affecting the micromechanical properties of the tissue. Finally, bone tissue contains water, bound to both phases, organic and inorganic[11,12]. Comparing the composition of different types of bone in terms of the mineral and water contents may be tricky, given that those contents change during the mineralization process, that is, they depend on the age of the tissue. Thus, the differences could be reflecting a difference in age rather than a different composition pattern. To understand better this idea, it is necessary to explain briefly the mineralization process. The material secreted by osteoblasts, called osteoid, contains only organic phase and water. Mineral is deposited later, displacing water, so that the volume fractions of mineral and water vary inversely to each other[13]. If so, the volume fraction of organic phase should remain approximately constant throughout the mineralization process, unless the tissue is remodelled. Therefore, the content in

organic phase is practically independent of the age of the tissue (unless it is remodelled) and could indeed be a distinctive feature to be used for comparison purposes.

Composition of lamellar tissue is well known, with a volume fraction of organic phase of about 40%[14,15]. However, the composition of woven tissue has not been measured yet, as far as we know, though some signs suggest that its organic content may be higher than in lamellar tissue. Certainly, it is known from histomorphometric studies[16] that the concentration of osteocytes is almost double in woven tissue ($1050\,\text{osteocytes}/\text{mm}^2$) than in lamellar tissue ($540,\,\text{osteocytes}/\text{mm}^2$). It is well-known that some of the osteoblasts are buried in the bone matrix by the osteoid secreted by other osteoblasts and the former can undergo apoptosis or differentiate into osteocytes. Then, a higher concentration of osteocytes could be related to a higher concentration of osteoblasts and, consequently, to a more abundant organic phase, synthesized by osteoblasts.

Mineralization of lamellar tissue starts with the so called primary phase, a very quick phase in which the tissue reaches 70% of its mineral content in a few days[13,17] and then the process slows down as the tissue becomes saturated with mineral, during the so called secondary phase. The process can last from 6 months[18] to several years until complete saturation[13,19], unless the tissue is remodelled in the meantime. To our knowledge, the mineralization rate of woven tissue has not been measured, but it could be influenced by the collagen concentration. Indeed, collagen is not a passive scaffold and seems to play an important role in bone mineralization by facilitating the deposition of mineral, as confirmed by several *in vitro* studies[20–22].

The aims of this paper are to measure the volumetric composition of woven bone tissue and to compare it with that of lamellar bone tissue to find differences that could explain the biomechanical features of woven bone. Secondly, the stoichiometry of the mineral phase of both tissues will be compared in search of impurities that might explain differences in the shape of crystals. Finally, the mineralization rate of woven tissue will be estimated and compared with data available in the literature for lamellar tissue.

Materials and methods

Bone transport. Formation of woven bone.

All the experiments complied with the European Directive 2010/63/EU for animal experiments and the study protocol was approved by the Medical Ethics Committee of the University of Seville. The bone transport process was performed in 9 ewes (merino breed, 3 to 5 years old). Three osteotomies were performed transversely in the right hind metatarsus of each animal, so to separate two pieces from the bone: one of 15 mm which was removed to create the defect and the other of 25 mm, which was the transportable segment. An instrumented Ilizarov type distractor (Fig. 1) designed for this study[23] was installed and the following bone transport protocol was applied[24,25]: a latency period of a week; a transport phase of 15 days in which the bone transportable segment was displaced 1 mm each day to fill the defect and a consolidation phase during which the callus ossifies completely and can be eventually remodelled. The animals were slaughtered at different time points (17, 22, 29, 35, 37, 51, 79, 98 and 161 days after surgery)[25]. The operated metatarsus were harvested, stored in airtight containers and frozen at $-80°$C. Before the composition analysis, the metatarsus was cut in pieces which were used for different studies: histological, nanoindentation and the composition study presented here. The posterior-lateral quarter was selected for this study. The skin and periosteum was removed locally from the extraction site and then the transport callus was removed. The proximal and distal ends of the callus were trimmed to analyze its central part, so that the pieces had an approximate length of 10 mm in the longitudinal direction of the bone. Given that the formation of the transport callus was not homogeneous, some of these pieces presented regions which appeared to be fibrous tissue (the name usually given to the fibrocartilaginous tissue that predominates in calluses of non-union cases) rather than woven bone tissue. These areas were removed before proceeding. Next, the pieces were arbitrarily divided in smaller samples of approximately the same weight (the larger pieces produced more samples) using a sterilized saw. In total 11 samples of woven bone were used for the composition analysis (table 1). The calluses from the

animals slaughtered earlier (17, 22 and 29 days) were not used, for these animals presented an excessively immature callus still composed mainly of fibrous tissue instead of woven bone.

The histological study revealed bone remodelling activity in the callus[26] of variable intensity for the different animals, depending on the time elapsed from surgery. The existence of bone remodelling is undesirable for the present study, as it implies the presence of lamellar tissue in the callus, deposited in the formation phase of the remodelling process. To ensure the callus specimen just contained woven bone, only the animals slaughtered at 36, 37, 51 and 79 days after surgery were selected for this study. Indeed, López-Pliego et al.[26] confirmed that the number of osteoclasts were very small at the beginning of the consolidation phase and drastically increased around 50 days after surgery. It is well known that osteoblasts appear in the remodelling site after osteoclasts have finished their activity, more precisely after the reversal period has elapsed. Counting the duration of the resorption phase and the reversal period (24 and 8 days approximately[27]), osteoblasts are expected to be numerous after day 82 (50+24+8) and that is the reason why the animals slaughtered at 98 and 161 days after surgery were excluded from this study.

Samples of woven bone were extracted from the calluses of the chosen animals as explained before. Additionally, samples of lamellar bone were extracted from the diaphyseal cortical shell of the operated metatarsus of the same animals. More precisely, from the end located between the docking site and the metaphysis. A transverse section of this end was cut for each animal after removing the skin and the periosteum and divided into smaller pieces to produce the samples of lamellar bone (10 samples altogether). A summary of the samples of woven and lamellar bone with indication of the animal it was extracted from is given in table 1

Animal	1	2	3	4	5	6	7	8	9
Days after surgery	17	22	29	35	37	51	79	98	161
Woven samples	-	-	-	W_1	W_2, W_3	W_4, W_5 W_6, W_7	W_8, W_9 W_{10}, W_{11}	-	-
Lamellar samples	L_1	L_2	L_3	L_4	L_5	L_6	L_7, L_8	L_9	L_{10}

Table 1. Samples of woven and lamellar bone with indication of the animal it was extracted from.

Measurement of the composition

To facilitate the drying and ashing processes the samples were manually ground with sterilized pestle and mortar to obtain a characteristic particle size of around $1\,mm$. Composition was measured right after grinding to prevent drying of the samples in contact with air. First, each sample was weighed to obtain the total mass, m_t, which includes the masses of water, organic and mineral phases, called m_w, m_o and m_m, respectively, i.e.:

$$m_t = m_w + m_o + m_m \tag{1}$$

Next, the samples were dried in a heater for 1 hour at $105 \pm 2°C$ and weighed. The heating and weighing were repeated in cycles of 15 minutes until constant weight was attained. This drying process removes water from the samples, so that the dry mass m_d results:

$$m_d = m_o + m_m \tag{2}$$

Finally, the samples were ashed in a furnace following this protocol: 1) 30 minutes during which the temperature was linearly increased from the room temperature to $250°C$; 2) this temperature was kept constant for 1 hour; 3) 30 minutes of linear increase up to $650°C$; 4) this temperature was kept constant during 2 hours; 5) the sample was weighed and 6) introduced again in the furnace at $650°C$ and kept at this temperature during 30 minutes. Steps 5 and 6 were repeated until constant weight was attained. This process ashes the organic phase and thus allows obtaining the ash mass, m_a, which coincides with the mass of mineral:

$$m_a = m_m \tag{3}$$

The ash fraction, α, commonly used as a measurement of the mineral content, is given by:

$$\alpha = \frac{m_m}{m_o + m_m} \tag{4}$$

The volumetric fractions of each phase i are calculated by:

$$v_i = \frac{m_i}{\rho_i \left(\frac{m_m}{\rho_m} + \frac{m_o}{\rho_o} + \frac{m_w}{\rho_w}\right)} \tag{5}$$

where ρ_i is the density assumed for phase i, namely, $\rho_m = 3.12\text{g/cm}^3$, $\rho_o = 1.43\text{g/cm}^3$ [28] and $\rho_w = 1.0\text{g/cm}^3$.

Once each sample was ashed, an elemental analysis was performed on the ashes to find impurities in the mineral phase. In bone, the mineral phase is composed mainly of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, but it can have substitutions of carbonate (${CO_3}^{2-}$) and hydrogen phosphate (${HPO_4}^{2-}$) ions for the original phosphate ion (${PO_4}^{3-}$)[29–31]. The mass percentage of carbon, phosphorus and calcium was measured to evaluate the presence of these impurities.

The carbon content was obtained through elemental analysis using a TruSpec Micro analyzer (LECO Corporation, Saint Joseph, MI, USA). The calcium and phosphorus contents were obtained through ICP-OES (*inductively coupled plasma - optical emission spectrometry*). The samples were first dissolved in hydrochloric acid and then analyzed with an ICP atom emissions spectrometer Ultima 2 (HORIBA Jobin Yvon, Edison, NJ, USA).

The results of volumetric composition and elemental analysis were compared for lamellar and woven tissue using a statistical analysis. The independent categorical variable (ICV) type of tissue has 2 levels: lamellar and woven and there are 4 dependent variables (DV) in the volumetric composition: v_w, v_o, v_m and α; and 4 DVs in the elemental analysis: $\%C$, $\%P$, $\%Ca$ and the ratio $\%Ca/\%P$, which is sometimes used as a measurement of stoichiometry.

For the statistical analysis a Kolmogorov-Smirnov test was used to check normality of the samples. The Levene test was used to check for differences between the variances of groups In the cases with unequal variances and when the groups have unequal sample sizes, the Welch's t-test is more reliable than Student's t-test to compare the means of two samples. The effect size was also quantified using Cohen's d statistic[32].

Results

The average ash fraction and average volume fractions of lamellar and woven samples are compared in Fig. 2. The corresponding averages of the elemental analysis are compared in Fig. 3.

The null hypothesis of being normally distributed could not be rejected in any DV using the Kolmogorov-Smirnov test ($p > .05$). However, the Levene test showed significant differences ($p < .05$) between the variances of both groups in all the DVs except for $\%C$ ($p = .063$). The p-values of Welch's t-test are given in tables 2 and 3 of the Supplementary Material.

The volume fraction of water and mineral phases are significantly lower in woven tissue than in lamellar tissue ($p < .001$ in both cases), with a large effect size.* In contrary, the content in organic phase is significantly higher ($p < .001$, large effect size, Cohen's $d = 9.27$) in woven tissue, almost twice (see Fig. 2).

Regarding the composition of the mineral phase, it must be said that the carbon content is significantly smaller in woven tissue ($p < .001$, large effect size, Cohen's $d = 2.94$), while the contents in calcium and phosphorus are similar and, indeed, no significant differences were found ($p = .052$ for %Ca, $p = .067$ for %P, see Fig. 3). The effect size in comparisons of %Ca and %P could be considered large following Cohen's definition[32] and the fact that the differences between the groups are not significant (though they are close) could be due to the small sample size. However, Cohen admits that "the terms small, medium and large are relative, not only to each other, but $\{\ldots\}$ to the specific content and research method being employed in any given investigation". So, the conclusion that the effect size is large should be drawn cautiously in the cases of %Ca and %P (not in the volumetric composition nor in %C where the effect size is well above 0.8). In summary, the differences found in %Ca and %P are not conclusive, as also shown by the ratio %Ca/%P ($p = .776$ and small effect size, Cohen's $d = 0.13$).

*Cohen[32] regards the effect size as: small (if $d \sim 0.2$), medium (if $d \sim 0.5$) and large (if $d \sim 0.8$), so that the effect sizes in the comparison of the volumetric fractions could be regarded as more than large.

Discussion

The differences found in the volumetric composition of both tissues can be partially explained by the mineralization process. The osteoid laid *de novo* by osteoblasts contains only water and organic phase. It is during the mineralization process, starting shortly after the tissue is deposited, that mineral is precipitated, by displacing water[13]. Thus, the water content decreases as the mineral content increases. In other words, v_m and v_w should be negatively correlated for a given type of tissue. For this reason, the difference found in the mineral volume fraction of woven and lamellar tissue could be reflecting a different age of the tissues rather than an intrinsic difference between them. Lamellar bone of the samples extracted from the cortical shells (with an average $v_m = 0.398 \pm 0.014$, Fig. 2) is very likely older than the recently formed woven bone (with an average $v_m = 0.280 \pm 0.041$).

On the contrary, if the mineral is deposited by replacing water, v_o must remain practically constant during the mineralization process, and, therefore, it must be independent of the tissue age (unless it is remodelled). For this reason, v_o can truly reflect an intrinsic difference in the composition of the tissues, being more abundant in woven bone (average $v_o = 0.584 \pm 0.027$ against $v_o = 0.392 \pm 0.009$ in lamellar samples).

Finally, water content is lower in woven samples (average $v_w = 0.136 \pm 0.031$ against $v_w = 0.209 \pm 0.010$). This seems to be in contradiction with the idea expressed above (v_m and v_w being negatively correlated). That way, younger tissues (woven), should contain more water on average. But, that correlation is only true for a given type of tissue. If both types are compared, another variable must be taken into account: the initial v_o is larger in woven tissue and this leaves less space for water. That can be the reason why v_w is smaller in woven tissue despite being younger.

The more abundant presence of organic phase is probably linked to the faster deposition rate in woven tissue, which is, in turn, related to a more intense recruitment of osteoblasts to the callus. This conclusion is supported by the fact that the density of ostecytes is twice greater in woven tissue, as measured by Remaggi et al.[16] in histomorphometric studies. Certainly, more osteoblasts would be buried in the bone matrix and differentiated into osteocytes if the population of tissue forming osteoblasts was higher.

Though this explanation of why v_o is larger in woven tissue might seem plausible, it is yet to be confirmed by other types of studies.

The dispersion of measurements is quite remarkable in woven samples, with a coefficient of variation $CV = 22.9\%$ for v_w and $CV = 14.6\%$ for v_m. These values are high, particularly if compared with that of lamellar samples ($CV = 4.6\%$ for v_w and $CV = 3.6\%$ for v_m). The dispersion could be due to the fact that the samples were extracted from different animals, but it could also be explained by how the mineralization process occurs: with a very quick primary phase, followed by a secondary phase during which the mineral is deposited at an exponentially decreasing rate, as the tissue becomes saturated with mineral[17]. The temporal evolution of the mineral content (specifically, the ash fraction) was modelled in a previous work by García-Aznar et al.[19] through eq. (6) and is qualitatively depicted in Fig. 4:

$$\alpha(t) = \begin{cases} \alpha_{prim}\,\dfrac{t}{t_{prim}} & if \quad t < t_{prim} \qquad \text{primary phase} \\ \alpha_{max} - (\alpha_{max} - \alpha_{prim})\, e^{-\mathrm{k}(t-t_{prim})} & if \quad t >_{prim} \qquad \text{secondary phase} \end{cases} \tag{6}$$

where t_{prim} is the length of the primary phase measured in days, α_{prim} is the ash fraction reached at the end of this phase, α_{max} is the maximum ash fraction, reached when the tissue is saturated with mineral and the constant k is related to the mineralization rate during the secondary phase. In eq. (6), t is the time elapsed since the formation of the non-mineralized tissue. In fracture healing or distraction osteogenesis, the initial clot must be resorbed before the fibrous tissue is deposited, which takes about 3 days to occur[33]. Therefore, $t = T - 3$ should be used in the equation, with T the number of days elapsed after surgery.

It can be deduced from Fig. 4 why the dispersion of the mineral content of woven bone can be so high, due to its youth. It is very likely that the age of the woven samples span the range depicted in Fig. 4 for two reasons: 1) because the days elapsed from surgery varied from 35 to 79 days and 2) because, the woven tissue is continuously formed during the bone transport process. The callus grows in longitudinal direction as the bone transportable segment is moved towards the docking site (Fig. 1). This makes

the tissue within the callus intrinsically heterogeneous in age along that longitudinal direction. Small variations in the age of this young tissue can lead to great variations in its mineral content, due to the shape of the mineralization curve. On the contrary, lamellar bone is generally older and it consequently has a higher and more uniform mineral content, as confirmed by the experimental results.

Opposite to water and mineral, the dispersion of the content in organic phase of woven samples is quite low ($CV = 4.64\%$) and more similar to that of lamellar ($CV = 2.32\%$). This is in accordance with the fact that the organic phase remains practically unaltered during the mineralization process.

The values of the parameters of eq. (6) given in the literature are: $\alpha_{prim} = 0.45$, $\alpha_{max} = 0.7$, $t_{prim} = 22$ days, k $\in [0.0003, 0.005]$ [19,34]. The value of k controls the mineralization rate during the secondary phase. This phase can last several years [13,19], though other authors have stated that it can be quicker and last around 6 months [18]. Figure 5 compares the temporal evolution of the ash fraction predicted by eq. (6) for different mineralization rates: k $= 0.005$ (thin solid line) and k $= 0.0003$ (thin dashed line), with the ash fraction measured experimentally for the woven samples (circles and linear regression of those circles). The figure shows that eq. (6), together with the parameters given above, which were proposed to describe the mineralization process of lamellar tissue, clearly fail to model this process in woven bone, as this tissue seems to get mineralized much faster. So, most of the points measured for woven bone have an ash fraction higher than that predicted by eq. (6) even with the highest mineralization rate parameter, k.

This faster mineralization rate could be related to the more abundant presence of organic phase. Indeed, it is well known that Type I collagen (the most abundant in woven bone [26]) is not a passive scaffold during mineralization, but it actively controls and templates hydroxyapatite formation by directing amorphous calcium phosphate (ACP) infiltration and mediating its nucleation into the crystalline phase [35]. Therefore, the fact that collagen is more abundant in woven tissue would facilitate its mineralization.

Besides, it is generally accepted that mineralization starts in the collagen gap region [36,37], the space between tropocollagen molecules that is periodically repeated within the collagen fibril structure. If collagen fibrils were not aligned in preferential directions, but more disorganized, like they are in woven

tissue[4], they would expose the gap region more clearly, so enabling and accelerating the heterogenous nucleation of mineral crystals. Moreover, a more disorganized collagen network might lead to a larger volume of extrafibrillar space, which could also favour the homogeneous nucleation of crystals, thus accelerating the mineralization rate. Nonetheless, these explanations should be confirmed in future studies by microscopy observation.

The stoichiometry of the mineral phase was analyzed in search of substitutions. Calcium deficient hydroxyapatite, $Ca_{10-x}(PO_4)_{6-x}(HPO_4)_x(OH)_{2-x}$, results from the substitution of hydrogen phosphate ions, ${HPO_4}^{2-}$, for the original phosphate ions, ${PO_4}^{3-}$, of pure hydroxyapatite, where $x \in [0, 1]$[38]. This substitution results in a weight ratio Ca/P $\in [1.941, 2.157]$, being the upper bound of that interval the stoichiometric value. The lamellar samples presented a Ca/P ratio closer to stoichiometric, though the differences were not significant (see Fig. 3).

The presence of carbon in the ash samples denoted the substitution of carbonate ions, ${CO_3}^{2-}$, for ${PO_4}^{3-}$ ions, which represents another typical substitution of hydroxyapatite. In this case, the differences between both tissues were significant, being the content in carbon smaller in the woven samples.

Pure hydroxyapatite crystals have a platelet shape, which is altered by substitutions that have been reported to modify the lattice parameters of the crystals[10], usually leading to amorphous crystals. This fact may have important implications in the micromechanical behaviour of bone. However, no clear conclusions can be drawn from the results obtained here since both types of tissue present substitutions.

It is noteworthy that the dispersion of the elemental composition of the mineral phase was, again, greater in the woven samples. This can be explained by the heterogeneous distribution of mineral within the woven tissue, which is transformed into different phases with time. *In vitro* studies[39] showed that under near physiological conditions, early precipitates correspond to ACP and later transform first into octacalcium phosphate (OCP) and only then into hydroxyapatite. It must be admitted that the reported transformations were observed *in vitro*, but more recent studies have also shown *in vivo* evidence of the same transient precursor phases[40–42]. ACP has a formula $Ca_xH_y(PO_4)_z \cdot nH_2O$ with a very variable ratio Ca/P $\in [1.294, 2.847]$, while OCP is $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$ with a ratio

Ca/P=1.725[43] smaller than that of pure hydroxyapatite. Therefore, a sample of woven tissue, which is just developing, may have a heterogeneous distribution of mineral of different ages, viz. at different stages of this transformation sequence (ACP $\rightarrow$ OCP $\rightarrow$ hydroxyapatite). This might explain the dispersion of elemental composition in woven samples, in contrast to lamellar samples in which most of the mineral phase is mature and would predominantly be hydroxyapatite.

Conclusions

Differences were found in the volumetric composition of lamellar and woven tissue, extracted, respectively, from the cortical shell and the bone transport callus in sheep. These differences might have an important influence on the micromechanical properties of both tissues, as recently discussed in a multiscale homogenization study[9].

The results showed an intrinsic difference in the volume fraction of organic phase which is more abundant in woven tissue, as a likely result of a more numerous population of osteoblasts during the formation of the tissue. This leaves less space for water in the osteoid, a fact that would provide higher stiffness to the forming tissue in the short-term, before mineralization starts. In addition, mineralization seems to occur faster than in lamellar bone, probably thanks to the more abundant presence of collagen and to its more disorganized arrangement. Ultimately, this disorganization is counterproductive in terms of stiffness and mechanical features, as confirmed by García-Rodríguez and Martínez-Reina[9], but all the previous ideas lead to conclude that woven tissue is formed very quickly (no matter the disorganized microstructure) to restore the continuity of the organ as soon as possible.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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Supplementary material

	α	v_w	v_o	v_m
L_1	0.7080	0.2005	0.3787	0.4209
L_2	0.6965	0.2137	0.3832	0.4031
L_3	0.6926	0.2018	0.3927	0.4055
L_4	0.6954	0.2150	0.3836	0.4014
L_5	0.6925	0.2081	0.3897	0.4022
L_6	0.6849	0.2163	0.3925	0.3912
L_7	0.6684	0.2167	0.4071	0.3762
L_8	0.6733	0.2134	0.4045	0.3821
L_9	0.6804	0.2201	0.3947	0.3852
L_{10}	0.6943	0.1892	0.3972	0.4136
Mean	0.6886	0.2095	0.3924	0.3981
SD	0.0119	0.0096	0.0091	0.0142
W_1	0.5053	0.1428	0.5838	0.2734
W_2	0.4164	0.2096	0.5956	0.1948
W_3	0.4481	0.1476	0.6212	0.2311
W_4	0.4915	0.1370	0.5980	0.2650
W_5	0.4796	0.1175	0.6205	0.2621
W_6	0.5402	0.1080	0.5798	0.3122
W_7	0.5539	0.1130	0.5653	0.3217
W_8	0.5612	0.1141	0.5585	0.3274
W_9	0.5429	0.1691	0.5380	0.2929
W_{10}	0.5527	0.1273	0.5571	0.3156
W_{11}	0.5078	0.1084	0.6053	0.2862
Mean	0.5091	0.1359	0.5839	0.2802
SD	0.0472	0.0311	0.0271	0.0409
p	$\mathbf{< .001}$	$\mathbf{< .001}$	$\mathbf{< .001}$	$\mathbf{< .001}$
Cohen's d	5.10	3.13	9.27	3.77

Table 2. Results of ash fraction, α, and volume fractions (of water, v_w, organic, v_o, and mineral, v_m, phases) of the samples of lamellar, L_i, and woven bone, W_i. The mean and standard deviations, SD, of each group are shown together with the p-values of Welch's t tests and the effect size (Cohen's d).

	$\%Ca$	$\%P$	$\%C$	$\%Ca/\%P$
L_1	38.3705	17.8732	1.3093	2.1468
L_2	37.9751	17.4264	1.5610	2.1792
L_3	36.5157	17.6306	1.2483	2.0712
L_4	36.2068	17.7053	1.0240	2.0450
L_5	37.8625	17.1349	1.2470	2.2097
L_6	38.3644	18.4183	1.2413	2.0830
L_7	37.4017	17.1511	1.1370	2.1807
L_8	36.7341	16.8571	1.1483	2.1791
L_9	35.7115	17.1155	1.1287	2.0865
L_{10}	37.3567	18.3402	1.1460	2.0369
Mean	37.2500	17.5653	1.2191	2.1218
SD	0.9246	0.5299	0.1455	0.0640
W_1	37.7915	22.3468	0.2700	1.6911
W_2	36.0874	19.5499	0.9130	1.8459
W_3	35.3156	18.0189	1.2055	1.9599
W_4	37.0140	17.6416	0.2480	2.0981
W_5	39.2579	18.7355	0.2305	2.0954
W_6	38.6008	17.3158	0.3257	2.2292
W_7	38.5508	19.6988	0.2943	1.9570
W_8	41.5066	16.9804	0.5427	2.4443
W_9	39.5250	17.3334	0.6090	2.2803
W_{10}	41.0371	17.5641	0.3700	2.3364
W_{11}	41.6896	19.2245	0.4400	2.1686
Mean	38.7615	18.5827	0.4953	2.1006
SD	2.1262	1.5735	0.3105	0.2237
p	.052	.067	$<$ **.001**	.776
Cohen's d	0.91	0.85	2.94	0.13

Table 3. Results of the elemental analysis for the samples of lamellar, L_i, and woven bone, W_i. The ratio $\%Ca/\%P$ is also given since it is used as a measurement of the stoichiometry of bone mineral. The mean and standard deviations, SD, of each group are shown together with the p-values of Welch's t tests and the effect size (Cohen's d).

Caption of figures

Fig. 1. Scheme of the bone with the distractor: (a) during the bone transport phase and (b) during the consolidation phase. (1) Bone transport callus ($c = 15mm$); (2) bone transportable segment ($d = 25mm$); (3) docking site. Taken from[24].

Fig. 2. Comparison of the average ash fraction, α, and the average volumetric fractions of water, v_w, organic phase, v_o, and mineral phase, v_m, of lamellar and woven samples. The p-values such that $p < .001$ are denoted by ***.

Fig. 3. Comparison of the average content in Ca, P and C and the average ratio %Ca/%P of lamellar and woven samples. The p-values such that $p < .001$ are denoted by ***

Fig. 4. Temporal evolution of the ash fraction of bone provided by the model of García-Aznar et al.[19].

Fig. 5. Comparison of the temporal evolution of the ash fraction: (a) values measured for the woven bone samples, circles and linear regression of those circles (thick solid line); ash fraction predicted by eq. (6) for different mineralization rates: (b) $k = 0.005$ (thin solid line), (c) $k = 0.0003$ (thin dashed line).

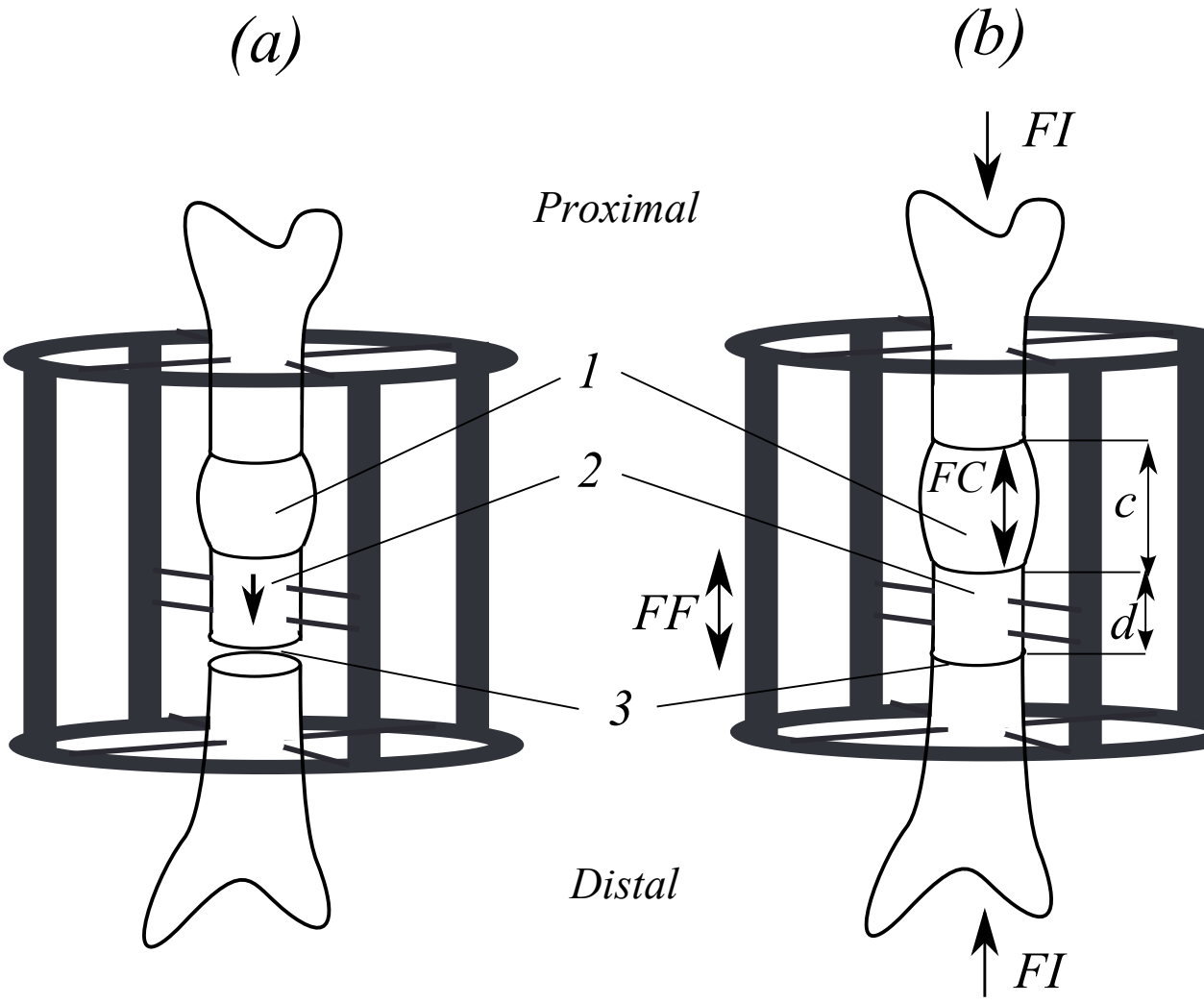

Figure 1

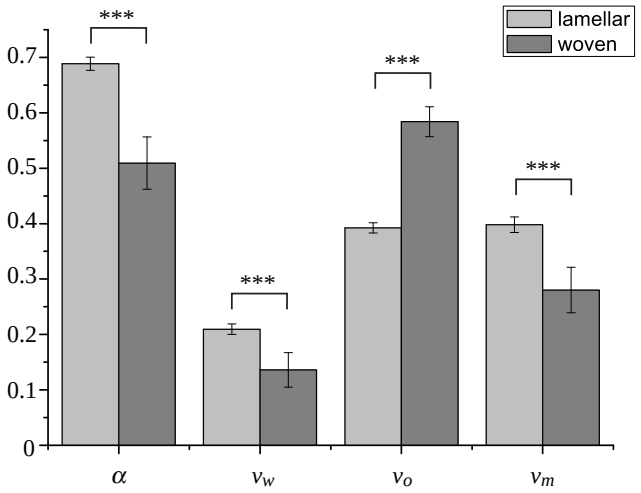

Figure 2

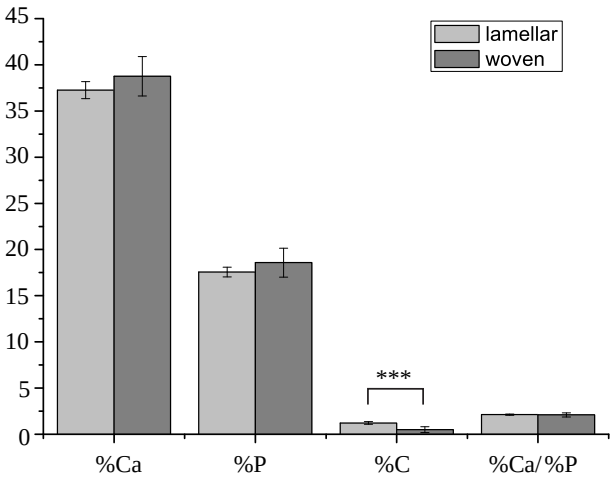

Figure 3

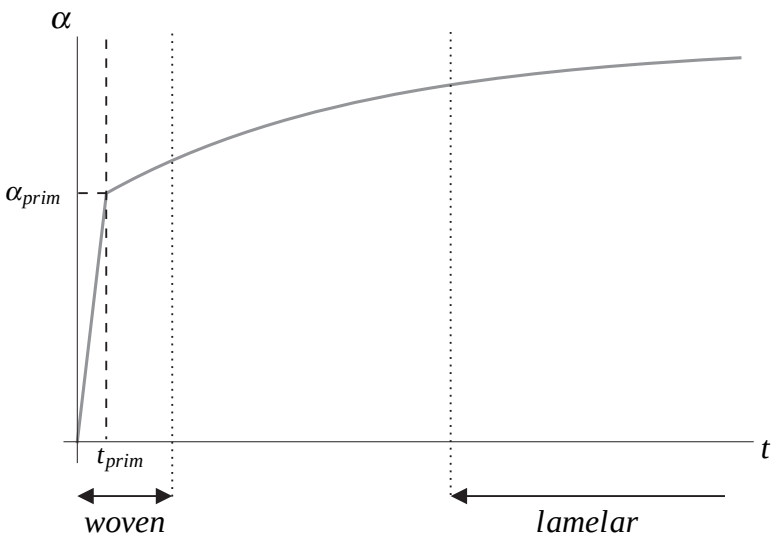

Figure 4

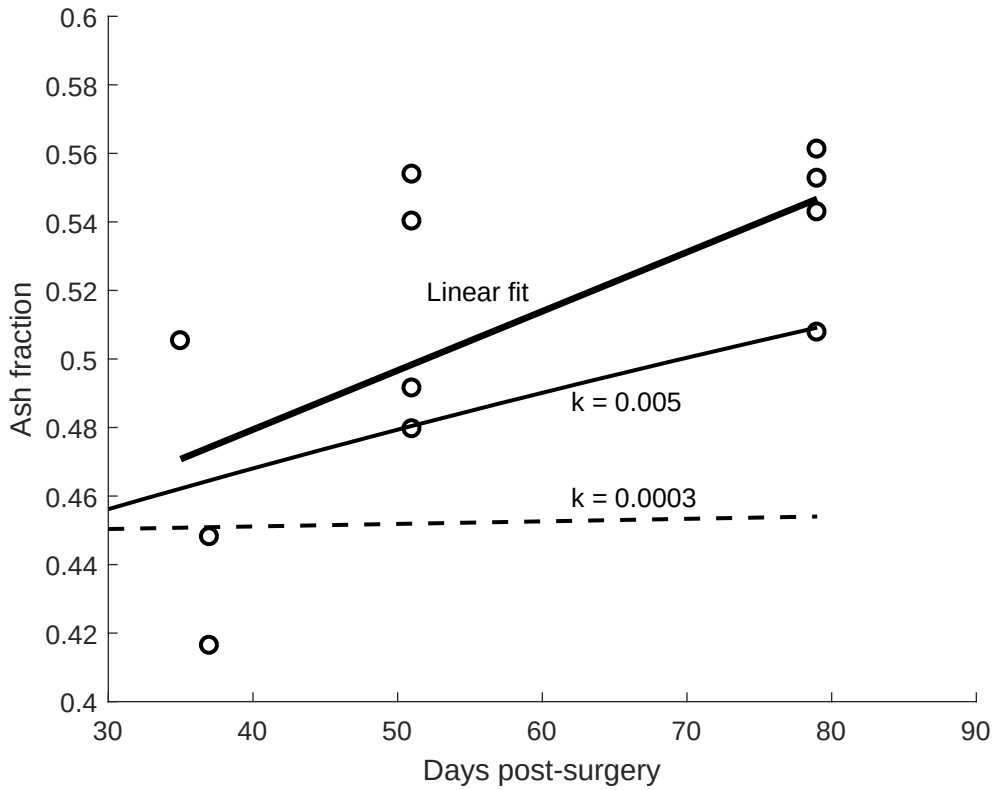
0.6
0.58
0.56
0.54
0.52
0.5
0.48
0.46
0.44
0.42
0.4
Ash fraction
30
40
50
60
70
80
90
Days post-surgery
Linear fit
k = 0.005
k = 0.0003

Figure 5