

# **Anisotropy of demineralized bone matrix under compressive load**

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Two groups of cubic specimens from diaphysis of bovine femur, intact and completely demineralized, were axially compressed. One half of the samples from each group were loaded along the axis of the femur (L) and the other – perpendicularly (T). Intact samples were characterized in terms of elastic modulus; for demineralized samples secant modulus of elasticity was calculated. During compression an acoustic emission (AE) signal was recorded and AE events and energy were analyzed.

Samples of intact bone did not reveal any anisotropy under compression at the stress of 80 MPa. However, AE signal indicated an initiation of failure in samples loaded in T direction. Demineralized samples were anisotropic under compression. Both secant modulus of elasticity and AE parameters were significantly higher in T direction than in L direction, which is attributed to shifting and separation of lamellae of collagen fibrils and lamellae in bone matrix.

*Key words: acoustic emission, anisotropy, bone matrix, compression, cortical bone*

## **1. Introduction**

A major challenge in bone research is to relate the composition and structure of bone tissue to its mechanical function and dysfunction. Even though the subject has been exploited for many years, further studies are needed to find the respective roles of mineral and collagen in bone strength and as well as in fracture mechanisms under different types of loading. Another important approach to the studies into basic properties of bone tissue can be explained by their applications in tissue engineering. Capacity for bearing loads is one of desired features of scaffolds used for regeneration of tissues and organs. Of many scaffold materials being investigated, type I collagen has been shown to have many advantageous features and the collagen-based materials have been used to support in vitro growth of many types of tissues [8]. So, information about the respective roles of mineral and

collagen in bone strength is important for the understanding of bone function as a load-bearing structure and for the designing of artificial tissues to substitute for bone and other types of connective tissue. A few previous attempts have been made to characterize mechanical behaviour of collagenous matrix obtained from demineralized bone [2], [5], [14], [15], [17], [21]. Using different experimental approaches the studies have demonstrated a wide range of values for measured properties, depending on level of the bone structure studied, the type of bone and the type and procedure of loading.

In bone subjected to external load, a sudden redistribution of stress can generate transient elastic waves in the form of acoustic emission (AE). The applications of AE analysis is generally focused on establishing a relationship between signals and failure mechanisms. Several attempts have been made to apply AE measurements to studying bone fracture [3], [7], [18]. AE signals were usually recorded in the non-

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linear region of material deformation and were attributed to the formation or propagation of microcracks. However, AE signal can be generated from sources not only involving failure but also responsible for friction or debonding between phases or layers of the material [1].

The purpose of the present work was to establish the ability of anisotropic bone matrix to transfer compressive loads and to test a potential of AE in studying properties of that material.

## 2. Material and methods

32 regular cubic specimens (5 mm) were wet-machined under constant manual irrigation from the cortical part of proximal diaphysis from one bovine femur with edges directed along main anatomical axes of the bone. Samples were divided into two groups, 16 samples in each. In one group, the samples were completely demineralized in formic acid [21] and in the other, they were left intact in saline solution at 4 °C. Samples in each group were coupled in pairs from adjacent locations.

All samples were axially compressed at the strain rate of  $0.033 \text{ s}^{-1}$  to obtain a 3-mm deformation (demineralized – D) or 2 kN (intact – I). The beginning of compression was determined for the force exceeding 0.1 N. Compression tests were done without any preconditioning. One sample from each pair was loaded along the long axis of the femur (L) and the other – perpendicularly to that direction (T). All the procedures of sample preparation and machining were performed with the samples fully wet.

The compression test was done with a universal testing machine (Lloyd LRX) with a 2500 N load cell and Nexygen software (Lloyd Instruments Ltd, Hampshire, UK) provided with the apparatus. The compression plate was made of duralumin with an M5 standard thread at the end to attach to the rest of the system (figure 1).

Intact samples were characterized in terms of the secant modulus of elasticity in the toe-region and an elastic modulus in proportional region of the stress–strain curve; for demineralized samples the secant modulus of elasticity at 40% strain was calculated.

During compression an acoustic emission (AE) signal was recorded as shown in figure 1. The AE head consists of two parts; the top part is made of ertacetal and the bottom part is made of duralumin and the two parts are screwed together. An AE sen-

sor is glued to the top surface of the duralumin part. The 4381V sensor (Bruel&Kjaer, Narum, Denmark) was used with a maximum sensitivity in the audible range of 1–16 kHz. The sensor was connected by a 2-m cable to the AE signal amplifier (EA System S.C., Warsaw, Poland) with an adjustable amplifier, which additionally filters the signal within 1–20 kHz. After filtering, the signal is converted into a digital signal by the A/D board Adlink PCI 9112 (Adlink Technology Inc., Taipei, Taiwan). The sampling rate was 44,000 samples/second at a resolution of 16 bits/ $\pm 1.25 \text{ V}$ . The second channel of the A/D board is used for recording an analogue signal of force delivered from the Lloyd LRX machine in order to trigger both measurements simultaneously.

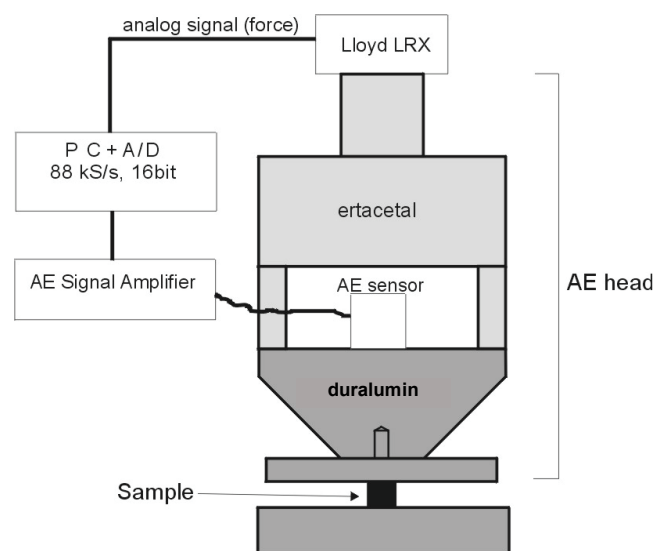


Fig. 1. Experimental setup

Since the acoustic signal during propagation from a source of AE to the AE sensor undergoes different wave phenomena, like diffraction and reflection, the system has its own characteristic and an analytical description of the wave propagation for the system is not possible. The results obtained are characteristic of the shape of the AE head, the material investigated, the sensor used for the AE head and the shape and material of the compression plate. So, to analyze AE signal certain descriptors are usually applied. In this work, AE events and AE energy were used for the characterization of material.

The AE event is the section of the AE signal where oscillations with amplitudes higher than a preset threshold, called the discrimination level, are measurable [22]. The AE discrimination level was found by running the Lloyd LRX machine with the

speed and other settings (including amplification) as in the proper experiment, but without the material tested. The desired level was the minimum voltage value of the signal from the 4381-V sensor at which no AE event was detected in this test. The energy of the AE signal was calculated as follows:

$$E = 0.5 V^2 t_1, \quad (1)$$

where  $V$  is peak amplitude of the signal and  $t_1$  is duration of a signal event.

AE events' number and AE energy were counted every 0.1 s during the entire test and cumulative values of both descriptors were calculated. Cumulative AE energy and average energy per AE event were analyzed; however, for demineralized bone AE parameters were calculated only to 40% strain. Differences between groups L and T were analyzed in terms of  $t$ -test.

### 3. Results

Intact bone cubes of 5-mm size did not reveal any anisotropy under compressive loading to 2 kN (~80 MPa of stress) (table 1), all parameters analyzed were similar for the samples loaded in longitudinal (I–L) and in transverse (I–T) directions. A considerable acoustic emission from intact bone samples was registered only in the first, nonlinear range of deformation. Also the distribution and energy of AE from samples were similar in both directions (figure 2 and table 1). However, in 5 of 8 transversely loaded intact samples, AE signal appeared again at the end of the test, probably as an indication of initiating failure.

The stiffness of the bone samples decreased dramatically after demineralization (groups D–L and D–T). Moreover, bone matrix revealed its anisotropic nature (table 2). As demineralized bone behaved nonlinearly in the entire range of deformations in both directions of loading (figure 3), the secant modulus of elasticity was used to characterize stiffness. A 3-mm deformation applied in the experiment was not enough to induce failure of samples loaded longitudinally. However, almost all the samples loaded in the transverse direction failed at the deformation somewhat larger than 2 mm (~40% of strain), so the secant modulus was calculated at 40% strain.

Table 1. Secant modulus of elasticity in toe region, elastic modulus in proportional region and AE parameters for intact (I) bone samples during compression; L, T – directions of load; mean values (s.d.) in groups are given; 8 samples in each group; n.s. –  $p$ -value > 0.05

	Secant modulus (MPa)	Elastic modulus (MPa)	AE energy $\times 10^{-3}$ (a.u.)	AE energy/event $\times 10^{-3}$ (a.u.)
I–L	153 (20)	336 (31)	304.5 (227.0)	138.6 (109.8)
I–T	176 (27)	375 (46)	358.9 (204.5)	98.1 (56.3)
$p$ (L–T)	n.s.	n.s.	n.s.	n.s.

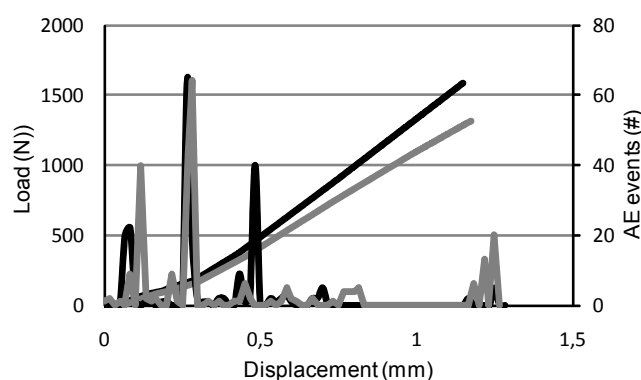


Fig. 2. Typical load–deformation relationships for intact bone samples compressed in longitudinal (black line) and transverse (grey line) directions and AE events during loading

Table 2. Results of compression test for demineralized (D) bone samples; secant modulus of elasticity and AE parameters are calculated at 40% strain; L, T – directions of load; mean values (s.d.) in groups are given; 8 samples in each group

	Secant modulus (MPa)	Stress at failure (MPa)	AE energy $\times 10^{-3}$ (a.u.)	AE energy/AE event $\times 10^{-3}$ (a.u.)
D–L	9.2 (2.2)	–	10.9 (4.5)	4.7 (1.2)
D–T	32.0 (8.3)	13.0 (5.2)	27.4 (5.5)	7.4 (1.4)
$p$ (L–T)	< 0.001	–	< 0.001	< 0.001

Emission of acoustic energy occurred in the whole range of deformations of demineralized bone for both directions of loading (figure 3). However, both cumulative energy and energy per AE event in the considered range of deformations were significantly higher in the transverse direction than in the longitudinal one (table 2). Moreover, in each pair of samples, a considerable increase of AE energy in the sample loaded transversely appeared at strain lower than that in the sample loaded longitudinally (figure 3).

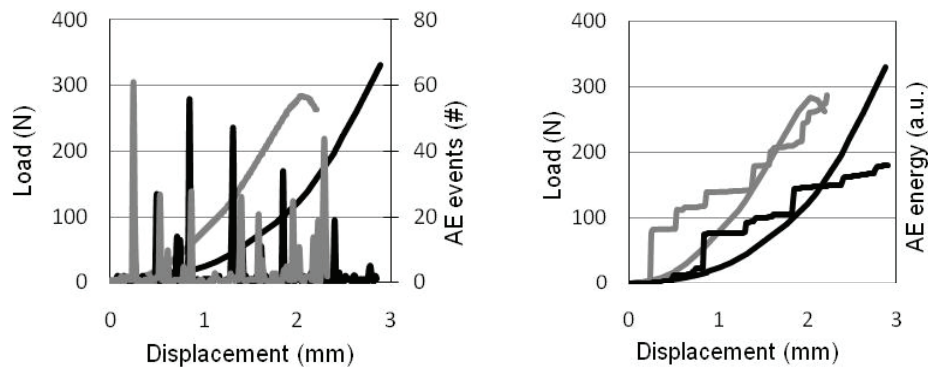


Fig. 3. Typical load–deformation relationships for demineralized bone samples compressed in longitudinal (black line) and transverse (grey line) directions and AE events (left) and cumulative energy of AE (right) during loading

## 4. Discussion

The work was aimed at establishing the ability of collagenous bone matrix to transfer compressive loads in two perpendicular directions. As a reference the intact bone samples from the same part of the bone were tested under the same conditions. The intact samples did not reveal anisotropy under compression in terms of all the parameters under study. Moreover, the samples tested in our experiment were very compliant with very low elastic modulus as compared to the results reported previously [6], [9]–[12], [16], [19], even assuming that fully hydrated bone is more compliant than a dry one [12], [20]. A possible reason for very large deformability of our samples can be explained by a complex and heterogeneous structure of compact bone together with the procedure of samples' preparation. Since the demineralization procedure involved two weeks of treatment in acid solution, the “intact” samples were stored in physiological saline solution during the same period of time. It was recently reported that in a saline solution without addition of calcium ions bone mineral can partially dissolve which influences its viscoelastic properties [13]. Compact bone contains a large number of structures like osteons of different degree of mineralization, an interstitial tissue between them and weak and ductile matrix–osteone interfaces (cement lines) [4]. It is presumable that weakly bonded nonmature mineral from cement lines was rinsed in part during the storing of samples, resulting in the relative shifting of osteons and other tissue structures during compression. Based on an intensive and continuous AE signal during the first nonlinear part of deformation (figure 2) it can be

inferred that a kind of shifting or debonding between the layers of the material occurs (AE signal that appeared again at the end of the test in majority of intact samples loaded in T direction was probably an indication of initiating failure). After the initial shifting between osteons or/and other structures, the tissue resistance to compressive load was provided by its stiffer mineral phase.

Demineralized bone was extremely susceptible to deformation (figure 3). Moreover, bone matrix revealed its anisotropic nature (table 2, figure 3). Transversely loaded samples were stiffer and all failed at the deformation slightly exceeding 40% strain, while none of samples loaded longitudinally failed up to 60% strain (3-mm deformation). The extremely high deformability in longitudinal direction may result from buckling of longitudinally aligned layers of soft collagen fibers in the absence of mineral.

There is a certain contradiction between the results of HASEGAWA et al. [10] and the anisotropy of decalcified bone obtained by us, while intact samples from the same place of bone are isotropic. Hasegawa et al. concluded that the collagen matrix within compact bone has little directional orientation and the orientation of mineral crystals is the primary determinant of bone anisotropy. No directional differences in compression in intact samples in our experiment would indicate that the distribution of mineral in and between collagen fibrils was not direction-dependent. Moreover, TAKANO et al. [16] stated that in mineralized tissues, collagen anisotropy and mineral anisotropy are not necessarily correlated. Bone strength and stiffness are related not only to mineralization, but also to porosity, other structural factors and the orientation of collagen fibers which depends on anatomical location of a bone sample [2],

[5], [6], [15], [16]. A relative effect of structural heterogeneity and collagen orientation becomes stronger after decalcification and leads to the changes in the distribution of bone mechanical parameters.

Strength at failure obtained in our experiment for transversely loaded bone matrix is in agreement with those published by CATANESE et al. [5] and SUMMITT et al. [14] in tensile test, though BOWMAN et al. [2] reported a value that is a few times higher. The stiffness of our samples is much lower than that reported in other experiments [2], [5], [14]. The difference can be at least partially explained by different conditions of the experiments, because mechanical behaviour of bone and other collagenuous tissues is strain rate-dependent [9]. Additionally, in all the papers cited above [2], [5], [14], preconditioning procedures were in contradiction to our method.

Acoustic emission was recorded in the first, non-linear part of deformation for intact samples and in the whole range of deformations for demineralized bone for both directions of loading (figures 2 and 3). Nevertheless, the energy of AE from demineralized matrix was more than one order of magnitude lower than that in intact samples (tables 1 and 2). The amount of energy released by an acoustic emission is related to the magnitude and velocity of the source events [1], [3], [22]. Large, discrete crack jumps possible during the shifting of rigid mineralized structures in intact bone will produce larger AE signals than events that propagate slowly over the same distance like the occurrences produced by the sifting of soft and compliant collagen layers.

Both cumulative energy and average energy of a single AE event were significantly higher in the samples loaded in the transverse direction than in the longitudinal one (table 2). For both directions of loading, a continuous uniform emission of acoustic signals of low energy was accompanied by regular occurrences of high energy. That pattern of AE distribution may result from both shifting and friction between collagen lamellae and their gradual separation and debonding. The difference in the rate of accumulation of AE energy for two directions we attribute to the differences in alignment of bone collagen lamellae and fibers.

In summary, implications of the results obtained in this study may be particularly relevant to preparing both artificial and collagen-based tissue implants and to explaining loss of bone mechanical competence in diseases resulting in abnormal mineralization of bone tissue. Moreover, the results encourage us to apply more frequently AE analysis to the studies of bone and bone-derivative materials.

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