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The effect of trabecular bone storage method on its elastic properties

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Purpose: The purpose of the study was to evaluate the effects of different methods of trabecular bone storage on changes in its elastic properties. *Methods*: 186 porcine trabecular bone samples were divided into 6 groups, approximately 30 samples each. Five groups were stored using the following methods: in buffered 10% formalin solution at room temperature, frozen at –21 °C, in the open air at room temperature, in 96% alcohol solution and in 50% alcohol solution at room temperature. The samples were subjected to compression test to measure the elastic modulus. The samples after the first measurement were subjected to further measurements for 14 weeks, every 2 weeks. The sixth group was used to determine the effects of 10 freeze-thaw cycles on changes in the elastic modulus. A Kolmogorov–Smirnov test at significance level $p = 0.05$ was used to determine the significance of changes in time. *Results*: The changes in elastic properties caused by the different storage methods were statistically insignificant, except for the group of samples stored in the open air. The changes in elastic modulus after 10 freeze-thaw cycles were also statistically insignificant. *Conclusions*: Except for the storage method in the open air, other storage methods did not significantly affect changes in elastic properties of the trabecular bones after 14 weeks. No effects of 10 freeze-thaw cycles on changes in elastic modulus were observed.

Key words: *statistical analysis, elastic modulus, trabecular bone, freeze-thaw cycle, storage method*

1. Introduction

Issues related to the storage of human and animal trabecular bone samples are currently being widely researched, and the studies cover two main aspects. In the first aspect, the authors focus on the evaluation of the storage methods with regard to medical applications, due to a high and growing number of trabecular bone grafts. It is, thus, vital to evaluate how the storage method affects the properties significant from the medical point of view, i.e., osteogenic cell vitality, active bone enzymes [16], probability of bacterial infection of sampled fragments [2], safe storage time [12], damage due to the storage method used or damage during transport from the tissue bank to the hospital [12]. The methods can be quite expensive due to the strict requirements. The bone fragments for grafting, stored in the tissue banks, are subjected to various storage procedures, including freezing at –20 °C or –40 °C, or deep, freezing at below –70 °C [3], [8], [12]. The bone fragments may also be sterilized before freezing [3], [27]. The type of procedure used depends on many factors, e.g., type of graft (autograft, allograft or xenograft).

In the second aspect, the authors focus on the trabecular bones stored to evaluate its different properties, e.g., structure, density [4], [8], [13], [26] or mechanical properties, using different methods [11], [13], [14], [24], [29]. In those cases, the methods most commonly used include freezing, storage in formalin or alcohol [8], [9], [12]. However, the results presented are limited, since either the number of samples is small or samples are taken from a small number of donors [10], [14], [21], [29]. This approach does not allow to completely eliminate the effects of individual traits of sample donors on the measurement results and reduces their statistical reliability. It is, thus, diffi-

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cult to determine with an absolute certainty how a specific storage method affects mechanical properties of the bone tissue.

The purpose of the study was to determine how the storage method affects the changes in elastic properties of the trabecular bone. 154 samples of porcine trabecular bone stored using five different methods to ensure high statistical reliability of the results. The effects of freeze-thaw cycles on the elastic properties of trabecular bone were also determined for a group of 32 samples.

2. Materials and methods

2.1. Preparing the samples

Porcine trabecular bone samples were used in the tests. The samples were taken from 186 heads of porcine femoral bone. All animals were at similar age of 6÷6.5 months and came from the same species and herd. From the slaughter to the sample preparation, the bones were stored in a cold store at 4 °C.

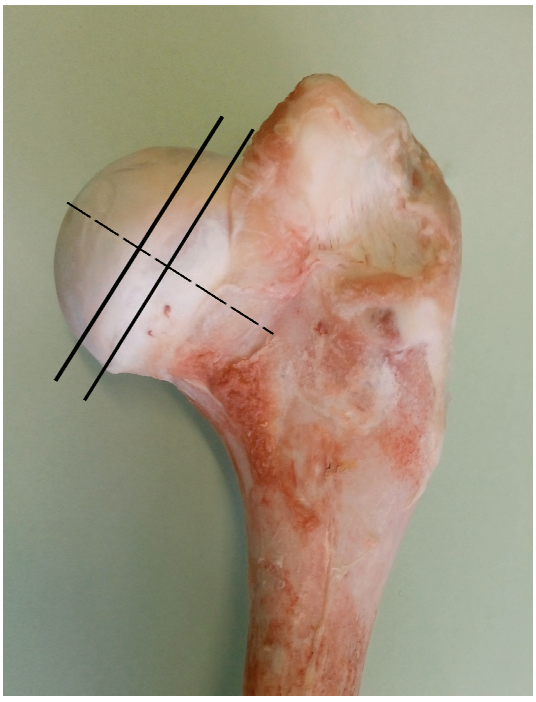

Fig. 1. Method of cutting sample slices from the head of the bone

12 mm slices were cut from the epiphysis of the femoral bone (Proxxon MBS 240/E, Proxxon GmbH, Foehren, Germany, with using self-designed holder). Figure 1 shows the method of cutting sample slices from the head of the bone. 2 to 3 cylindrical samples 10 mm in diameter were cut from each slice (Proxxon FF 230, Proxxon GmbH, Foehren, Germany) with the use of self-designed core drill. The face surfaces were ground to make cylinders with a height of 10 mm. The cut material was placed in a bath (0.9% NaCl solution at ambient temperature). The final sample diameter and height was 10 mm. The samples were subjected to microscopic and quality testing, and the samples with surface damage or cracks were rejected. A single sample from an individual, selected based on the above procedure was used for further analysis. The samples for each group were selected at random. The elastic modulus was measured immediately after sampling. No more than 8 hours passed from the slaughter, the first measurement and placing the sample in storage conditions. The following tests were carried out after 2, 4, 6, 8, 10, 12 and 14 weeks, respectively.

2.2. Sample storage methods

Samples were divided into 6 groups marked as G1 to G6, each containing approximately 30 samples. G1 – samples stored in a buffered 10% formalin solution at constant room temperature (n = 31). G2 – samples frozen at –21 °C (n = 32). To avoid dehydration before freezing, the samples were wrapped in a compress soaked in 0.9% NaCl solution and placed in a closed plastic bag. G3 – samples stored at room temperature at constant air access and 35% humidity (n = 30). The method was called “dry” method. G4 – samples stored in 96% alcohol at room temperature (n = 30). G5 – samples stored in 50% alcohol solution at room temperature (n = 31). Group G6 (n = 32) was used to evaluate the effect of freeze-thaw cycles on elastic properties.

The samples were stored for 14 weeks. After the first test, the following tests were carried out every 2 weeks.

Before the successive test, the samples were removed from the containers and the excess liquid was removed. The frozen samples were put in a container with 0.9 NaCl solution at room temperature for 3 hours to thaw.

2.3. Compression test

The test was conducted on Instron ElectroPuls E 3000 Test System (Instron, High Wycombe, England). The samples were placed on the testing machine between two polished steel surfaces and subject to initial force 3N for 15 seconds. Force and dis-

placement channels after initial loading were reset to zero. Three cycles were carried out, until strain equal to 0.65% [1] was obtained. To guarantee static load conditions and reduce the surface area of a hysteresis loop during sample loading and unloading, the duration of a single cycle time was 1 minute – 30 seconds loading followed by 30 seconds unloading. It corresponded to the strain rate of approximately 0.02% ε/s [9], [15]. The interval between cycles was 5 seconds. Value of a secant modulus of a loop for the 3rd cycle was assumed as a sample's elastic modulus.

2.4. Statistical calculations

A Shapiro–Wilk test at significance level $p = 0.05$ was carried out to determine the type of module distribution. A Kolmogorov–Smirnov test was used to compare the significance of changes in module values for each group in relation to first measurement. Significance level of the test was also $p = 0.05$. All statistical calculations were performed using "R" software [19].

2.5. The effects of freeze-thaw cycle

A group of 32 samples marked as G6 was subjected to 10 freeze-thaw cycles. To reduce the effects of freezing on the modulus values, the tests were carried out for the next 10 days, i.e., a single free-thaw cycle per day. The storage and thawing conditions were the same as previously described for a group of frozen samples – G2. The normality of module distribution for each group and significance of changes in module value after each cycle was evaluated using Shapiro–Wilk and Kolmogorov–Smirnov tests at significance level $p = 0.05$.

3. Results

The modulus values measured after sampling for group of 186 samples were characterized by a normal distribution. After grouping, group of samples stored in formalin (G1) and frozen (G2) were characterized by a normal distribution. The values for samples stored in the dry conditions (G3), in 96% alcohol (G4) and in 50% alcohol solution (G5) were characterized by a log-normal distribution. All other measurement results for group G1 and G2 after 2, 4, 6, 8, 10, 12, 14 weeks were characterized by a normal distribution, whereas for group G3÷G5, they were results were characterized by a log-normal distribution.

In Table 1 the test results are collected. The average modulus values for each group, measured immediately after sampling, are included and marked as initial modulus. The initial modulus values for group G1-G5 were between 1557.9 and 2205.6 MPa. Relative standard deviation values are shown in parentheses. The remaining columns show percentage changes in average value of modulus for each group, in relation to the initial modulus for the tests carried out after 2, 4, 6, 8, 10, 12 and 14 weeks. For groups G1–G2 and G4–G5, the percentage changes in average value for all tests were between –4.9 and +8.3%. For group G3, a maximum increase in the modulus value was 84.1%, and the average modulus has increased for 6 weeks, after which it has stabilized at relatively constant level. The average value of modulus and

Table 1. Changes in average modulus values for group G1-G5.
The initial modulus column include relative standard deviation value in parentheses

Group	Inital Modulus, MPa (RSD, %)	Change in modulus, %						
		Week						
		2	4	6	8	10	12	14
G1 (formalin)	1828.6 (58.9)	–1.8%	–5.8%	–4.9%	+2.8%	–2.1%	–3.7%	+3.5%
G2 (frozen)	1557.9 (59.5)	+0.1%	+3.5%	+7.4%	+2.9%	+8.3%	+8.2%	+6.6%
G3 (dry)	1615.8 (64.1)	+22.8%	+52.7%	+79.9%	+84.1%	+81.0%	+79.3%	82.5%
G4 (96% alcohol)	1846.2 (72.8)	+0.4%	+4.5%	+4.2	+2.9%	0.0%	+1.8%	–0.3%
G5 (50% alcohol)	2205.6 (64.1)	+0.9%	–2.2%	–2.4%	+1.2%	+2.0%	+0.7%	+3.3%

standard deviation for each measurement are shown in the bar graphs (Figs. 2–6).

For samples stored in formalin (Fig. 2), frozen (Fig. 3), stored in 96% alcohol (Fig. 4) and 50% alcohol solution (Fig. 5), the statistical test results show that the differences between the results obtained for each study are not significant, compared to the first reference test. For a group of samples G3 stored using "dry" method (Fig. 6), significant differences in module values in time were observed.

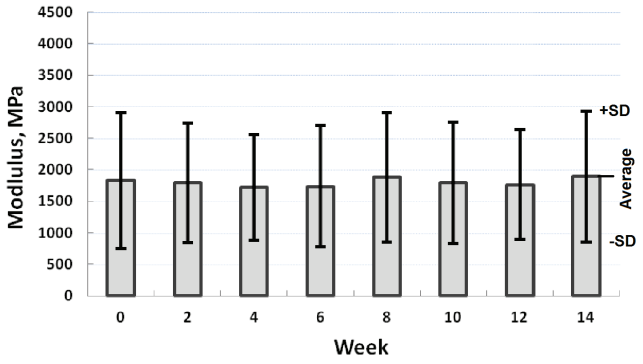

Fig. 2. Changes in average modulus and standard deviation values for G1 group (formalin)

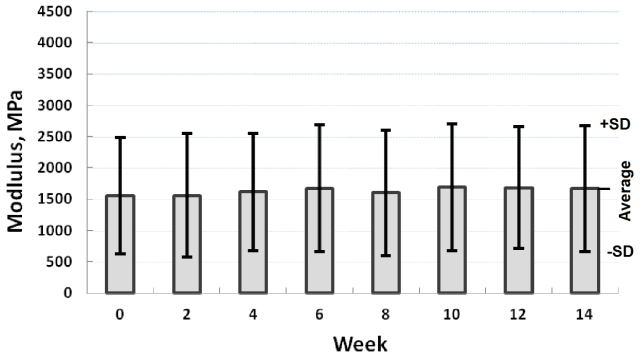

Fig. 3. Changes in average modulus and standard deviation values for G2 group (frozen)

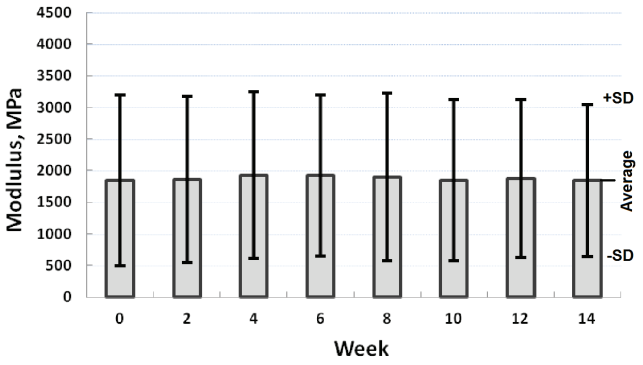

Fig. 4. Changes in average modulus and standard deviation values for G4 group (96% alcohol)

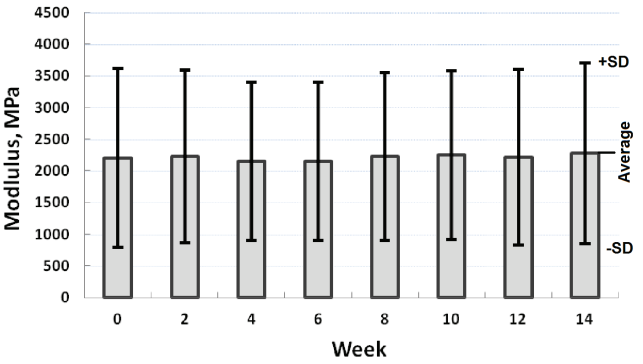

Fig. 5. Changes in average modulus and standard deviation values for G5 group (50% alcohol)

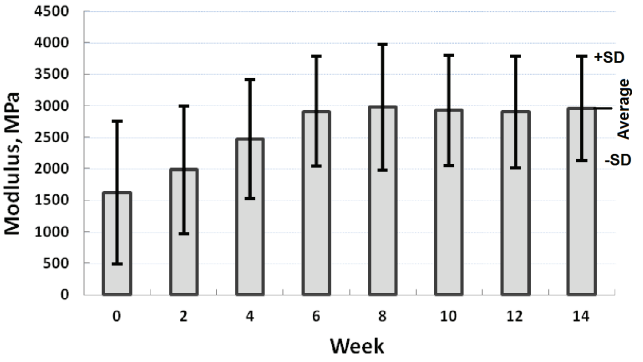

Fig. 6. Changes in average modulus and standard deviation values for G3 group ("dry" method)

No statistically significant effects on sample modulus were observed during the evaluation of the effects of 10 freeze-thaw cycles on changes in elastic modulus. The values of modulus after each measurement were characterized by a normal distribution. Figure 7 shows the average modulus and standard deviation values for subsequent measurements for group G6.

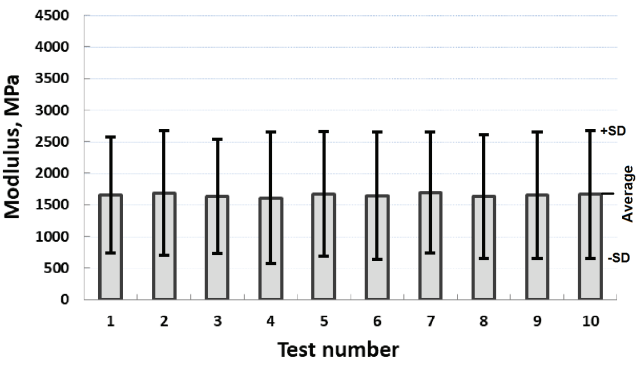

Fig. 7. Changes in average modulus and standard deviation values for G6 group (10 freeze-thaw cycles)

4. Discussion

The main strength of the study compared to other studies is that the tests were carried out on a large number of samples. 154 samples of porcine trabecular bone stored using five different methods were used in the tests. The number of samples in each group – approximately 30, was selected to ensure the most statistically reliable results. Since each sample comes from different individual, the effects of individual traits, i.e., trauma or past diseases, on the results can be reduced. All animals were part of the same herd, the same species and were at similar age. The feeding and living conditions were also similar to ensure the best possible uniformity of population the samples were taken from. The samples were not classified by sex, anatomical position of the sampling point within the head of femoral bone, relative sample density, structural indices and other factors. The test procedure aimed to ensure good statistical reliability of the results.

The test program aimed to answer which trabecular bone storage method causes the least changes in its elastic properties. Average initial elastic modulus for each group was between 1557.9 and 2205.6 MPa. The differences in average values for each group are the result of random selection of samples in each group. According to the Wolff's law, the density, thickness and quantity of the trabeculae changes locally depending on the position of the trabeculae path in the volume of the head of femoral bone. Average module value for each group may be a result of the number of samples included after cutting from each area of the head. Some of the samples were characterized by a very low elastic modulus of several MPa, which differs from the data available in the literature [1], the samples were nonetheless included in further tests. It was due to the difficulties in stating whether it has been caused by the position of the sub-volume of the sample within the head area or the errors in sample preparation or measurement technique. Random errors during measurements of the samples were excluded for those samples, since the values in subsequent measurements were comparable.

The measurement results may be affected by sample preparation accuracy. All samples were checked for accuracy, surface and internal defects, and only the samples which passed the quality check were qualified for further tests.

Distribution of average modulus value for each group after first measurement was normal in groups G1 and G2 or log-normal in groups G3-G5. However, the values of initial modulus for group 186 have a normal distribution. It enabled the evaluation of significance of the changes in time using the Kolmogorov–Smirnov test. Based on the statistical tests, changes in values for groups G1 and G2 and G4-G5 are not statistically significant. Similar results were recorded for the cortical bone. Wieding et al. [28] have shown that freezing and storing sheep cortical bone in alcohol and formalin for 14 days did not affect its elastic properties. Thus, those methods can be used to store cortical bones or whole bones for 14 days. Since the authors of the study [28] did not carry out any tests after 14 days, the results cannot be compared to a longer storage period for both types of bone tissue.

Errors occuring due to the measurement method were studied by Keaveny et al. [6] and Un et al. [25]. They have shown that the error of evaluating modulus measured based on the platen-to-platen displacement may differ up to 50%, compared to the direct strain measurement. The testing method is commonly used, and any possible systematic errors should not affect the trends in changes. As part of the study, the modulus was measured at the strain rate of 0.02% ε/s. Linde and Hvid [9] and Odgaard and Linde [15] have shown that the lower the test rate, the higher the determination accuracy of the modulus. The modulus measured at the strain rate of 0.02% ε/s differs by 16% from the modulus measured at the strain rate of 0.1% ε/s [9]. However, obtained values of modulus and standard deviation were compared with the results obtained by other researchers [1], which verified that the correct test methods were used.

Significance of changes in the module values in time was observed for group G3 only, which includes samples stored at room temperature and constant humidity 35%. For this group, an average initial module was 1615.8 MPa, after that it increased and finally stabilized at 2800÷2900 MPa after 6 weeks. It was probably related to the reduction in sample's moisture content. After 6 weeks, the moisture content was equal to the ambient humidity 35% and remained constant. The results of tests carried out by other authors show that the dry bone is characterized by a higher elastic modulus, compared to the wet bone [1]. Similar effects were not always observed for the cortical bone. For example, Wieding et al. [28] stored the sheep cortical bone samples for 14 days in the air at room temperature without preservative method. No statistically significant changes in the bending elastic modulus values were recorded, compared to the measurements taken immediately after sampling. The results for the trabecular bone

stored in similar conditions showed an increase in average modulus value for trabecular bone after 14 days by 23%, from 1615.8 to 1984.1 MPa. Different behaviour of both types of the bone tissue may be caused by the different structure. The cortical bone is characterized by a higher mineral content and lower porosity (approx. 5–8%) compared to the trabecular bone, with a porosity of up to 75–85%. Since the pores between the trabeculae are filled with liquid, i.e., bone marrow and blood, the loss of volume caused by drying at room temperature is significantly higher than in the cortical bone. It results in a significant increase in elastic modulus and strength of the trabecular bone due to the loss in moisture [1], compared to the cortical bone.

No effects of 10 freeze-thaw cycles on elastic modulus were observed. The values recorded for 32 samples were statistically insignificant. The present research validate the results of similar studies on human and animal bones reported by other authors [7], [8].

The test results show that all the methods of sample storage, except for the storage in the open, do not significantly affect changes in elastic properties in 14 weeks. All methods can be used to store bone samples for the above-mentioned period. Each method has its advantages and disadvantages. Freezing of the samples requires thawing before test and re-freezing. The evaluation of 10 freeze-thaw cycles did not show any effects on elastic modulus, however, preparing the samples stored with the use of this method is more time consuming, compared to the samples stored using other methods. For samples stored in 96% alcohol and 50% alcohol solution, it was necessary to check the liquid level periodically. Since the samples were stored at ambient temperature, the alcohol could evaporate, what would result in drying of the samples. The level of liquid was checked once a week. Storage in formalin seems the most optimal sample storage method. The advantage of using this method is that, in most cases, human cadaver for medical purposes is stored in formalin for up to several months. Thus, formalin may be present in the bone tissues taken from the cadaver stored in formalin. It allows to sample and use bone preparations for mechanical properties testing even after several months. The method is cost-effective and easy to use in laboratory conditions, however, no consensus on the effects of formalin on changes in mechanical properties of bone tissue is reached. Some authors state that storage in formalin does not affect the properties of the trabecular bone tissue, but the others show that the effects are present [5], [18].

5. Conclusions

The usability of the results has some limitation, since only porcine trabecular bones were used in the tests. The author aimed to use large number of samples to ensure good statistical representation of the results. Obtaining the samples of human bone from the same number of donors would be time consuming and complicate, since it would require additional selection due to the variables including age, sex, physical activity level, past diseases and other. However, other authors [13], [17], [20], [22], [23] show similarities in human and porcine trabecular bone, regarding their different properties, which enables to relate obtained results to the human trabecular bone.

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