ORIGINAL ARTICLE

Muscle metabolism during tibial lengthening with regular and high distraction rates

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Abstract

Introduction Muscle regeneration is promoted when the Ilizarov method is used for limb lengthening and deformity correction, but the regenerative ability of muscles decreases when achieving large amounts of elongation. Much research has been dedicated to studying the capabilities of muscles under lengthening, but no reports are available that investigate the muscle metabolism. We supposed that energy turnover would be activated in skeletal muscles under lengthening as a response to distraction, and the activity of the energy turnover would grow in proportion to the increase in the distraction rate or amount.

Materials and methods We compared the metabolism of canine anterior tibial muscles (ATMs) by regular and 3-mm high-frequency bone distraction in 30 dogs to obtain 14.5 ± 0.8 % lengthening from the initial tibial length.

Group 1 ($n = 12$) had manual lengthening with a rate of 1 mm per day. Three millimeters per day was produced with 120 increments in automated mode in group 2 ($n = 12$). An intact group ($n = 6$) served as controls. ATMs were harvested at the end of distraction, after 30 days of fixation, and 30 days after frame removal. We assessed the activity of lactate dehydrogenase, creatine phosphokinase, glucoso-6-phosphate dehydrogenase, and catalase and calculated the concentration of malone dialdehyde, sarcoplasmic and contractile proteins in the ATM extract.

Results Energy turnover reactions were activated in the ATM as a response to distraction forces, but the activity of the energy turnover did not grow proportionally to the increased distraction rate. Levels of sarcoplasmic and contractile proteins in the ATM decreased insignificantly in both groups.

Conclusions High-frequency 3-mm daily lengthening results in compensatory energy turnover changes in the muscle, sufficient for prevention of catabolic processes.

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Introduction

The Ilizarov method of distraction osteogenesis has become the leading method used for limb lengthening and simultaneous deformity correction [1–5]. The author of the method scientifically grounded the daily rate of bone lengthening equal to 1 mm that is accomplished with four increments in a manual mode [1]. However, studies have been undertaken to reveal the effects of a faster frequency of increments or greater daily amount of lengthening in order to speed up the process of distraction osteogenesis [6–9]. One of the methods, an automated high-frequency distraction with a rate of 1 mm per day implemented

continuously with 60 increments for 24 h, was used clinically with the aid of an automated distractor [6]. Bone regeneration in humans with a rate of up to 2 mm per day produced bifocally or at multiple levels has become more common in several orthopedic situations, and the regeneration in such conditions was proven to be successful [3, 7, 8]. Our orthopedic centre has studied bone regeneration experimentally with a rate of 3 mm per day at one level, which was completed with 180 steps using automated distraction, and showed that the bone regeneration could be valid and consistent [10].

However, large amounts of daily bone distraction can limit the functions of the skeletal muscles attached to the bone under lengthening as the soft tissues are exposed to great tensile stress [7]. Therefore, much of the basic research has been devoted to muscle tissue. A number of experimental models have reported that muscle growth during distraction is not adequate for bone tissue growth and leads to impairment or loss of function due to stretching [11], whereas the acceleration of the process results in greater degenerative changes in muscle tissue [12]. Most of the reports showed structural, morphometric, and histological changes in animal muscles during distraction, which was performed with different lengthening rates, including 3 mm per day [11–18].

It is not clear whether the marked changes in muscles are reversible and how they can limit the use of higher daily rates and a high frequency of distraction. No studies are available assessing the relationship of different bone distraction rates or modes and metabolic conditions in skeletal muscles according to their application. Nevertheless, a study that investigated the effects of different distraction frequencies on neuromuscular recovery in a rabbit model showed that microtrauma due to high-frequency distraction resulted in improved recovery of muscle and nerve functions [19]. In clinical studies, it was also shown that patients experienced a significantly lower level of tensile forces during a high-frequency lengthening procedure [6, 9].

It is well known that several biochemical processes determine the functional activity of muscles, namely, the reactions of energy turnover that are regulated by enzymes. Therefore, we aimed to compare the metabolic changes that can occur in the canine anterior tibial muscle (ATM) by using the regular and high-speed lengthening with the Ilizarov method. We hypothesized that (1) the energy turnover reactions would be activated in the skeletal muscle under lengthening as a response to distraction forces; (2) the activity of the energy turnover in the skeletal muscle would grow proportionally with the increase in the distraction rate.

Materials and methods

Tibial lengthening was carried out using 30 adult mongrel dogs divided into three groups. Group 1 consisted of 12 animals that underwent bone lengthening at the rate of 1 mm per day (4 increments, 0.25 mm each) in a manual mode. Group 2 included 12 dogs that had bone lengthening at the rate of 3 mm per day done with 120 steps in an automated mode. We obtained 14.5 ± 0.8 % lengthening from the initial tibial length. The animals were killed at the end of distraction ($n = 4$), after 30 days of fixation ($n = 4$), and 30 days after frame removal ($n = 4$) in each group. There were six dogs in the control intact group (IG). The age range of the animals was from 1 to 3 years. They weighed from 15 to 30 kg. The average length of the tibia was 18.6 ± 1.3 cm.

The number of dogs in the groups was chosen according to the requirement of the ethics board, which prescribes minimizing the number of experimental mammal animals used in invasive experiments to numbers sufficient for statistical analysis.

Lengthening protocols

In the experimental lengthening groups, the Ilizarov apparatus was used and comprised two arches proximally and two rings distally as external supports used for attaching eight crossed wires that transfixed the tibia, with two wires at each level. The arches and rings were connected to form the proximal and distal subsystems. An open transverse osteotomy in the middle third of the fibular diaphysis was performed using a chisel while the tibia was cut at the same level using a closed technique of flexion osteoclasis. Flexion osteoclasis results in minimal damage to the periosteum, cortex, and surrounding soft tissues. The bone marrow and the nutrient artery remain intact. Then, the proximal and distal apparatus subsystems were connected with threaded rods in group 1, receiving regular manual lengthening of 0.25 mm with four daily steps, while in group 2, an automated distractor was used between the subsystems, including a programmed control unit to produce 120 lengthening steps for 24 h (Fig. 1).

Bone distraction started on day 5 following the operation after the acute invasion signs had subsided. Previous studies revealed that the optimal time to start distraction was the 5th day after the operation. An earlier start resulted in poorer regeneration, while a later one resulted in premature consolidation [20]. Group 1 dogs had distraction for 28 days and group 2 animals for 10 days. The final amount of lengthening achieved in all the animals was similar (mean, 14.5 ± 0.8 % of the initial tibial length). The post-
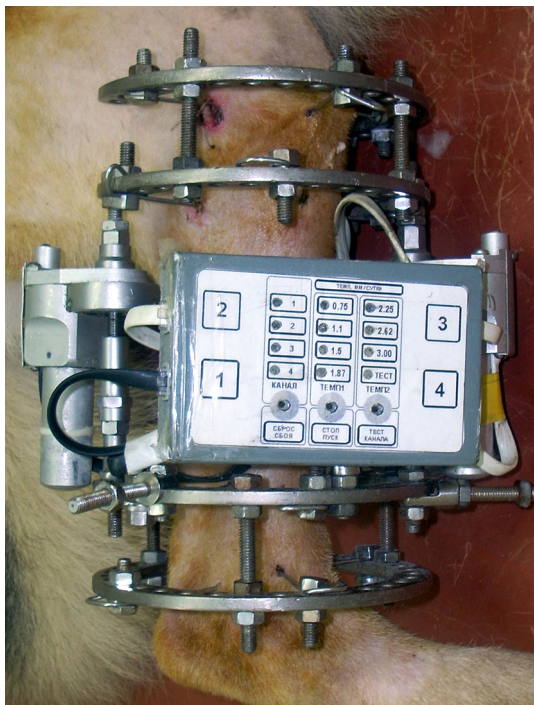
Fig. 1 Diagram of the Ilizarov frame for tibial lengthening with an automated control

distraction period or fixation for regenerated bone maturation with the frame on continued approximately 30 days.

The regeneration quality was assessed with radiography. Coronal and lateral views were taken on the day of the operation, every 7th day of distraction, every 15th day of fixation, and after the removal of the apparatus using a Toshiba E-7239X system (Toshiba Electron Tubes and Devices Co., Ltd., Japan).

Preparation of ATM material

The ATM was harvested immediately after euthanasia at three time points: at the end of distraction, after 30 days of fixation, and 30 days after the Ilizarov apparatus removal. The ATM material from the control animals was harvested at a single time point.

To study the metabolic processes in the ATM, a sarcoplasmic extract was prepared. Erythrocytes were washed from the ATM tissue with a cooled 0.03 M KCl solution, then it was weighed, ground, and homogenized. The homogenate was centrifuged for 15 min at 14,000g using a Beckman Coulter ultracentrifuge (Optima^{TM} XE, Beckman Coulter Inc., USA) to obtain a supernatant or sarcoplasmic extract for studying. The sarcoplasmic extract represented the myocyte-derived sarcoplasm. The remaining precipitate was used to separate miofibrillar proteins. It was dried, weighed, saturated with an equivalent volume (1:5) of 0.6 M KCl solution, and centrifuged for 10 min at 5,000g. The content of proteins according to Lowry was determined in the supernatant, which was a solution of miofibrillar proteins such as actin, myosin, and actin-myosin complex.

The sarcoplasmic extract was used to study the activity of the key enzymes that regulate energy turnover: lactate dehydrogenase [LDH, EC (enzyme code): 1.1.1.27], which is the main enzyme regulating glycolysis in muscles; creatine phosphokinase (CPK, EC: 2.7.3.2), which regulates the synthesis of creatine phosphate, a macroergic compound of muscle energy accumulation; glucoso-6-phosphate dehydrogenase (G6PDH, EC: 1.1.1.49), which regulates the intensity of the pentose phosphate pathway of carbohydrate oxidation in muscles; and catalase (EC: 1.11.1.6), which is the key antioxidation enzyme in muscles, which inactivates hydrogen peroxide. LDH was studied for the isoenzyme spectrum. The concentration of malone dialdehyde (MDA), the final product of peroxide oxidation, was assessed in the supernatant.

A semiautomatic chemical analyzer, STAT FAX$^{®}$ 1904+ (Awareness Technology, Inc., USA), and reagent kits from Vital Diagnostics SPB (Russian Federation) were used to define the activity of LDH and CPK. Catalase activity in the supernatant was assessed by hydrogen peroxide subsiding in the incubation medium. G6PDH activity was determined by the accumulation of restored NADP coenzyme in the incubation medium. Tissue enzyme activity was calculated per 1 g of sarcoplasmatic protein, which was determined according to Lowry. Electrophoretic division of LDH was performed using the Paragon Electrophoresis System (Beckman Coulter Inc., USA) and reagent kits and plates from the same company. MDA was determined by the reaction with tiobarbituric acid in the deproteinized sarcoplasmic solution.

The findings in group 1 and 2 were compared with the findings in the intact dogs. The reliability of differences was estimated using the Wilxoson-Mann-Whitney U test. The reliability of differences between the groups was assessed using a nonparametric Kruskal-Wallis test followed by multiple comparisons with the Dunn criterion.

The study was approved by the ethics board of the institution. Interventions, animal care, and euthanasia conformed to the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 18.03.1986), Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985), and the national laws.

Results

The radiographic findings by the end of distraction in group 1 showed active endosteal and periosteal osteogenesis, and the regenerated bone featured well-developed bone portions, which had similar widths as the adjacent bone fragments. The intermediate layer in the regenerated bone was not high (Fig. 2a). After a month of fixation, the regenerated bone was homogeneous and the cortex formed was uninterrupted (Fig. 3a). By the end of distraction in group 2, the regenerated bone featured weak bone portions, and its intermediate zone took up much space (Fig. 2b). Active bone formation was noted after 30 days of fixation, which did not differ from the new bone regenerated in the gap in group 1 (Fig. 3b).

LDH activity in the ATM of group 1 was significantly higher than in the intact animals at all time points studied (Fig. 4), while in group 2 it was significantly higher relative to the intact animals after 30 days of fixation. Reliable differences between experimental lengthening groups 1 and 2 were not revealed for LDH.

CPK activity in both experimental lengthening groups was significantly higher relative to the intact dogs by day 30 of fixation and 30 days after the apparatus removal. Reliable differences between the experimental lengthening groups were noted at both mentioned time points: CPK was higher in group 2 than in group 1.

Low activity of G6PDH was observed in group 1 at all experimental time points, similar to the intact dogs. Interestingly, it was considerably increased in group 2 as compared to the intact group and group 1 at all time points of the experiment.

The LDH isoenzyme spectrum in group 2 at the end of distraction had a statistically significant growth of LDH1 (Fig. 5), but by day 30 of fixation the LDH2 percentage grew in both groups. Thirty days after the removal of the apparatus, group 2 dogs had a similar LDH spectrum as the intact group. However, the isoenzyme profile of the ATM in group 1 retained a high aerobic fraction.

In both experimental lengthening groups, the levels of contractile proteins and sarcoplasmic proteins tended to decrease at all studied time points (Fig. 6). No significant difference was noted between those groups.

Mean MDA concentrations in groups 1 and 2 increased until the end of distraction, but no reliable variance between those two groups was noted (Fig. 7).

The catalase activity reached the maximum by day 30 of fixation in group 2, while it was the maximum by the end of distraction in group 1.

Discussion

Acceleration of distraction osteogenesis has been much studied and discussed as it ultimately results in the
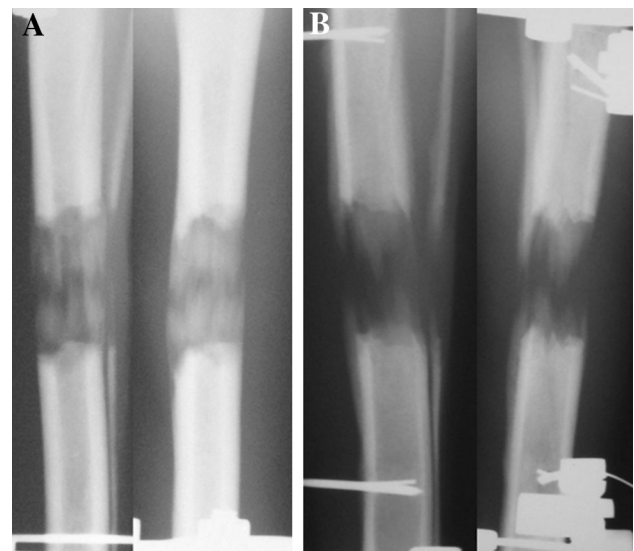

Fig. 2 Radiographs at the end of tibial distraction. **a** Manual mode with the rate of 1 mm per day in group 1; **b** automated mode with the rate of 3 mm per day in group 2
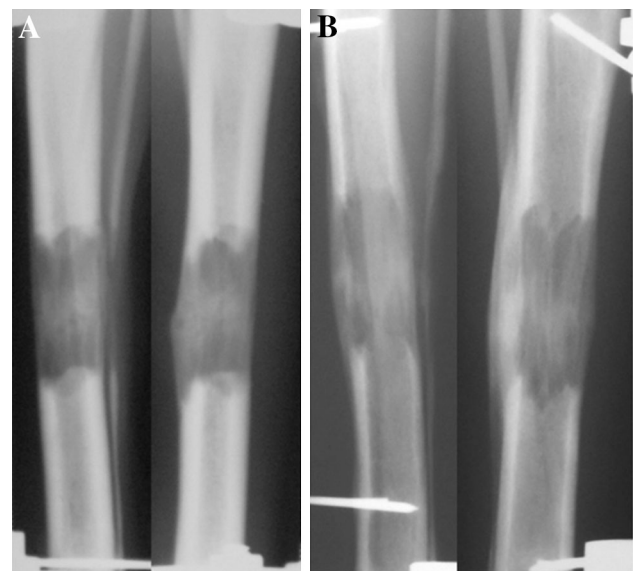

Fig. 3 Radiographs after 30 days of fixation. **a** Manual mode with the rate of 1 mm (4 increments) per day in group 1; **b** automated mode with the rate of 3 mm (120 increments) per day in group 2

reduction of treatment time for patients who undergo lengthening procedures for various pathologies [21]. Bifocal and multifocal bone lengthening procedures have been advocated as well as an increase in the amount of daily bone distraction [7, 8]. Ways to speed up distraction osteogenesis may include high-frequency distraction with a greater daily amount of stretching, which should also be further studied [10, 19].
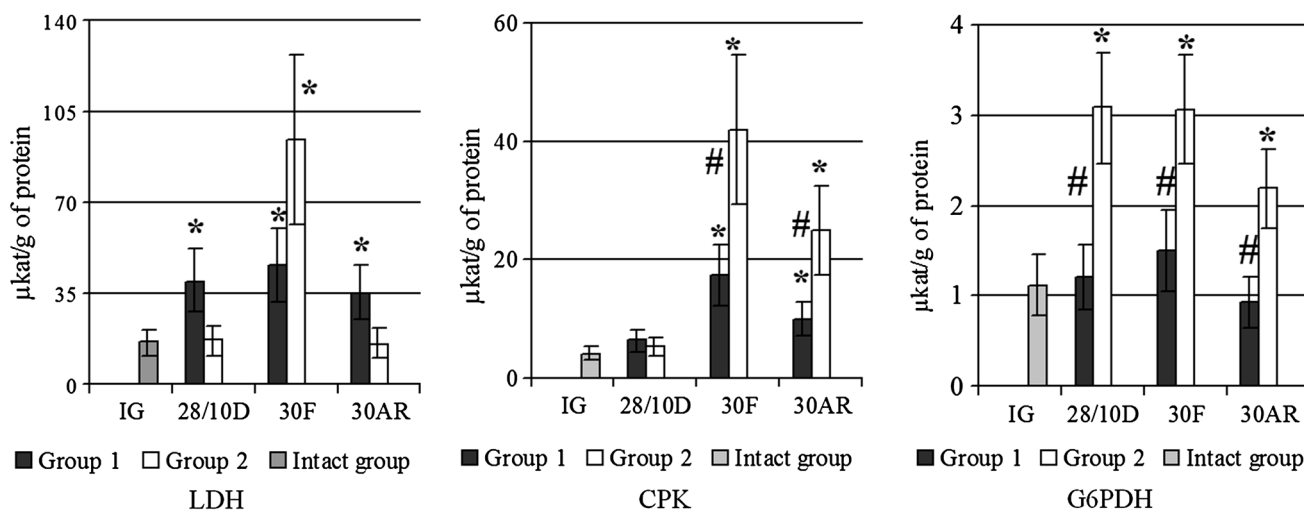
Fig. 4 Activity of enzymes of energetic cycles in the ATM. Note: 28/10D, 28 days of distraction in group 1, 10 days of distraction in group 2 (end of distraction); 30F, 30 days of fixation; 30AR, 30 days after apparatus removal; IG, intact group. *Asterisk* indicates a significant difference from IG, $p < 0.05$. *Hashed area* indicates a significant difference between the lengthening groups, $p < 0.05$
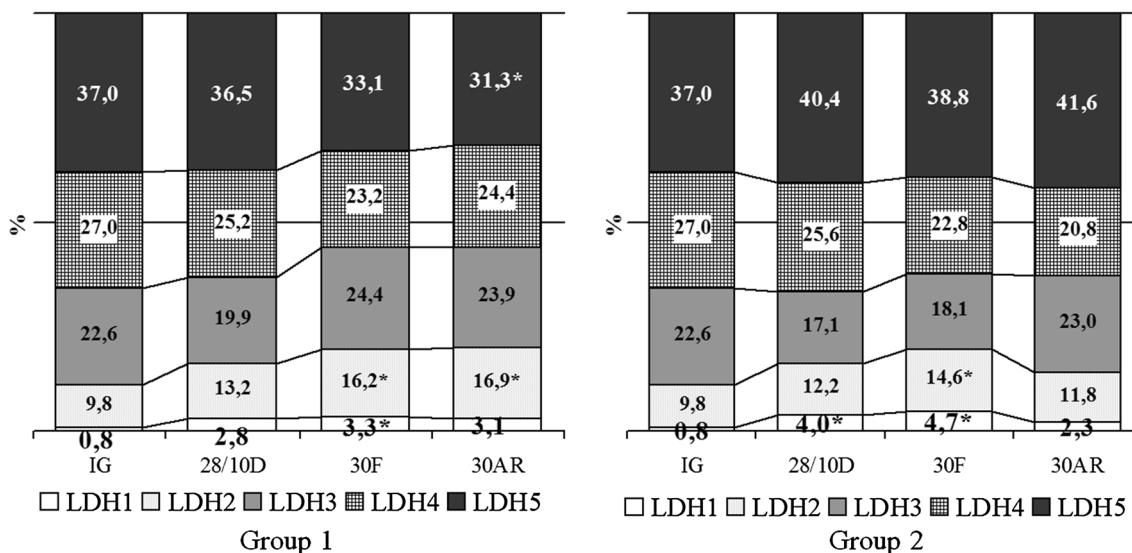

Fig. 5 LDH isoenzyme spectrum (% from the total activity) in the ATM. Note: 28/10D, 28 days of distraction in group 1, 10 days of distraction in group 2 (end of distraction); 30F, 30 days of fixation; 30AR, 30 days after apparatus removal; IG, intact group. *Asterisk* indicates a significant difference from IG, $p < 0.05$

However, the response of skeletal muscles to bone lengthening is not fully understood, especially as it refers to muscle metabolism, which has been poorly studied. Therefore, we investigated the metabolic changes in canine muscle tissue under lengthening and compared these changes by using a regular manual mode (1 mm/day) and a fast (3 mm/day) high-frequency mode in order to detect the energy turnover disorders that may accompany these procedures.

We expected that both LDH and CPK activities in the ATM would grow significantly in group 2 because of the increased tensile force during distraction. An increase in the enzymes that are responsible for energy turnover in the ATM would be required for proliferative activity in the muscle tissue under lengthening. Increasing numbers of proliferating cell nuclei were noted in the muscles during bone distraction, but the proliferation was shown to cease when the distraction was over [22]. Our findings show that no considerable increase in the activity of LDH or CPK was noted by the end of distraction in group 2. However, the increase in their activity continued in group 2 after completion of distraction on both day 30 of fixation and
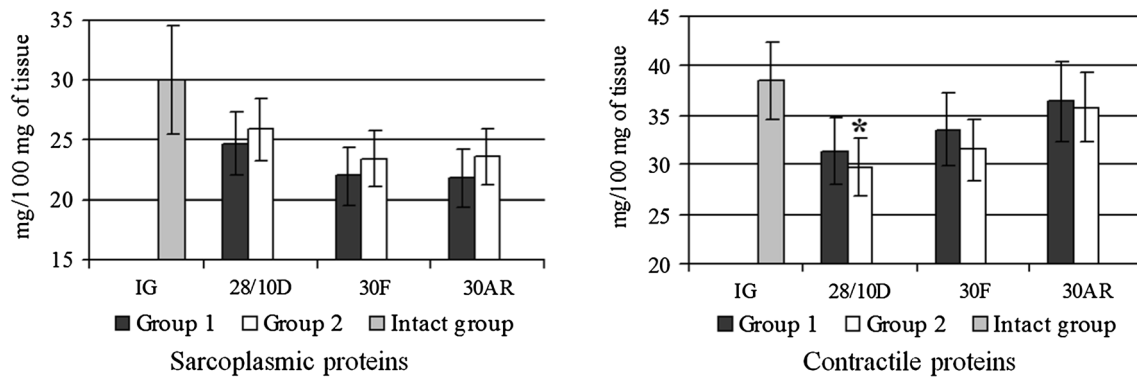
Fig. 6 Content of proteins in the ATM. Note: 28/10D, 28 days of distraction in group 1, 10 days of distraction in group 2 (end of distraction); 30F, 30 days of fixation; 30AR, 30 days after apparatus removal; IG, intact group. *Asterisk* indicates a significant difference from IG, $p < 0.05$
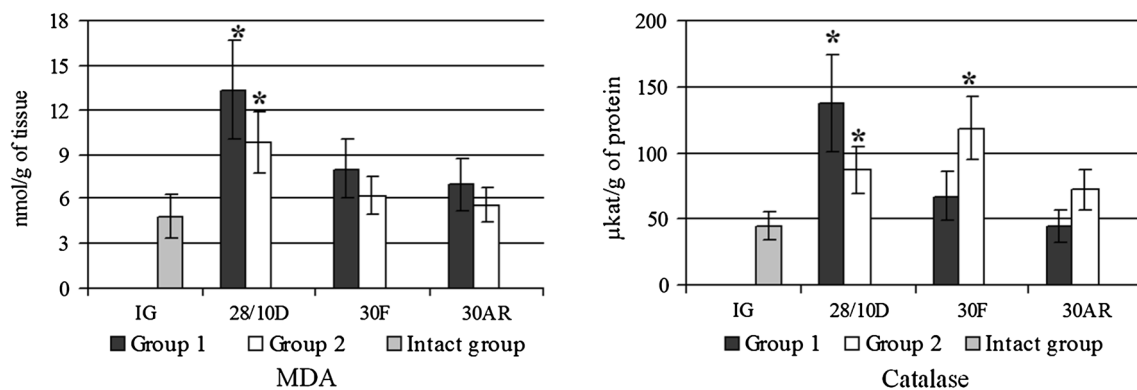

Fig. 7 MDA content and catalase activity in the ATM. Note: 28/10D, 28 days of distraction in group 1, 10 days of distraction in group 2 (end of distraction); 30F, 30 days of fixation; 30AR, 30 days after apparatus removal; IG, intact group. *Asterisk* indicates a significant difference from the IG, $p < 0.05$

day 30 after the apparatus removal. On the contrary, an increase in the LDH activity in the ATM of group 1 was observed only at the end of distraction. The growth of the LDH during the distraction stage in group 1 was caused by the activation of the glycolytic pathway of carbohydrate oxidation. Such an activation of the glycolysis in that group was associated with a temporary factor since the distraction period was longer than in group 2.

It appears that for the first time we observed the phenomenon of increased G6PDH activity in the ATM of the group 2 dogs, which proved that the pentose phosphate pathway of carbohydrate oxidation was considerably activated in the muscle tissue of these animals. The growth of the pentose phosphate pathway could have provided the proliferation that was noted in the ATM during distraction [16, 19] because the metabolites of the pentose phosphate pathway, known as pentoses, participate in the synthesis of the nucleic acids. It probably continued during the post-distraction period in group 2 as the activities of the energy enzymes studied were high. Moreover, the activation of the pentose phosphate pathway of carbohydrate oxidation in

the ATM under the conditions of 3-mm-per-day lengthening have a compensatory character and may be related to the decrease in the glycolytic pathway of carbohydrate oxidation.

However, our findings reveal that the increase in glycolytic activation occurred because of the high rate of distraction at the expense of the shift in the LDH isoenzyme spectrum, which showed the growth of aerobic fractions. Such changes would provide adequate functions of the oxidative muscle fibers of the first type. Their increase in the ATM during distraction has been observed in previous studies [13, 18].

Thus, the energy turnover reactions were activated in the ATM under lengthening as a response to distraction forces and confirmed our first hypothesis, while the activity of the energy turnover in the ATM did not grow proportionally with the increase of the distraction rate as we had assumed when planning our study.

However, our study was the first to reveal the following phenomenon: the character of energy turnover in the ATM underwent changes by high-frequency lengthening of 3 mm

per day. Specifically, alongside the increase in the efficiency of glycolysis, the main metabolic method of tissue turnover, the alternative universal pentose phosphate pathway of oxidation was activated. Its universal character is related not only to its high energy effectiveness, but also to its important role in the synthetic process. This interrelationship of the energy turnover pathways prevented excessive proteolysis as well as a non-controlled increase in peroxidation in the ATM.

The phenomenon of G6PDH activity growth in the ATM of the dogs that were undergoing the high-frequency lengthening procedure requires further study. It was previously considered that the activity of this enzyme and of the general pentose phosphate pathway of carbohydrate oxidation in the skeletal muscle was low [23]. Our findings revealed that considerable activation of this metabolic pathway in the ATM was possible under certain conditions of stretching, which was 3 mm per day in our series.

Thus, our findings showed that high-frequency tibial lengthening at the rate of 3 mm per day was accompanied by compensatory changes in the energy turnover in the ATM that were sufficient to prevent catabolic changes. We conclude that the studied high-frequency rate and a lengthening amount that does not exceed 15 % from the initial tibial length do not result in considerable disorders in the metabolism of the ATM. Our results correlate with those of other authors who concluded that lengthening up to 15 % in rabbits was safe for the muscle and did not result in permanent metabolic damage [24].

However, our findings show that the accessibility of energetic and plastic substrata is an important factor for supplying and maintaining the energy reactions in the muscle of the segment under lengthening. In clinical practice, this condition may be realized by stimulating muscle blood flow and with a complex of nutritional and pharmacological corrections of the metabolic status of the patients during treatment. We assume that the 3-mm high-frequency daily rate in an automated mode could be applicable in clinical trials for cosmetic reasons in achondroplasia or posttraumatic bone shortening where the muscle stock is large enough, provided that our experimental conditions are reproduced (daily rate, frequency of distraction steps, and percentage of the lengthening amount in relation to the initial length). Further basic research is required in order to study soft and bone tissues with high rates of bone lengthening for relevant use in clinical settings for different orthopedic pathologies.

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