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Mechanical properties of the mouse femur after treatment with diclofenac and running exercises

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Purpose: The flexible properties of the bone are essential for the movement and protection of vital organs. The ability of a bone to resist fractures under the influence of large muscles and physical activity depends on its established mechanical properties. This article discusses how exercise such as treadmill running and taking non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, affect the musculoskeletal system by modifying the elastic and thermal properties of the left femur of a mouse. *Methods*: The research was conducted using 9-week-old C57BL/6J female mice. In order to investigate the elastic and thermal properties of bones, dynamic mechanical analysis (DMA) and differential scanning calorimetry (DSC) were performed. *Results*: The study of elastic properties, followed by in-depth statistical analysis, shows that taking diclofenac slightly reduces the elastic parameters of the bones under study. These changes are more pronounced in DSC studies, the shift of the observed endothermic peaks is on the order of several degrees with a simultaneous increase in the enthalpy of this process. *Conclusions*: The opposite effect of the applied factors – diclofenac and running – on the elastic properties of the bones of the examined mice was found. The external factors – running and diclofenac – modify the basic parameters of the endothermic process associated with the release of water.

Key words: *mechanical properties, mouse femur, NSAID, treadmill running, dynamical mechanical analysis, differential scanning calorimetry*

1. Introduction

Understanding of bone formation, growth and disease are important for improving the quality of life and functioning. One of the vital biomechanical parameters characterizing bones is the complex elasticity modulus, which gives the information about the storage modulus and loss modulus [37]. This depends mainly on collagen content its mineralization and the microarchitecture of porosity [7], [8]. In the situation of inflammation, cause non-steroidal anti-inflammatory drugs (NSAIDs) are administered, which, by reducing the inflammation cause side effects, including the form of a weakening of the skeletal system [27]. This weakness may be manifested by disturbances in the bone fractures healing [6]. The weakening of the bone is evident in the change of the elastic properties. The elastic properties of bones also change with individual age [24], [25].

However, the investigation of elastic parameters for mouse bones is complicated partly due to a small size of the bones. Important is also the fact that the bones are not regular and homogenous in their shape. Numerous articles describe various methods used to quantitatively measure elastic modulus of murine bones [21], [32], [33], [35].

Thermal analysis can also provide important information about the physical properties of the material

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as well as about the phase transitions occurring when the examined bones are heated [12].

Hereby, we present the results of Dynamical Mechanical Analysis (DMA) and Differential Scanning Calorimetry (DSC) investigations of mouse femora. One of the DMA modes of work is three-point bending, which we used in the study. This kind of bending is a simple, repeatable test, which makes it the preferred method of mechanical testing in small animals [9], [20], [37]. This bending method gives us information about the modulus of elasticity and viscoelasticity. The changes in elastic properties can also be seen in DSC tests in the form of a shift in the maximum thermal effects as well as in the appearance of anomalies in the observed thermograms [11], [18]. The change in elastic properties is stimulated by the presence of water and its various physical and chemical properties caused by different forms of bonds in the tested material [12], [16], [39].

The physical activity can change a mineral density of bones so it also influences elastic and thermal parameters. Bone adaptation to exercise and loading is mediated by the increase of prostaglandin production. Prostaglandin production is blocked by NSAIDs such as diclofenac (DF) [31], [34].

The aim of this work is to determine the influence of diclofenac and enforced treadmill exercise on mechanical properties of mouse bones. Our study investigates the relationship between parameters such as modulus of elasticity, temperature of the dehydration phase, the enthalpy of this process in mouse bones treated with diclofenac and physical activity.

The study attempts to answer the question whether there is a mutual influence of diclofenac and physical activity on the mechanical properties of bones.

2. Materials and methods

2.1. The samples

Animals

The research was conducted using 9-week-old C57BL/6J female mice housed in standard polycarbonate cages and under controlled conditions (light – dark cycles 12/12 h; temperature 294 ± 1 K). The mice were divided into the following four groups, 10 individuals in each group, based on body weight and fasting glucose level: (1) control (C); (2) running (R); (3) diclofenac (DF); (4) DF plus running (DFR). The diclofenac (5 mg per kg of body weight) was injected intraperitoneally every day for 30 consecutive days. One group from each treatment was subjected to the running procedure (12 m/min by 30 min a day) using a rodent horizontal treadmill with 5 degrees' slope, detailed information is provided in our previous work [26].

The mice were euthanized in accordance with institutional guidelines at the age of nine weeks After the decapitation of the mice, their left femora were thoroughly removed using scalpels. The bones were protected with aluminum foil and transferred to sealed tubes. The tubes were placed at 250 K. We decided to freeze the samples as other methods of bone storage sometimes used can change the biomechanical measurement and cause a change in bone stiffness [13]. The main value of femoral weight for DMA measurements were 52 ± 3 mg and the bone length were 13.7 ± 0.5 mm.

The animal protocol was approved by the Local Ethical Commission for Investigation on Animals, Poznań University of Life Sciences (Permission No. 39/2017). All methods were performed in accordance with the relevant guidelines and regulations.

2.2. Experimental setup

The measurements of mechanical properties were performed by using a three-point bending setup of the Dynamic Mechanical Analysis – DMA 242C (Netzsch, Selb, Germany) and the associated data collection software [29]. The DMA can be described as an applied oscillating force and the analysis of the material's reaction. Because the bones of the mice are small, they are tested as whole entities. The sample is supported on two edges, while the load end of the push rod applies a load to a sample from above, Fig. 1. The arrangement of the sample and the experimental setup make the push rod perpendicular to the long axis of the tested femur.

Our DMA software enables us to select a measuring frequency from 0.01 Hz to 100 Hz. Very low frequencies cause a slow change in the applied force and the obtained results are characterized by a very wide range of measurement errors. Therefore, it was decided to perform the measurements in a sequence of six frequencies: 0.5, 1, 2, 5, 10 and 20 Hz, assuming that events with such a frequency occur in the normal life activity of the examined objects. Long-term test measurements (3 hours) showed no significant changes in the measured elastic values. Therefore, the time of 30 minutes was adopted for the implementation of one measurement series. The short-term measurement had an additional task to exclude changes in

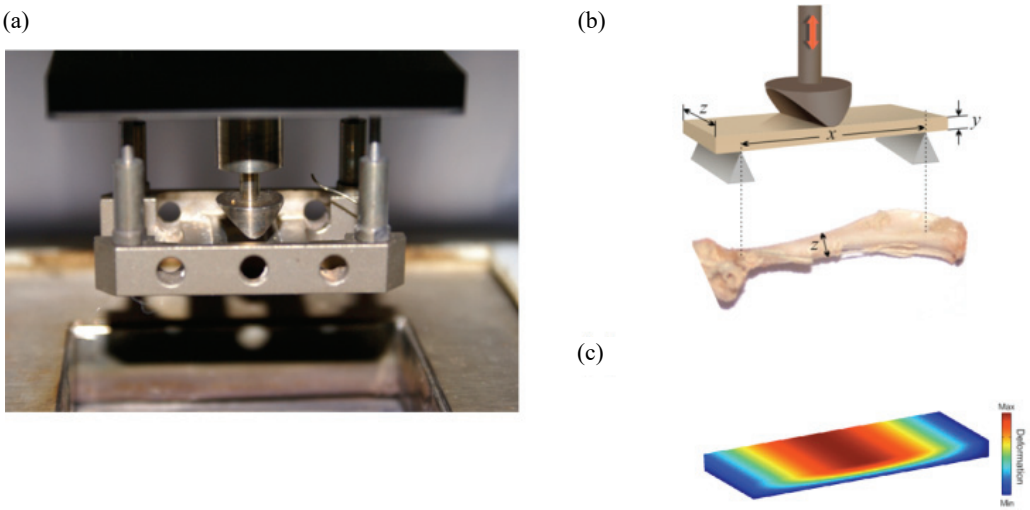

Fig. 1. The idea of the three-point bending: (a) real view of experimental setup, (b) schematic presentation the idea of bone mounting during measurements in DMA; x = support length, y = mean depth of bone, z = mean width of bone, (c) a typical deformation of the femur of mice in in three-point bending DMA measurements

water loss. This means that, for a given frequency, 15–17 measurement readings were obtained. The frozen femurs were stored at 250 K. Two hours before the DMA measurement, the femora were thawed to room temperature at sealed tubes.

After placing the bone in the measuring holder, the temperature of the measuring chamber was heated up to 308 K at a rate of 1 degree per minute. Then, after 5 minutes of temperature stabilization, a proper 30-minute measurement was made. No bones were broken during the measurements. In order to determine the value of the modulus of elasticity, it is necessary to adopt a model of the shape of the examined bone. In the literature, we can find information about the use of the isotropic model of an empty cylinder [30] or the isotropic model of an empty cylinder filled with a viscous liquid [2]. The idea that we are dealing with an empty cylinder is too far from the actual bone structure. Another approach is to adopt a solid model with a constant thickness [25].

In our analysis, it was assumed that the sample had the shape of a slab. The mean value of the width of the samples obtained on the sample of 40 bones was 1.85 mm, the standard deviation of the mean value was 0.18. The mean value of the thickness of the samples was 1.22 ± 0.15 mm. The difference between the average values is 66%, which prompted us to adopt the model of the slab and not the model of the rod in the analysis.

The bone lengths were around 14 mm so both of the capitula were always outside the measuring range. The distance between the edges (support span, x) was 10 mm. The samples were not processed and the sample arrangement was the same for each measurement.

The geometric factor for the adopted shape of a slab is defined as: $x^3/4zy^3$, where: x is the value of the support span, y is the value of a sample height, z is the value of the sample width [29] (Fig. 1).

The DMA gives the information about a complex elasticity modulus E^* which can be defined as:

$$E^* = E' + iE'', \tag{1}$$

where E' is the storage modulus and E'' represents the loss modulus.

The ratio of the loss to the storage modulus is called damping and it is given by the following equation [28], [29]:

$$\tan\delta = E''/E', \tag{2}$$

where δ is the phase lag between stress and strain sine waves.

The scanning thermal analysis allowed us to obtain information about the thermodynamic processes taking place during the supply of heat to the sample. Our DSC measurements were made using a Perkin–Elmer Heat Flow double furnace DSC 7 setup. Thermal analysis was performed immediately after the completion of testing of mechanical properties of each bone. The applied loading force in DMA measurements did not cause any fracture or rupture of the femur under examination. The following standardization for thermal measurements was adopted. After the DMA measurement, a part from the central bone fragment was cut out of each femur sample and then this small part was closed in a 5 mm diameter DSC measuring vessel. We used 30 μL aluminum pans. An empty pan of the same

capacity was used as the reference vessel. The net weight of a single sample was several mg. The individual mass of the tested samples was considered when determining the thermal parameters.

The calibration was verified using indium. All DSC measurements were taken at a rate of 20 degrees/minute in the temperature range of 323–623 K. Helium gas was used as the working medium.

After placing the sample in the measuring chamber, several minutes passed to equalize the temperature conditions, and then, after achieving a power level of 20 mW, the proper measurement started. At the rate of temperature change applied, the measurement lasted less than 20 minutes. After the last point, the temperature was returned to the start value of 298 K. For the DSC temperature measurements, the sample must be exchanged at room temperature. Therefore, after each completed measurement, the bone was cooled down to 298 K. None of the measuring capsules exploded or were destroyed. The presented results start from the first temperature scan for each tested sample. This approach to data analysis is typical of temperature studies [17], [18] because subsequent thermograms obtained in the studied temperature range are significantly changed.

2.3. Statistical analysis

The obtained results of the mechanical and thermal examination have been processed in a digital form, illustrated and analyzed with the Origin Pro 10.0 software. The statistical analysis was performed with the Statistica 13.0 package. The significance level was set at 5% ($p < 0.05$). The one-way ANOVA followed by Tukey's multiple pairwise comparisons was performed.

3. Results

The basic values for discussing results are the storage (elastic) modulus E' and the loss modulus E'' which are presented in Fig. 2a and 2b, respectively. The results are presented for the frequency of 2, 5 and 10 Hz.

The results are presented for four investigated groups of mice: control group (C, grey), running control group (R, red), the group treated with diclofenac (DF, blue) and group with diclofenac and running (DFC, green). We observe the influence of running on both groups: control and with diclofenac – the values of E' and E'' increased compared with non-running groups. The lost modulus had smaller values for running groups for the frequency of 2 Hz but it increased to 5 and 10 Hz. For the smallest frequency these effects are not so clearly visible. The most interesting is the fact that diclofenac, in comparison with control group, has a harmful effect on the examined mouse bones – the values of E' decrease for almost all frequencies. We have to notice that the statistical disper-

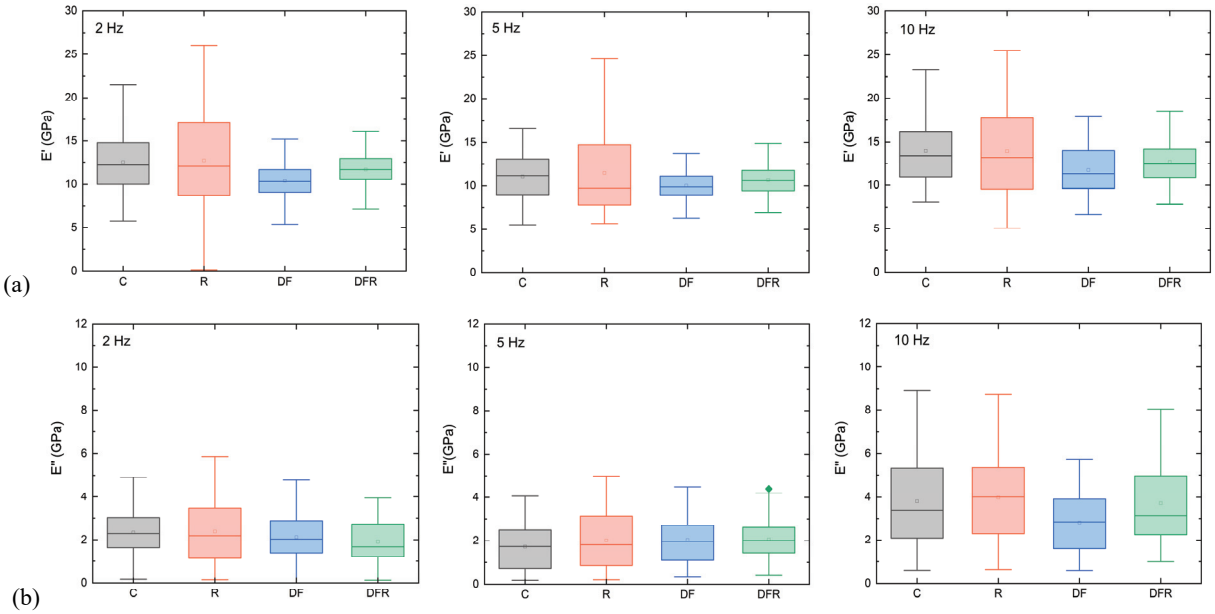

Fig. 2. Frequency-dependent elastic properties: (a) storage (elastic) modulus E' and (b) the loss modulus E'', presented for left femurs of mice (C – control, R – running, DF– diclofenac, DFR – DF plus running) for the frequency of 2, 5 and 10 Hz

sion of the results is greater for the group of running mice.

The elastic properties were measured at one stable temperature of 308 K, so the question arises whether it is possible to perform temperature tests for the bones tested. It is difficult to carry out the above tests as a function of temperature, because the elastic properties of the heated bone in the many-hour test cycles are significantly modified. We decided to perform DSC thermograms of femur fragments that followed directly the DMA measurements.

A typical thermogram for individual groups of femurs examined is shown in Fig. 3. The initial monotonic increase in the energy stream is followed by an anomaly area in the temperature range of 373–473 K. In the first part of this temperature range, we can observe exothermic peaks, with the enthalpy at the level of several J/g. They are often double or even triple maxima lying close to each other. The next phase of thermoanalys is shows a strong endothermic peak. In the control group, the endothermic peak is only about 12 degrees wide. The enthalpy of this process is around 40 J/g.

Analyzing the parameters of endothermic process in the next groups, it was noticed that the location of the extreme point shifted under the influence of the applied factors (running, diclofenac) modifying the examined physical parameters (temperature, enthalpy). Consequently, for the control group, the endothermic peak was 395.2 K, for the running group, this point has shifted by several degrees up to 400 K (Fig. 4). For the group of running mice, the average value of the minimum point was 398.5 K. In two successive groups of mice receiving diclofenac, the value of this temperature is even slightly higher and was about 400 K. Diclofenac administration to mice stabilizes this value at 400 K regardless of whether the mice were running or not.

A typical thermogram shape for the control group above the anomaly temperature shows a linear relationship. In the remaining three groups of mice, endothermic processes do not end at a single peak, but continue

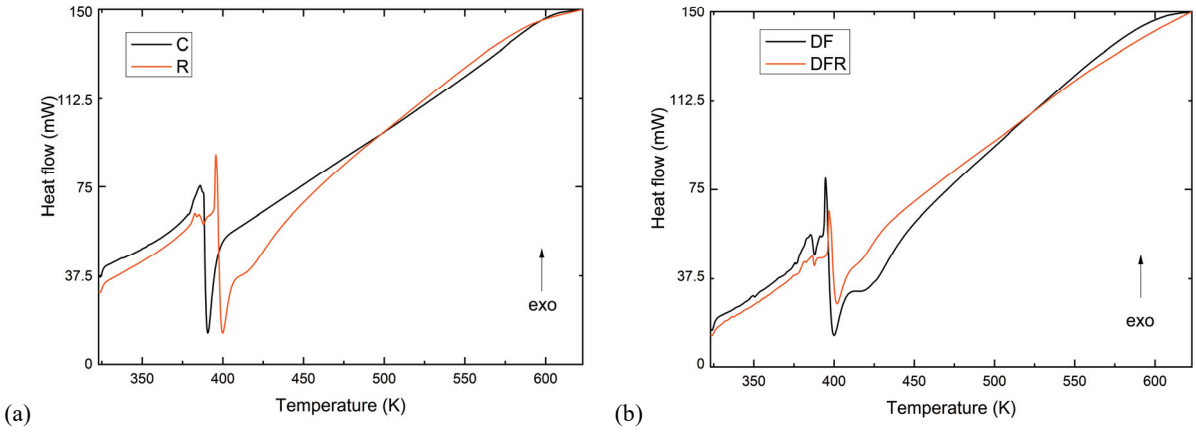

Fig. 3. Typical DSC thermograms for individual groups of mice: (a) C – control group, R – running group, (b) DF – the group with diclofenac and DFR – the group with diclofenac and running

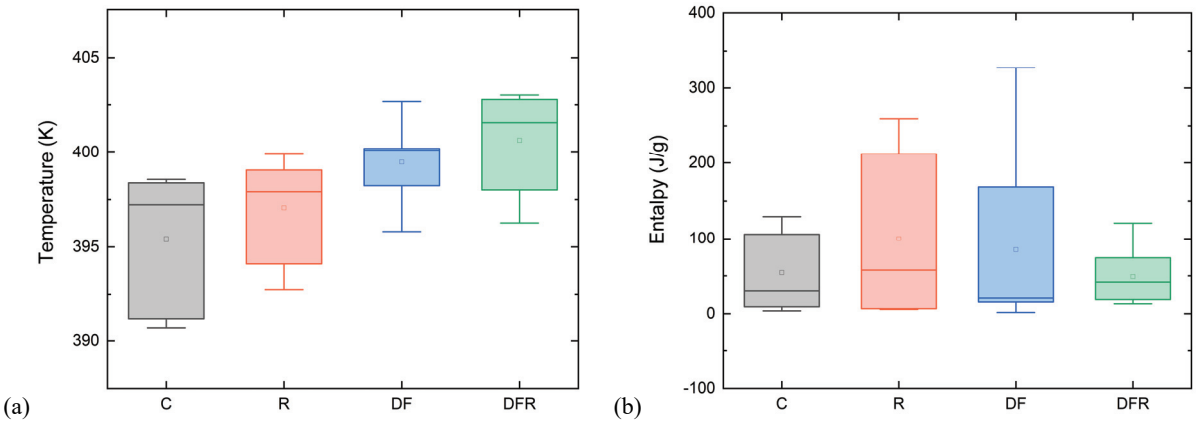

Fig. 4. Temperature of endothermic peak (a) and enthalpy (b) for individual groups of mice presented for left femurs of mice (C – control; R – running, DF – diclofenac, DFR – DF plus running)

at higher temperatures. We observe a second endothermic peak at a temperature of about 417.6 ± 1.9 K. Both endothermic processes are so close together that it is difficult to separate them. Endothermic processes in these three groups are very widely extended on the temperature scale. The determined enthalpy value for the whole observed endothermic process is greater than 250 J/g.

The presented range of the DSC temperature measurements covers the area in which we observe exothermic and endothermic processes. In the control group we initially observe one small exothermic process, then a strong endothermic process, and there are no traces of other anomalies up to the border of the temperature range tested. The heat of the reaction of the small exothermic process is at the level 10 J/g. The enthalpy of the endothermic process around 396 K is on the border of 40 J/g (Fig. 5).

4. Discussion

To determine the influence of running and diclofenac on bone elastic properties, we decided to perform a statistical analysis (the significance level was set at 5% ($p < 0.05$)). In our case, it was Tukey's multiple pairwise comparisons, the results of which are presented below (Fig. 5).

Using Tukey's test, the means of every treatment to the means of every other treatment (squares in Fig. 5) cam be compared, that is, it applies simultaneously to the set of all pairwise comparisons – in our situation is relation between DF, C, DFR and R. The difference between two means that is greater than the expected standard error (the whiskers).

A one-way ANOVA test was conducted in conjunction with Tukey's pairwise comparisons to determine with 95% confidence whether the percent of storage (elastic) modulus E' and the loss modulus E'' determined in different groups of mice are statistically different. For storage modulus E' (Fig. 5a) it was found that there is statistical difference between the group of mice which received diclofenac in comparison with control and running groups. Opposite reactions occur for the group with diclofenac and running – there are no significant differences between the mentioned group and the control group. This demonstrates that the effect of diclofenac can be leveled through physical activities – in our case, it was running. Such an effect is observable in the frequency of 2, 5 and 10 Hz. The results of the Tukey's pairwise comparison of the loss modulus E'' (Fig. 5b) do not show such clear results as they do in the case of E'.

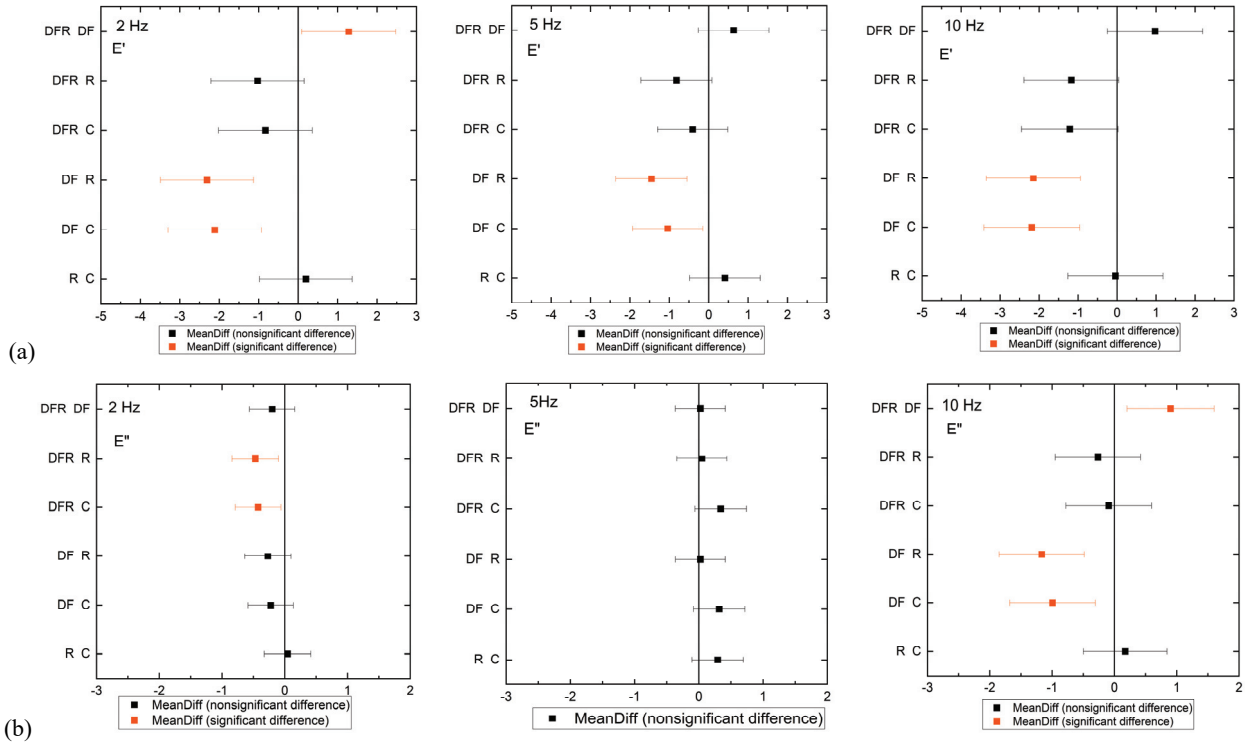

Fig. 5. Frequency-dependent elastic properties: (a) storage (elastic) modulus E' and (b) the loss modulus E'', presented for left femurs of mice (C – control, R – running; DF – diclofenac; DFR – DF plus running) for the frequency of 2, 5 and 10 Hz

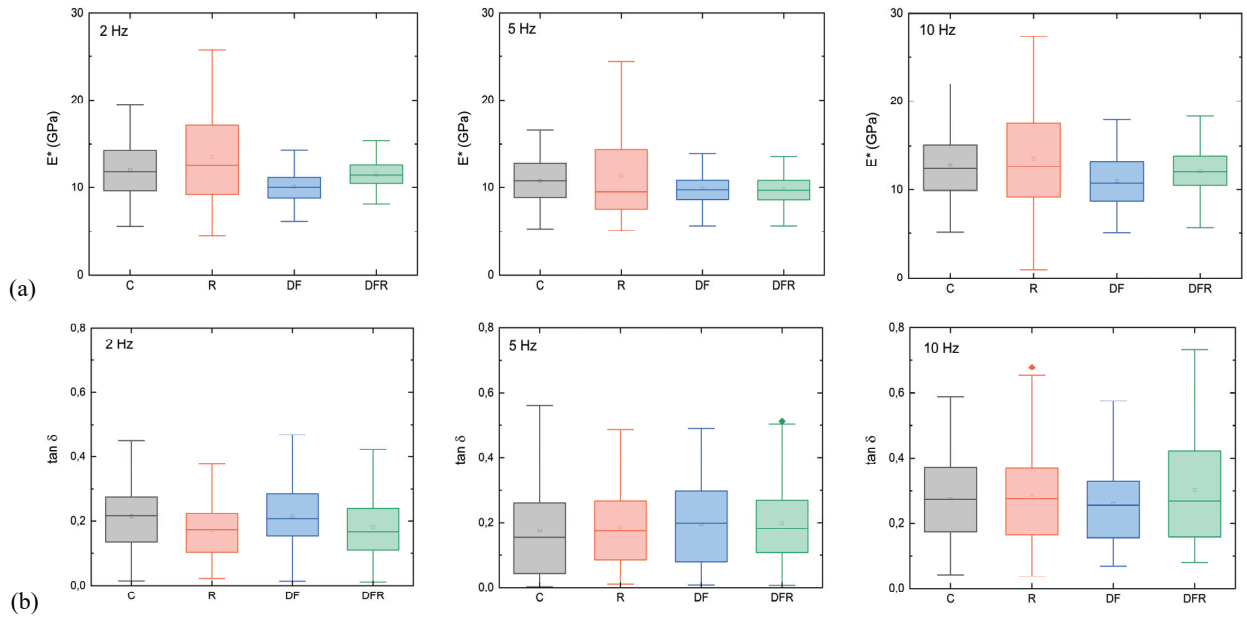

Fig. 6. Frequency-dependent elastic properties, (a) complex elasticity modulus and (b) damping, presented for left femurs of mice (C –control, R – running, DF – diclofenac, DFR – DF plus running)

For the frequency of 10 Hz, the comparison between the groups of mice for E'' shows result similar to E', but 2 and 5 Hz frequencies do not confirm such statistical results.

The obtained results, according to the Eqs. (1) and (2), can be confirmed positively by determining changes in values of E^* and tan δ which are presented in Fig. 6.

We observe the influence of diclofenac on both groups: control and running – the values of complex elasticity modulus (E^*) decreased. The damping had smaller values for running groups, which confirms the positive influence of running. The most interesting is the fact that diclofenac, in comparison with control group, has a harmful effect on the examined murine bones – the values of E^* decrease and the values of tan δ increase for almost all the frequencies. The relation between relative percentage change of elastic modulus for different groups in a few frequencies of applied force has been shown in Fig. 7a. The X axis in Fig. 7a corresponds to the values of E^* for the control group of mice. This graph precisely shows the positive influence of running and the negative influence of diclofenac in the three selected frequencies.

Such a clear view is not observable when we discuss tan δ (Fig. 7b). The X axis in Fig. 7b corresponds to the values of tan δ for the control group of mice. Interesting is the fact that the percentage changes in tan δ increase for different frequencies for the group of mice with diclofenac. For the rest of the groups, the tendency is not so clearly visible.

Diclofenac, which is routinely prescribed in patients after bone surgery as an analgesic, unfortunately also has inhibitory effect on bone reparing [32]. Demonstrating that physical activity carried out in the form

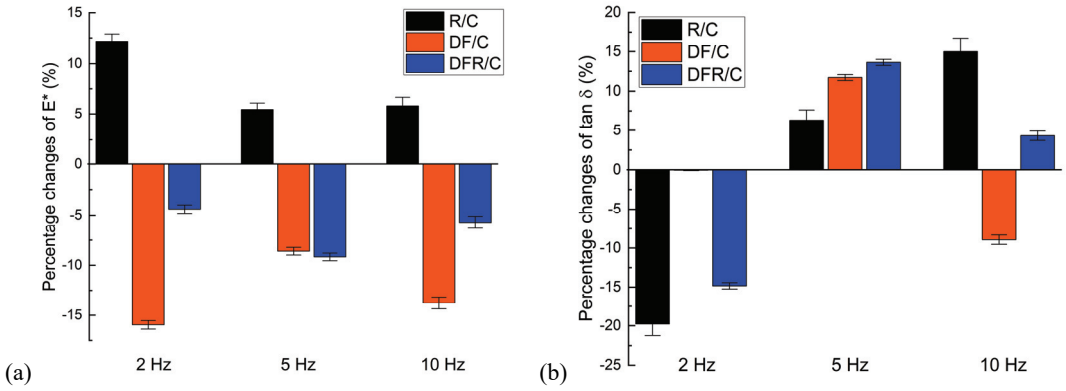

Fig. 7. The relative percentage change in E^* (a) and tan δ (b) for different groups of mice at 2, 5 and 10 Hz of loading force (C – control, R – running, DF – diclofenac, DFR – DF plus running), control – 100%, corresponds to 0 on Y axis

of running improves the elastic properties of bone is a positive aspect of our research. The three-point DMA method used for the elastic study of femur in small animals can be used, in different scope of research – also for much higher values of the acting forces leading to a fracture of the femur [1]. The values of modulus of elasticity of the femora of small animals are generally very scattered, which causes that the average values are burdened with a large errors. Also, the fact that the species, age and individuals weight are differet makes it difficult to compare the obtained values for individual studied groups of mice, which causes some discomfort in the scientists conducting the tests.

Bones of mice are not homogenous in their shape and their construction is not uniform either. Due to this fact we have to consider both elastic and viscous properties. The femur was mostly deformed in the area directly affected by the force. However, this deformation spread throughout the bone volume. This is not only due to the elastic properties of bones but also because of their viscous properties. Conclusions about the viscoelastic nature of a bone can be drawn based on the results of tanδ. The relation between the loss and the storage modulus of the material can be basically characterized by tanδ (Fig. 6b). Delta (δ) should be in the range of 0–90° and as δ approaches 0° it also approaches a purely elastic behavior. Received values of tanδ are close to zero so it means that the investigated bones of mice have elastic behavior with little viscous contribution. We have to remember that bones are not uniform in their construction so viscoelastic behavior should be expected. Crucial is the fact that diclofenac influences such behavior – a greater contribution of viscosity. Thus, the bones of mice treated with diclofenac have larger capacity for dissipated energy than the control group of mice (because of movement (segmental in solid state), the friction between molecules and the heat generated such mechanisms). According to the results, the frequency-dependent complex viscosity $\eta^* = E^*/\omega$ is presented in Fig. 8.

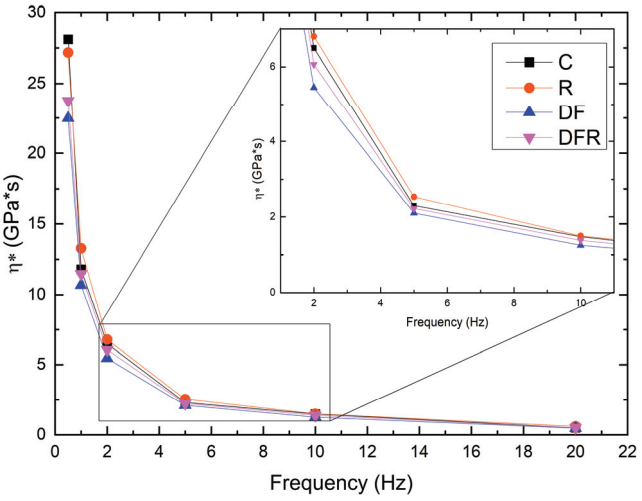

Fig. 8. Frequency-dependent complex viscosity for left femurs of the mice (C – control, R – running, DF – diclofenac, DFR – DF plus running)

It is clearly noticeable that as the frequency increases, the values of viscosity decrease for all the studied groups. The influence of running and diclofenac is also observable – similarly to elastic properties the running increases the values of viscosity while diclofenac decreases the η^* values. To discuss the influence of running and diclofenac on elastic properties of femora, we should consider the relationship between storage modulus and complex viscosity. Such a relation is presented in Fig. 9.

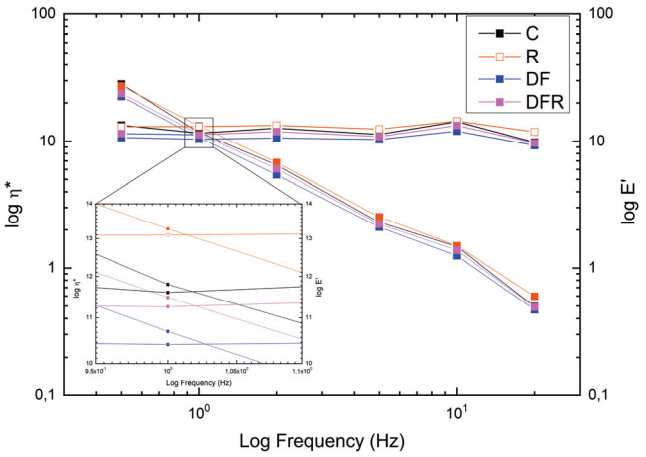

Fig. 9. Relation between complex viscosity and storage modulus depending on the frequency for left femurs of mice. In the insert the region of crossing between log η^* and log E' is presented (C – control, R – running, DF – diclofenac, DFR – DF plus running)

The region of crossing between complex viscosity and storage modulus is shown in the insert in Fig. 9. The position of crossing region does not change too much in the frequency axis (X axis) for different groups of mice. It is clearly visible that running in the control group moves the crossing point to higher values and diclofenac has the opposite effect. On the Y axis, the crossing point has the lowest position for the group with diclofenac (blue lines) and moves to higher values for the running group with diclofenac (magenta lines), the control group (black lines) and the control group with running (red lines).

Based on the results presented in Fig. 9, we can observe changes of molecular weight of the investigated samples. This piece of information is really vital

when discussing viscoelastic behavior of bones. Unlike local techniques such as nanoindentation, DMA investigations give a more precise piece of information about the whole bone structure. The limitation of the study is that we cannot determine on which level, from molecular to structural, it is the R or DF that rebuilt the femur. Diclofenac has negative influence on the femur structure but this influence can be partly reduced by physical activities of the mice.

Bones are a heterogeneous material in terms of structure and basically consist of three elements: mineral, organic and water. These elements are interrelated and fully correspond to the biomechanical properties of a bone [23]. The mineral phase, which consists mainly of hydroxyapatite crystals and is relatively brittle in nature [4], is largely responsible for the elastic properties of a bone. Viscoelastic properties, on the other hand, are related to the presence of collagen in bones, i.e., the organic phase. In particular, literature mentions the collagen type I, which is dominant in bones. Water present in the bones in various places and forms can affect both elastic and viscoelastic properties [3], [40].

The positive effect of physical activity in mice on the structure of their bones is achieved by increasing the periosteal bone formation and overall mineral density [19], [22], [38]. Literature data link the changes taking place in bones, especially with the changes in viscoelastic coefficients which indicate changes in collagen. In our research, this is clearly visible in the E^* values which are the highest for the group of running mice. At the same time, these bones show worse viscoelastic properties, which additionally confirms the implication of the increase in the periosteal bone formation and overall mineral density [14]. The unfavorable effect of diclofenac on the bone structure results from the changes occurring in the collagen, which can be concluded on the basis of the values of the viscoelastic coefficients and the unfavorable changes in bones mineral content. These changes are a result of the action of diclofenac on the body [3]. The opposing effect of both applied factors – diclofenac and systematic running – eliminates the negative influence of diclofenac on the elastic properties of the bones of the studied mice.

Thermograms for all the other tested mouse femora show two effects: a slight shift in the minimum peaks towards higher temperatures by a few degrees and an additional second endothermic process whose minimum is shifted by several degrees towards higher temperatures. The information about the second endothermic process is also confirmed by other authors, who associate it with the next stage of releasing water in bone structures [5], [11], [36]. Our research is not intended to show what is the form of water binding in the examined femur, whether it is only physically absorbed water [5] or embedded water [11]. To correctly determine the enthalpy value for the expanded endothermic process, deconvolution of double-minimum should be performed. Determining the integrated value for a wide temperature range in these endothermic cases gives a much higher value of enthalpy. Analyzing the shape of the obtained curves, it is difficult to draw a base curve for the R, DF and DFR groups in a comparable temperature range, which is easily manageable in the case of the control group.

5. Conclusions

Our DMA tests were carried out on the bones without mechanical treatment and to determine the modulus of elasticity, they were treated as a homogeneous slab with specific physical properties. Based on the DMA results, we can conclude that the viscoelasticity of the femur can be changed by physical activities and diclofenac. The bone is a hierarchical composite and the viscoelastic properties may differ between microstructural and macroscopic levels of the analysis. On the molecular scale, collagen and non-collagenous proteins can give rise to significant viscoelasticity [10]. From the macroscopic point of view, porosity plays a major role in the viscoelasticity of bone [15]. Of course, these two elements are not the only ones responsible for the viscoelastic properties. Based on our investigations, it is clearly visible that running and diclofenac have influence on viscoelasticity at the whole bone level. Macroscopically, we can observe that the elastic properties of the bones of mice receiving diclofenac are enhanced by running. All measurements were made in a strict time regime and under identical conditions.

Applied to subsequent groups of mice, these external factors – running and diclofenac – modify the basic parameters of the endothermic process associated with the release of water. As for now, we are unable to give any explanation of the observed small exothermic process preceding the strong endothermic processes discussed above.

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