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Trabecular bone remodelling in the femur of C57BL/6J mice treated with diclofenac in combination with treadmill exercise

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Purpose: Analgesic treatment with diclofenac deteriorates bone structure and decreases biomechanical properties. This bone loss has been though to be reversed by training. The impact of exercise on bone treated with diclofenac (DF) has reminded elusive. In the present study, we assayed the combined impact of exercises and DF on mouse femur. *Methods:* The femur samples we obtained from 30 days treated C57BL/6J female mice. The training group ran on a horizontal treadmill at 12 m/min by 30 min a day (5% grade/slope). The group of ten mice treated with DF received the drug subcutaneously every day (5 mg/kg of body weight/day). The combined group ran on the treadmill and obtained DF. After 30 days, we sacrificed mice and studied their femurs using microcomputed tomography (μCT), dynamic mechanical analysis (DMA) and nanoindentation. *Results:* We observed that treadmill running and DF decreased trabecular bone volume and mineral density. Combined effect of training and DF was not additive. A significant interaction of both parameters suggested protective effect of training on bone loss provoked by DF. The femur cortical bone shell remained untouched by the training and treatment. The training and the DF treatment did not alter the storage modulus E′ significantly. The unchanged storage modulus would be suggesting on the unaltered bone strength. *Conclusions:* We concluded that even relatively short time of training with concomitant DF treatment could be protective on trabecular bone. Although viscoelastic properties of the entire femur were not modulated, femur trabecular tissue was thinned by treatment with DF and protected by training.

Key words: *storage modulus, treadmill, femur, dynamic mechanical analysis, nonsteroidal anti-inflammatory drugs, diclofenac*

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1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used to control pain which accompanies musculoskeletal diseases, fractures, and osteoarthritis (OA) [4]. Chronic administration of NSAIDs, depending on the study protocol, adversely affects the skeletal system and bone healing or has a neutral impact [20]. The one of the most effective NSAIDs is diclofenac (DF), which has been reported to be a potent inhibitor of cyclooxygenase 2 (COX-2) and also cyclooxygenase 1 (COX-1) [5]. In rat osteomised tibia, DF reduces bone mineral density (BMD), decreases resistance to three-point bending and the maximum load to fracture [1]. Krischak et al. [23] demonstrated that DF diminished the appearance of osteoblasts at the site of bone healing in rats. DF treatment impairs fracture healing in the mouse femur. DF-treated mice show a reduced osteoblast number in bones compared to placebo-treated mice [15]. One of the most recent studies investigating the effects of DF on C57BL/6J mouse bone repair has shown that DF applied during the first two weeks after orthopaedic surgery disrupts the healing cascade [21]. Nevertheless, the structural and mechanical properties of unharmed mouse bones after treatment with DF have not been investigated.

Contrary to NSAIDs treatment, exercises has been thought to induce increase bone strength [8]. Most animal models have reproduced the training effect of an increase in bone strength. However, female C57BL/6J mice have exhibited a decrease in bone strength after four weeks of treadmill training [9]. The strength increase is observed after a two-week latency before termination [26]. Other studies of C57BL/6J female mouse treadmill training have shown no significant changes in architecture and strength of tibiae [25].

Despite the extensive literature, there are surprisingly few studies of combined treatment with NSAIDs and exercise in the mice. Combined studies have been rarely applied in unhurt mice with uninjured bones. Usually, various NSAIDs drugs has been studied in animals that have undergone bone injury procedures [4], [5], [7]. An effect of NSAIDs on bone composition has been rarely analysed in training mice [24]. Several groups reported the impact of NSAIDs and training on bone in humans and rats [13]. An interesting study has showed that ibuprofen treatment of intensively trained rats preserved trabecular bone quality by reducing osteoclasts and bone inflammatory cytokines [9]. In the present study, we aimed to test the hypothesis that DF differently shapes the bone tissue of active and inactive mice. To test this hypothesis, we injected DF into C57BL/6J mice put to forced physical activity on a treadmill. Using microcomputer tomography, dynamic mechanical analysis and nanoindentation, we analysed structural and mechanical properties of mouse femur.

2. Materials and methods

2.1. Materials

The Local Ethical Commission approved the animal protocol for Investigation on Animals, Poznan University of Life Sciences (Permission No. 39/2017). The research was conducted using 9-week-old C57BL/6J female mice housed in standard polycarbonate cages under controlled conditions (12 h dark/12 h light, temp. 21 °C ± 1 °C) with free access to water and the commercial rodent chow Labofeed B (Kcynia, Poland). The mouse model C57BL/6J Cmd, strain C57BL/6J, was obtained from the Mossakowski Medical Research Centre, Polish Academy of Sciences (Warsaw, Poland). Before the experiment, the mice were allowed to adapt to the laboratory environment for two weeks. Based on the literature, the experiment was performed using female mice due to data indicating that osteoporosis is more common in women. The experiments conducted were within the Polish animal welfare regulations and guidelines.

Experiment was performed on 40 mice divided into the four groups: control (Contr, $n = 10$); running (Contr(R), $n = 10$); diclofenac (DF, $n = 10$) and diclofenac running group (DF(R), $n = 10$). 55 mg of diclofenac per kg of body weight per day (Alfa Aesar, cat no. J62609, Haverhill, MA, USA) was injected intraperitoneally for 30 consecutive days mice from DF and DF(R) groups. The mice in the control groups were treated with equal amounts of saline (0.9% NaCl). During the experiment, body weights were recorded weekly.

In addition, mice from the Contr(R) and DF(R) groups were subjected to the running procedure using a rodent horizontal treadmill (Ugo Basile, Italy; cat no.47300). To avoid stress effects during the main experiment, the animals were acclimated to the treadmill over seven days before the main procedure. Animals ran on the horizontal treadmill 12 m/min for 30 min a day (5% grade/slope) according to the modified schedule described by Wu [29].

At the end of the experiment, after 30 days, mice were sacrificed by decapitation. The right and left femurs were dissected, and fragments of soft tissues were thoroughly removed using scalpels. Then, the bones were washed with PBS, dried on filter paper, protected with aluminium foil and transferred to sealed tubes. The tubes were kept at –20 °C till the day of tests.

2.2. Microtomography examination

The microstructural properties of cortical and trabecular tissue of the distal part in right murine femur bones were determined using a SkyScan 1172 micro-CT system (Bruker, Belgium). All 40 specimens of murine femora (10 from each group) were scanned with a resolution of 6 μm using the following scanning parameters: X-ray tube current set at 181 μA, tube voltage of 55 kV, 0.5 mm A1 filter and exposure time of 800 ms. During the scanning, bones were wrapped in paper tissue, placed in a polypropylene tube and stabilised using the foam material. Tissue paper was moistened around the bones with water.

To calculate the mineral density of the analysed tissues (BMD), Bruker-Micro-CT BMD calibration phantoms with CaHA concentrations of 0.25 and 0.75 g·cm^{-3} were used. After the reconstruction of the obtained images (NRecon, Bruker, Belgium), volumes of interest (VOI) were created within the distal femur (CtAn, Bruker, Belgium). Both trabecular and cortical VOIs were created regarding a growth plate, which was localized each time at cross-sectional images (Fig. 1). The trabecular region was located approximately 0.3 mm from the growth plate and extended for 2.4 mm, while the cortical tissue region was approximately 0.6 mm above the trabecular region and extended from this position for 0.9 mm.

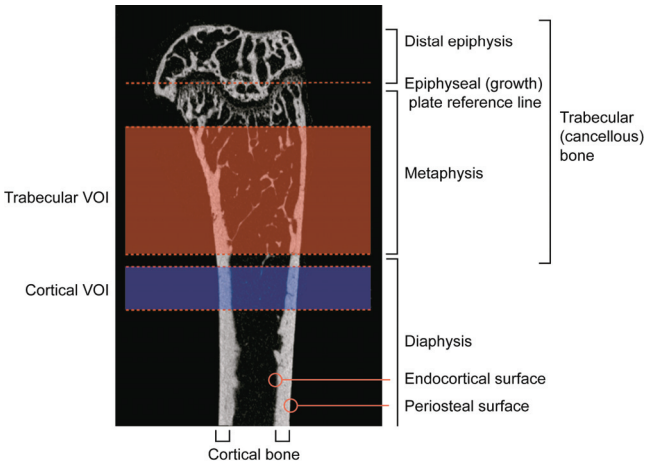

Fig. 1. Visualisation of the distal epiphysis of the mouse femur. There are marked regions subjected to the μCT analysis (VOI) for trabecular (cancellous) bone tissue (red area) and compact bone (blue area) with indicated growth plate as a reference level

The analysed parameters were as follows: percent bone volume (BV/TV); trabecular thickness (Tb.Th); trabecular separation (Tb.Sp); trabecular number (Tb.N); bone mineral density of trabecular bone (BMD); mean total cross-sectional cortical bone area (Ct.Ar); cortical cross-sectional thickness (Ct.Th); tissue mineral density of cortical bone (TMD).

2.3. Dynamic mechanical analysis – DMA

Using the DMA 242 C Dynamic mechanical analyser DMA 242C (NETZSCH, Germany), a three-point bending test was performed to measure the bones' macro-mechanical properties. Forty samples, ten from each group, were examined. In a measuring holder, defrosted bones were placed in a way so that the two edges were supported (10 mm distance) while the load was applied from above.

The analyser's measuring chamber was set to increase from room temperature to 35 °C at a ramp rate of 1 degree per minute. For each sample (n = 40, 10 from each group) we applied six oscillation frequencies: 0.5, 1, 2, 5, 10 and 20 Hz. Each frequency sample was measured 16–17 times, and the mean value of the repeats was applied as a single repeat in the calculation of the presented results. The frequencies changed sequentially, and the entire measurement lasted 30 min. The sample alignment was the same for each test, and no bones were broken during the measurements.

Parameters describing elastic properties of the examined mouse bones were analysed: the storage (elastic) modulus E′ and the loss (viscous) modulus E″. The analysis assumed that the sample has a bar shape. The geometric factor for the adopted shape is defined as $x^3/4zy^3$, where x is the value of the support span, y is the sample height, and z is the sample width.

2.4. Nanoindenteration – micromechanical analysis

In order to perform the nanoindentation test, samples required special preparation. According to the method described by Zhang and Jing [11], [31], the samples were dewatered in 80% ethanol after thawing.

Three millimetres bone slices were cut immediately behind the head of the bone and fixed in the fast setting epoxy resin. The post-set mass of bone was embedded with a low-temperature acrylic mass in a plastic mould and polished sufficiently deeply to reveal the bone tissue. For this purpose, abrasive papers with gradually decreasing grain size of 200, 400, 800, 1200, and fabric disc with 50 nm aluminium oxide grains were used. The obtained surface quality was checked using a LEXT OLS4100 laser confocal microscope (Olympus, Japan); the average surface roughness was Ra = 84 ± 1 nm.

Optical measurements of the measurement area were performed using a laser confocal measuring microscope LEXT 4100 (Olympus, Japan). Nanomechanical tests were carried out using a nanoindenter Agilent G200 with DCMII measuring head (Keysight Technologies, USA). The indenter registers the force versus depth curve by making an indent. The force applied to the indenter tip oscillated sinusoidally during the test, which allowed for quasi-continuous determination of the hardness value and bone elastic module. The measurement was carried out for indentation depth between 400 and 2400 nm from the cross-sectional area. On each bone (n = 20, five from each analysed group), 10–15 measurements in the middle of the bone wall cross-section were performed. The obtained results were used to analyse the hardness and modulus of elasticity.

2.5. Statistical analysis

The data were developed using GraphPad Prism version 9.0.2 software (GraphPad Software, USA). The normal distribution of the analysed parameters was examined using the Shapiro–Wilk test, equality of variance was tested with the Bartlett test, and outliers were rejected using the Grubbs test. The two-way ANOVA was used for identification the influence of the diclofenac and treadmill activity on analysed parameters. Statistica 13.0 software (TIBCO Software Inc., USA) was applied to prepare the box-whisker graphs. The significance level was set at 5% ($p < 0.05$).

3. Results

3.1. Microstructural analysis

Representative images of cortical and trabecular bone for each of the analysed mouse groups are presented in Fig. 2. A weaker structure of trabecular bone tissue, characterised by lower BV/TV, Tb/N, and BMD related to the control group, was observed after DF treatment (Fig. 2A).

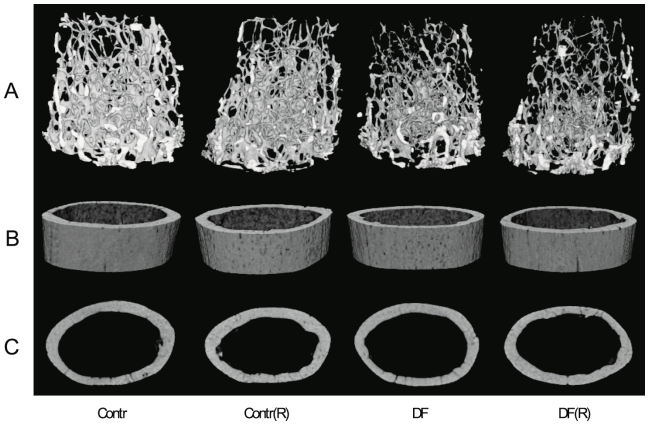

Fig. 2. Images of C57BL/6J mouse femur microstructure obtained by μCT after 30 days of treatment with diclofenac (DF), methylprednisolone (MP) and/or running for 30 min/day. A) trabecular bone; B) cortical bone (frontal view); C) cortical bone (transverse view) of the analysed areas, representing six groups

There was a statistically significant interaction between running and DF effects on the trabecular bone microarchitecture in BV/TV (p = 0.0263), Tb.Sp (p = 0.0242), Tb.N (p = 0.0301) and BMD (p = 0.0115) parameters. The simple main effect showed the differences in mentioned parameters among the control group's activity level. The running significantly decreased BV/TV, Tb.Th, Tb.N and BMD by 29.3, 7.9, 23.1 and 33.5%. Also, the statistically significant influence among DF presence in non-running mice was observed. In such case, we noticed a decrease of BV/TV, Tb.N and BMD by 36.6, 30.5, and 39.5%, consecutively, with increased Tb.Sp by 12.1% (Fig. 3A, C, D, E).

Moreover, we found a statistically significant difference in average Tb.Th caused by drug and (p = 0.0237) physical activity (p = 0.0229), though the interaction between these factors was not significant (Fig. 3B).

In the case of cortical bone, two-way ANOVA showed a statistically-significant difference in average Ct.Ar by DF (p = 0.0228), though the effect of running and the interaction between these factors was not significant. Analysed factors have not affected Ct.Th and TMD values.

3.2. Mechanical analysis

Depending on the group of mice being studied using DMA, the obtained dynamic elastic modulus E′ ranged from 9800 to 14500 MPa (Fig. 4). The lowest E′ values,

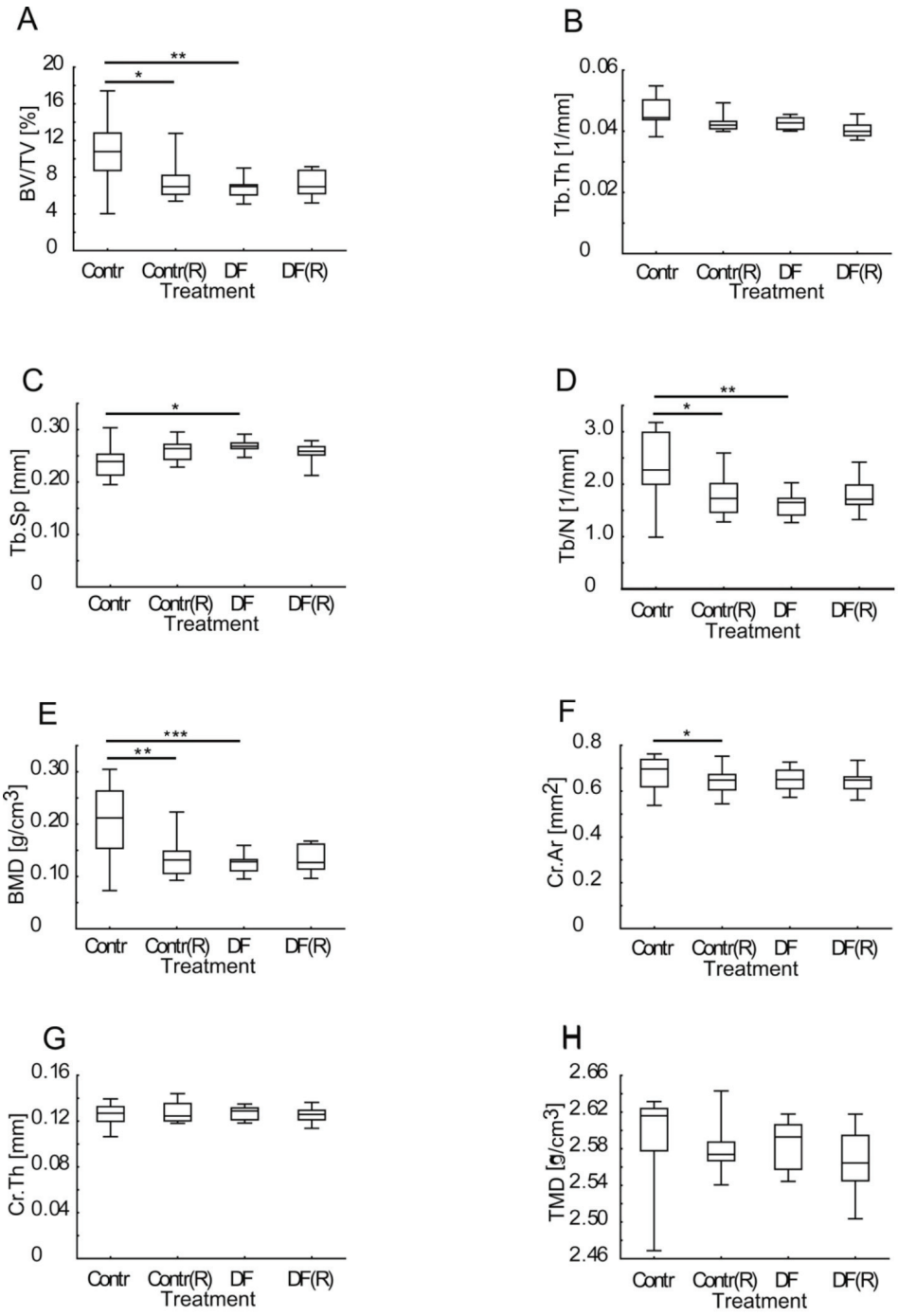

Fig. 3. Measurements of femoral microstructural parameters by μCT in mice after 30 days of enforced treadmill running and treatment with diclofenac (DF) among trabecular bone: (A) percent bone volume (BV/TV); (B) trabecular thickness (Tb.Th); (C) trabecular separation (Tb.Sp); (D) trabecular number (Tb.N); (E) bone mineral density (BMD). Cortical bone: (F) cortical cross-sectional bone area (Ct.Ar); (G) mean total cross-sectional thickness (Ct.Th); and (H) tissue bone density (TMD(c))

ranged 9200–12070 MPa, were observed in the group of mice treated with DF; mice treated with DF and running had E′ values that were approximately by 500 MPa higher.

The nanoindentation results are shown in Table 1. Mouse exposure to DF caused a slight increase in the static elastic modulus and hardness. However, when mice were exposed to running, we observed a slight increase in the static elasticity and hardness of 12% (p = 0.043) and 18% (p = 0.028), respectively, relatively to the control. In running mice treated with DF an increase of 11% was observed in the tested parameters.

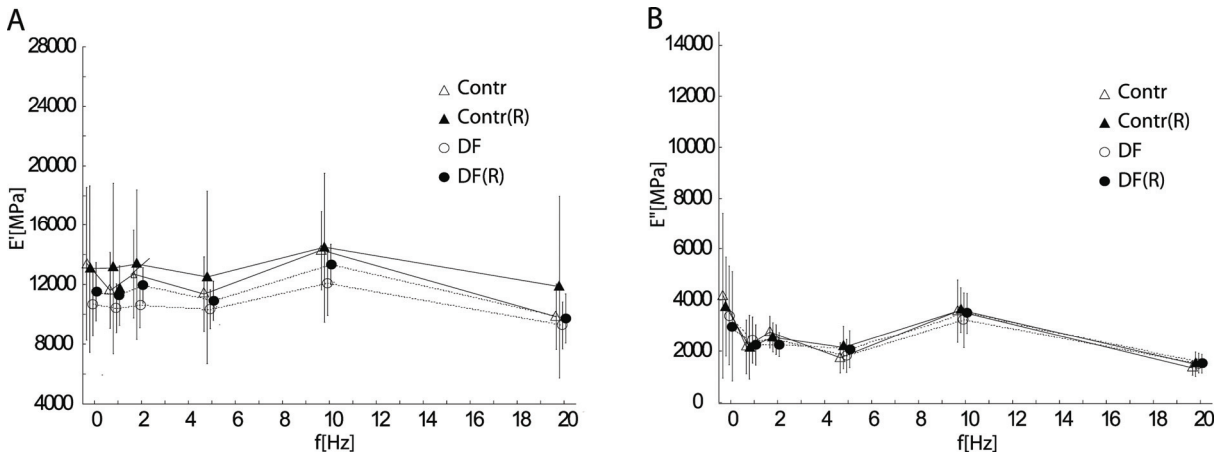

Fig. 4. Dynamic mechanical analysis (DMA) measurements of the storage modulus E′ (A) and the loss modulus E″ (B) of the femur of mice after 30 days of treatment with diclofenac (DF and/or running for 30 min/day on a treadmill (R)). Presented values in MPa assayed at vibration frequencies of 0.5, 1, 2, 5, 10, and 20 Hz. Each point represents the arithmetic mean of measurements of ten bones, with whiskers corresponding to the SD

Table 1. Bone storage modulus and hardness measured by nanoindentation

	Contr	Contr(R)	DF	DF(R)
Modulus [MPa]	21944 ± 1612[a]	22319 ± 452	22698 ± 1363	22036 ± 1414[b]
Hardness [MPa]	807 ± 65[a]	859 ± 64	850 ± 51	817 ± 73[b]

Quantitative analysis of the bone nanomechanical properties indicates statistically significant interaction between DF effects and running on the bone hardness (p = 0.0047). The simple main effect showed the differences in averaged hardness values among DF treatment in non-running mice (p = 0.0125).

4. Discussion

The effects of NSAIDs and training on the structural, biochemical and mechanical properties of bone tissue have been usually studied separately. Little attention has been paid to the combined effect of training and NSAIDs on viscoelastic parameters of bone. To the best of our knowledge, the analysis of DF impact on the mouse femur under enforced treadmill training has not been published. Various authors have shown in separate studies that NSAIDs impair bone healing, and the exercises increase bone density and strength [4], [5], [7], [10], [11], [16].

In the present study, we aimed to test the hypothesis that DF promotes the weakening of healthy mouse femur. According to our assumption, DF injections have a different effect on bone structure depending on mice physical activity. Since DF weakens the bone structure, running should strengthen the bone tissue. Therefore, physical activity should work as a bone protective factor for the harmful effects of DF. Combination of microstructural (μCT) and mechanical (nanoindentation, DMA) tests enabled us to fully investigate this effect quantitatively and qualitatively. During experiments, we tested femora because they show a better response than tibiae to loading in C57BL/6J mice [20].

In the group of non-running mice from our study, treated with DF, we identified a weaker bone structure. The other group of mice exercised on the treadmill 30 days. The conducted morphometric studies showed that in mice with enforced physical activity, which did not take the DF drug, a weakened microarchitecture of trabecular tissue was observed (lower BV/TV, Tb.N. BMD, and higher Tb.Sp). However, in the group of mice treated with diclofenac, the authors of this paper identified a weaker structure in non-running mice. Therefore, we can conclude that increased physical activity modifies the drug's action in this case, so it is a protective factor that weakens the negative effect of NSAIDs. However, treadmill training did not compensate fully for diclofenac injections' negative effect on the microarchitecture changes in the femur.

Using μCT, we found that DF promoted trabecular bone degradation and caused a decrease in BV/TV, Tb.N and BMD. Previous studies have already shown the harmful effect of DF on animal femurs [21]. Before treatment with DF, authors damaged mouse fe-

murs by drilling. DF treatment caused a decrease in bone volume and increase in bone porosity [21]. In another study, DF decreased the Tb.N and bone mass in the site of bone healing. Authors explained it by a reduced osteoblast number and activity [23]. DF also delayed fracture healing and decreased BMD in rats [1]. In line with our study, in mice C57BL/6J receiving NSAID drug, NS-398, trabecular number decreased but cortical bone remained intact [24]. In ovariectomised, 8–12 weeks old, C57BL/6J mice trabecular number loss also occurred and cortical bone was unaffected [32]. In COX-2 knockout mice, COX-2−/− female mice had healthy bone geometry and trabecular microarchitecture [22]. Most previous animal studies have concerned injured bone healing and NSAIDs treatment [20]. The novelty of our study was DF administration to mouse with unhurt bones. The μCT results raised the question of whether the observed structural changes after exercise and DF treatment implicate biomechanical effects.

The bone volume and mineral density are usually concomitant with mechanical properties [7], [17], [18]. Nevertheless, there are exceptions to this dependency. Wojtków et al. [27] presented that, at early stages of osteoporosis, morphological changes of trabecular bone structure (reduced trabecular thickness and atrophy of the smallest trabeculae) can be observed. Despite significant changes in trabecular microarchitecture induced by osteoporosis, no significant effect was reported on the mechanical parameters of bone tissue.

In our study with the use of DMA, we did not show a significant decrease in storage modulus E′ in the DF treated femur. DMA has been used previously to study the femur of C57BL/6J mice, but authors have not applied DF [12]. Our results are novel because of application of DMA to measure mouse femurs treated with DF. The advantage of DMA is an experimental simulation of repeated vibrations. The frequency of vibration is similar to the natural vibration of the mouse femur. These pre-yield repeated strains imitate usually repeated loading of femur without single damage incident. The most previous bone mechanical studies assayed strength using three-point bending. Nevertheless, DMA and other biomechanical studies are complementary. DMA determines viscoelastic properties and could shed light on bone strength [30]. The storage modulus (E′) is analogous to Young's modulus for monotonic loading. E′ may be an indication of changes in strength due to the correlation between modulus and strength of cortical bone tissue [30]. These assumptions allow for comparing our results with previous strength tests. Rat bone mechanical testing also revealed a diminished strength of the injured femora in the DF treated group [23]. Other authors have used a three-point bending test for rat tibiae and showed a decrease in bending stiffness and braking force after DF treatment [1]. The separate use of DF and physical exercise did not affect bone viscoelastic properties.

Despite the lack of influence of the applied running and DF on the viscoelastic parameters, we observed their statistically significant influence on the hardness of the cortical tissue. The same as for morphometric parameters, increased activity of mice on a treadmill turned out to be a protective factor for the negative effects of diclofenac. However, due to the short duration of the impact of physical activity (only four weeks), these changes did not fully compensate for the negative effects of DF, but revealed protective tendency.

Our results were consistent with data obtained by Hollinski et al. [8] for female and male C57BL/6J mice. These authors showed that four weeks of exercises provoked a net reduction of female bone mass. In another study, female mice trained on a treadmill did not show changes in the strength of tibiae [25]. Even so, many studies have shown the opposite effects. Bone mechanical properties increased after training [13], [14]. The discrepancies between earlier studies prompted us to design our experiments. Studies showing training effect on pre-yield elastic behaviour have been rarely reported.

In a study of Kodama et al., female 9-week-old C57BL/6J mice exercised for 28 days. Authors assayed right tibia and femur using 3-point bending [13]. They revealed exercise-dependent increases in periosteal bone formation but lack of increase in bone strength [13]. In the study of Wallace et al., male 16-week-old C57BL/6J mice ran on a treadmill for 21 days. Tibial strength increased without an increase in cross-sectional properties. The authors suggested that exercise improved bone strength by modifying the extracellular matrix [26]. In our study, although trabecular bone BV/TV and BMD decreased, the storage modulus E′ and loss modulus E″ of femurs did not decline. Resilience measured by DMA refers to the capacity of bone to store energy [7]. We concluded that due to unchanged cortical bone, the femur kept its resilience. The cortical bone receives the most of physiological loading [28].

In earlier studies, the BMD modified by training in humans remained unaffected by ibuprofen [10]. Yet, ibuprofen consumed immediately after resistance training has a deleterious effect on bone mineral content at the human distal radius [3]. Similarly to our study, in mice C57BL/6J receiving NSAID drug NS-398, inhibition of COX-2 was associated with reduced trab-

ecular number but did not influence cortical bone [24]. In contrast to our study, in Sugiyama's et al. study, loading has increased trabecular thickness and cortical periosteally enclosed volume. NS-398 has not modulated this response [24]. Discrepancies with some previous results and our observations suggest that bone response to training and NSAIDs may be the result of different drugs administered, their doses, and schedules of exercises.

The novelty of our work demonstrats that DF and training stimulats trabecular bone loss, but do not affect cortical bone. This observation resembled the effect of estrogens deprivation in ovariectomised mice [19], [32]. Trabecular bone loss did not impair bone elastic module because cortical bone was unaffected by training and DF. However, hardness of cortical bone was affected by analysed factors. We showed that bone loss was not accompanied by alteration of viscoelastic properties. The same as in the results obtained by other authors [2], [27], our experiment show that changes in the microstructure of trabecular bone tissue in femur occur faster than changes in the mechanical characteristics in cortical bone. It suggests that the rate of remodelling and drug response of cortical tissue is slower than that of spongy tissue. This difference may be correlated with a age-related trabecular bone loss and cortical bone increase in nine weeks old female mice [6].

The limitation of this study is the use of a single mouse strain C57BL/6J. Other strains, especially those with a thicker bone structure, should be investigated in the future. Their analyses could show bone response to the combined effects of DF and training. In the future, prolonged schedule of exercises could compensate the DF effect on the trabecular bone structure. The mechanism of the selective DF and training impact on femur cancellous bone remains to be elucidated in future study.

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