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ADAPTATION OF MYOSIN HEAVY CHAIN MRNA EXPRESSION AFTER IMPLANTATION OF POLY(3)HYDROXYBUTYRATE SCAFFOLDS IN RAT M. LATISSIMUS DORSI

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The aim of this study is to identify the synergistic effect between an ectopic bone substitute and surrounding tissues, in this case muscle tissue, which is known to have a considerable potential for adaptation. To describe this effect, changes of myosin heavy chain (MyHC) isoform mRNA content of 12 Wistar-King rats m. latissimus dorsi with implanted poly(3)hydroxybutyrate (PHB) scaffolds were examined after six and 12 weeks. At each time interval six rats were killed and implants and surrounding tissues prepared for genetic evaluation. Eight rats without any implants served as controls. After homogenisation of muscle tissue, RNA was extracted and reverse transcribed. Changes in mRNA content were measured by Real-Time PCR using specific primers for type I MyHC, IIa, IIb and IIx isoforms. The mRNA level of myosin isoform type I of the muscles surrounding the implant was significantly increased ($p<0.02$) compared to the control group. Further, the studied muscle tissue showed a significant decrease in MyHC isoform IIx mRNA compared to the controls ($p<0.02$). Implantation of PHB scaffolds into rat m. latissimus dorsi causes an increase of its' content of slow myosin isoforms indicating a synergistic effect between the PHB scaffold and the surrounding muscle tissue.

Key words: *myosin mRNA, muscle, rat, poly(3)hydroxybutyrate, real-time PCR*

INTRODUCTION

Previous studies have shown that bone defects can be a limiting factor in achievement of optimal orthodontic treatment, so that autogenous bone grafts, which develop functional muscle attachments and become invested with other normal soft tissues, are sometimes used. This requires a donor site, with the possibility of increased morbidity for the patient. A new approach is to create bone or a bone substitute ectopically in another convenient anatomical area of the subject with a good vascular supply (1, 2).

It has been demonstrated in previous animal studies that the m. latissimus dorsi muscle is a suitable *in vivo* bioreactor for the growth of heterotrophic bone. The anatomical postion of the *m. latissimus dorsi* is close to the thoracodorsal arthery, which allows spontaneous neovascularisation (2) and scaffold material forming a mandibular replacement has already been implanted in the m. latissiums dorsi of a human being. The scaffold material was cultivated in the m. latissimus dorsi of the patient for seven weeks and the technique provided a good three-dimensional outcome and a lower operative burden for the patient (2).

Scaffold materials are used in tissue engineering studies to imitate the extracellular matrix (ECM) in connective tissues. The structure of these electrospun scaffolds is designed to resemble the fibrillar structure of the collagen bundles in the ECM that are known to play a crucial role in tissue regeneration (3).

Several studies have hypothesized that poly(3)hydroxybutyrate (PHB) may serve as scaffold for tissue engineering strategies (1, 4, 5) due to its' form stability and low inflammatory response after implantation. PHB is an ideal biomaterial, because it completely degrades to release a normal component of blood and tissue, D,L-β-hydroxybutyrate (HB) (3). In recent studies PHB has been evaluated as a scaffold for ectopic bone formation *in vivo* (1), and it has also been examined *in vitro* as a scaffold material for skin and nerve regeneration (3).

Todate, the synergistic effect between an electrospun PHB scaffold and the surrounding tissues, in this case *m. latissimus dorsi*, have not been investigated. Muscles are known to have a considerable potential of adaption. The extracellular matrix (ECM) of the muscle tissue surrounding the implant can, for example, integrate changes of the mechanical load of the muscle and hereupon induce signalling cascades with a following adaption of protein synthesis and arrangement of the cytoskeleton (6). Myosins are the major components of the contractile apparatus of muscle and the content of slow and fast myosin isoforms is a key-indicator for the kind of adaption of a particular muscle group (7, 8).

The aim of this study was to measure the changes of MyHC mRNA content in that part of the m. latissimus dorsi which was in direct contact with the implanted PHB scaffold.

MATERIALS AND METHODS

Poly(3)hydroxybutyrate (PHB) embroidery

As raw material, biotechnologically produced PHB with a molecular weight of 540 000 g/mol was used. The powder was granulated using twin screw extruder equipment. PHB multifilaments were produced using a high-speed melt spinning and spin drawing process (1, 5). Round embroidery patches were generated using an embroidery automat and coated with calf skin collagen type I. The average macro mesh pore size of the embroidery was 200 µm. Implant discs with a diameter of 12 mm, a thickness of 1.2 mm and weight of approximately 12 mg were used. Before the surgical procedure all implants were ultrasonically cleaned in 70 % ethanol for 15 minutes and sterilised by ethylene oxide. A total of 24 implants were prepared.

Experimental design and surgical procedure

Twelve six-month-old adult male Wistar-King rats (200g) were used to implant the scaffolds. All surgical and experimental procedures were approved by the Animal Welfare Committee of the State Government (no. 24-9168.11-1-2004-2). For surgery, each rat was anesthetized with intraperitoneal pentobarbital at an approximate dosage of 75 mg/kg. For the intra muscular implants a 3 cm sagittal incision was made in the skin in the midline of the back. Blunt dissection away from the midline cranially and caudally to the left and the right of the spine was used to form 4 intramuscular pockets. In one of these a PHB embroidery was inserted (1). The muscle pocket was then closed around the implant with one resorbable suture and the skin closed with a continuous suture. For localisation of the implants a complete statistical randomisation was used. Eight rats without any implants served as control group.

MyHC mRNA content was measured 6 weeks and 12 weeks after implantation, which proved to be reasonable timeframes for tissue adaption and tissue engineering processes (1, 2). At the end of each period 6 rats were euthanized with carbon dioxide. After euthanasation, the implants with the surrounding tissues were retrieved and prepared for molecular genetic evaluation. Following a blunt dissection surrounding tissue was carefully cleaned off by trimming and placed in liquid nitrogen.

RNA-isolation and reverse transcription

The frozen muscle tissue was homogenised under liquid nitrogen using a mortar and pistle. The cells were lysed and the RNA released by centrifugation of the cell-homogenate through a biopolymer shredder (Qiashredder, Qiagen, Valencia, CA, USA). Total RNA was isolated using the Qiagen Fibrous Tissue Mini Kit and the Fibrous Tissues Protocol (Qiagen, Valencia, CA, USA). The quality and yield of the RNA was determined by spectrophotometry and the integrity examined by agarose gel electrophoresis with ethidium-bromide staining.

Immediately 1 µg of the isolated RNA was transcribed in cDNA according to the manufacturer´s instructions using the High Capacity cDNA Kit (Applied Biosystems, Foster City, CA, USA).

Primer design

Primers (Metabion, Munich, Bavaria, Germany) amplifying the four myosin isoforms were designed using the free online eprimer3 software. The input data for the primer search were, amongst others, a melting temperature of 60 °C, a primer length from 19 to 21 bases, and a GC content at about 50 %. Further the primers were designed according to the manufacturer´s

instructions of the Sybr-Green Real-Time PCR Master Mix (Applied Biosystems, Foster City, CA, USA), which was used to accomplish Real-Time PCR. The chosen primers specific for each myosin isoform and the housekeeping-gene β-actin are shown in *Table 1*.

Standard cloning

A Sybr-Green Real-Time PCR was performed using 12.5 µl Sybr-Green Master Mix, 8 ng cDNA template, and 200 nM of each forward and reverse primer in 25 µl total volume. For Real-Time PCR the 7500 Real-Time PCR-Cycler (Applied Biosystems, Foster City, CA, USA) was used, creating a dissociation curve for each run. Ten µl of each PCR-product were analysed for specificity and identified by revising the size on a 3 % agarose-gel. The PCR-Products were cut out of the gel and the DNA extracted and purified using Qiaquick Gen-Elute Gel Extraction Kit (Qiagen, Valencia, CA, USA), following the manufacturer´s guidelines. A new PCR was performed using the purified Real-Time PCR products as a template and the MyHC-specific primers. After revision of the PCR-products on an agarose-gel, the fresh PCR products were ligated into the pCR 2.1 TOPO-Vector (Invitrogen, Carlsbad, CA, USA) and transformed into competent One Shot TOP10 E. Coli using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and following the manufacturer´s instructions.

The clones were selected on LB-Medium containing 8 % Agar and 100 µM Ampicillin. To check them for inserts, clones were picked and a PCR was run, each containing 0.5 µl of one clone in liquid LB-medium as a template, using M13 sequencing primers. The PCR-products were analysed on an agarose gel. Positive clones were picked and cultured overnight at 37 °C and 200 rpm in 3 ml LB-Medium containing 100 µM Ampicillin for selection. The following day, plasmids were isolated using the S.N.A.P. Miniprep Kit following the manufacturer´s instructions (Invitrogen, Carlsbad, CA, USA). The concentration of the plasmids was determined spectrophotometrically. To linearise the plasmids, they were digested once with the restriction enzyme SpeI (Promega, Madison, WI, USA), completion of the digestion being checked on an agarose gel. The concentration of the linearised plasmids was again determined spectrophotometrically. With the help of the ascertained plasmid concentration, the number of plasmid copies for each standard per µl was calculated. Dilutions of the standards were prepared

Table 1. Real-time PCR primers.
Real-time PCR primers specific for the MyHCs and for the housekeeping-gene

Primer Name	Rat mRNA Target Sequence	Primer Sequence 5´-3
MyHC-I forward	NM_017240	ACCTGATGGTGGATGTGGAG
MyHC-I reverse		CTTCTGCTTCCACTCAACCA
MyHC-IIa forward	X72589	CCGCGAGGTTCACACTAAAG
MyHC-IIa reverse		TGCCTCTCTTCGGTCATTCT
MyHC-IIb forward	X72590	AAGAGCCGAGAGGTTCACAC
MyHC-IIb reverse		TGTCACCTTTCAACAGAAGGAA
MyHC-IIx forward	AF157005	GAACAGAAGCGCAATGTTGA
MyHC-IIx reverse		TTCTTGCGGTCTTCCTCAG
β-Actin forward	NM_031144	CGTCTTCCCCTCCATCGT
β-Actin reverse		GGAGTCCTTCTGACCCATACC

from 10^{10} copies/2µl to 10^{0} copies/2µl and frozen at -80 °C for conservation. Each standard and its dilutions were only thawed once.

Real-time PCR

The concentrations of the primers were adjusted as described in a manual for the Power Sybr-Green Master Mix (Applied Biosystems, Foster City, CA, USA). Double-appointments were measured for every sample and No Template Controls were accomplished for each primer pair on each 96-well plate. From each sample, Real-Time PCR with primers for MyHCI,- IIa,-IIb, IIx and β-actin was performed - the standard curves, two myosin isoforms and β-actin at the same time on one 96 well plate. In each well 12.5 µl of Master Mix was added and 300 nM of each primer, except that for MyHCI forward, which was used at 50 nM. 2 µl of 1:50 diluted cDNA of a sample served as a template and concerning the standards 2 µl of each concentration. The specifity of the reaction was examined by creating a dissociation curve for each sample and finally by checking the PCR products by agarose gel-electrophoresis.

The results were statistically tested for significance using the paired student´s t-test of the σ-Plot software. They are summarised and represented in Box Plots.

RESULTS

The initial state of the distribution concerning the MyHC mRNA in rat *m. latissimus dorsi* is presented in *Table 2* as the averaged fraction of each myosin fibre type in percent in the m. latissmus dorsi of the eight control rats. Consisting of only 8.4 % of MyHCI and 7.6 % of MyHCIIa, the *m. latissimus dorsi* of the rat has predominantly the properties of a fast muscle.

The twelve animals surviving the surgical procedure remained in good health and did not show any wound complications. The macroscopic shape of the PHB scaffold persisted six weeks and 12 weeks after implantation (1).

The relative expression of the MyHC isoform I of the control rats was measured as 100.0 % ± 34.4 %. Six weeks after surgery the mRNA amount of MyHC isoform I increased significantly to 142.9 % ± 89.4 % ($p<0.05$) (median ± standard deviation) and 12 weeks after the implantation the an expression of 202.8 ± 46.8 ($p<0.02$) was found. The relative expression of MyHCIIa isoform in the untreated control rats was 100 % ± 29.9 %. Six weeks after the implantation the expression rose to 159.1 ± 82.9 and after 12 weeks an expression of 164.2 % ± 66.1 % ($p<0.02$) was measured. The expression of the fast MyHC IIx isoform was set in the control rats to 100 % ± 24.8 %. Six weeks after the implantation the mRNA level of this myosin isoform decreased

Table 2. MyHC fibre type percentage in control rats average percentage of the MyHC fibre type fractions of the eight untreated control rats and the standard deviations

MyHC-I [%]	MyHC-IIa [%]	MyHC-IIb [%]	MyHC-IIx [%]
9.0 ± 2.93	7.2 ± 3.53	14.3 ± 8.45	69.6 ± 17.7

significantly to 55.9 % ± 36.9 % ($p<0.02$) and twelve weeks after surgery the mRNA level of MyHCIIx was still reduced to 73.5 % ± 34.0 %. In the control rats the expression of MyHCIIb was set to 100.0 % ± 60.7 %. Six weeks form the implantation an expression of 163.5 ± 65.9 was measured, and after 12 weeks an expression of 108.3 % ± 74.8 %. No significant changes were found regarding the expression of the isoform MyHCIIb (*Fig. 1*).

Based on these changes there was a shift from fast to slow MyHC isoform mRNA, so that by 6 weeks the *ratio* from slow to fast myosin isoforms doubled from 100.0 % ± 23.0 % to 203.4 % ± 54.9 % ($p<0.02$). This effect continued for the following six weeks and after 12 weeks, the ration from slow to fast muscle fibres was still doubled: 189.8 % ± 52.4 % ($p<0.02$). *Fig. 2* shows the mRNA ratio of slow to fast myosin isoforms of the treated animals after six and 12 weeks in comparison to the controls.

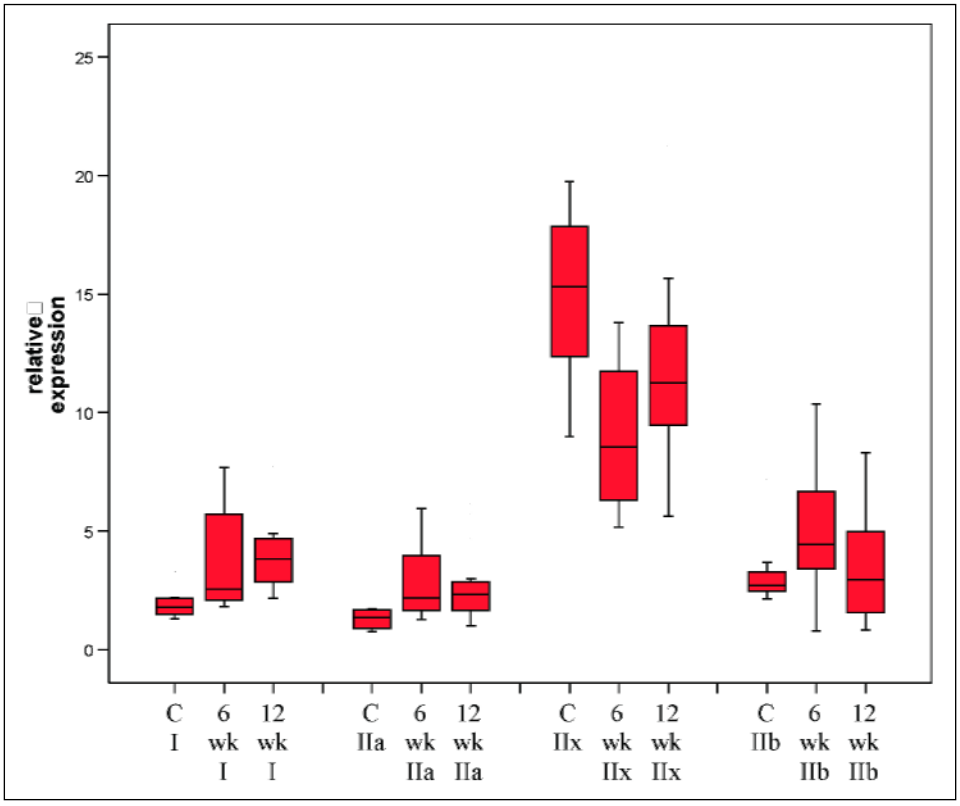

Fig. 1. Relative expression of the myosin isoforms.
Relative expression of the myosin isoforms I, IIa, IIx and IIb in the *m. latissiums dorsi* of control rats and in the *m. latissimus dorsi* of the rats carrying implants 6 weeks and 12 weeks after surgery. C: control group, I, IIa, IIx, IIb: myosin isoforms, 6 wk: 6 weeks, 12 wk: 12 weeks

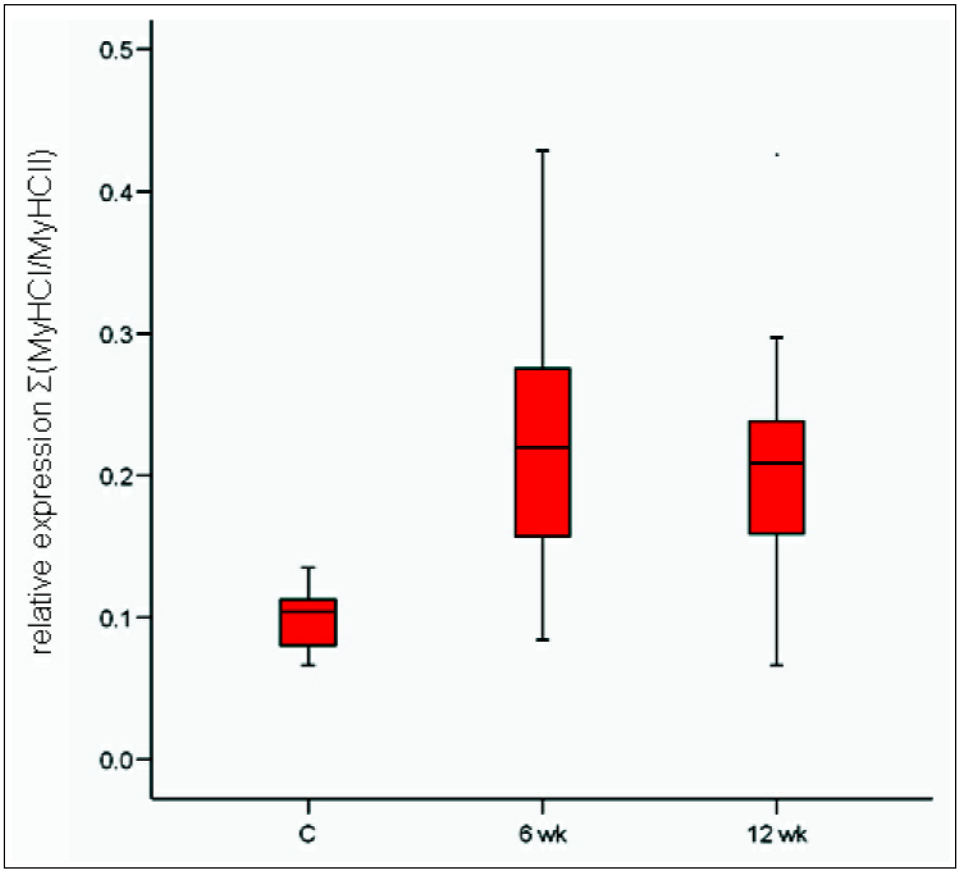

Fig. 2. Expression ration slow to fast myosin isoforms.
Expression ratio from slow to fast myosin isoforms (ΣI/ΣII) in the *m. latissimus dorsi* of the control rats and the animals with implants, 6 weeks and 12 weeks after surgery. C: control rats, 6 wk: 6 weeks after implantation, 12 wk: 12 weeks after implantation.

DISCUSSION

This is the first study that shows that PHB implants can have an influence on the myosin isoform composition of the surrounding muscle tissue. According to our results, the *m. latissimus dorsi* of rat is a predominantly fast muscle. This is in agreement with the findings of other studies, where it was shown that the smaller the mammal, the higher is the percentage of fast myosin isoforms in the muscle (12, 13). However, the *m. latissimus dorsi* of rat is not one of the very fast muscles as, for example, the *m. tibialis anterior* and *m. vastus lateralis*. These muscles do not contain the slow myosin isoform I and hardly the myosin isoform IIa (8).

When transforming, the myosin isoforms and the corresponding muscle fibre types usually follow the sequence IIb→IIx→IIa→I (10). In this study the mRNA of myosin isoform IIb was neither up-or downregulated by the implant. This result is in accordance with studies in the field of changed muscle activity, where changes in the expression of myosin isoforms I and IIx occurred, but no or very little changes in the expression of myosin isoform IIb were recorded (8).

Our results show an increased mRNA level of myosin isoform I and a decrease of mysoin IIx mRNA expression. The myosin isoform IIa is a so called "jump fibre" that could be skipped during changes of mysoin isoform expression (9). In this study, showing an increase of IIa myosin isoforms after 6 and 12 weeks, the shift of myosin isoform mRNA seems to follow the transformation sequence of muscle fibres. The myosin isoform mRNA indicates a transformation from IIx to IIa and I myosin isoforms, while the isoform IIb remains unchanged.

Comparing the results six weeks and 12 weeks after implantation, no significant changes of myosin isoform expression were recorded. This implicates that the most dramatic changes in myosin isoform expression seem to take place in the first 6 weeks after implantation of the scaffold and afterwards the new distribution of myosin isoforms stabilises. This result is in accordance to other experiments regarding implants and ectopic bone formation, where implantation periods of 7 weeks proved to be reasonable (1, 2).

Adaptive responses of myosin isoforms include transcriptional, translational and posttranslational regulation (11). This means the effects measured on mRNA level mostly, but not necessarily display on the protein level. Therefore, in this study, the changes of myosin mRNA level towards the slower myosin isoform I could indicate a change in muscle fibre types.

In this case, an increased number of slow myosin isoforms indicates an increased vascularisation (12) and therefore an increased blood flow and a higher oxidative capacity of the muscle due to metabolic changes. Thereby, especially increased vascularisation and blood flow are factors which can benefit muscle regeneration and ectopic bone formation within the implantation area of the PHB scaffold (14).

In further studies concerning PHB implants we would like to verify the results of this study on the protein level and focus on the expression of not only muscle fibre types, but also on factors that indicate inflammation and regulate vascularisation or interaction with the extracellular matrix.

Implantation of PHB scaffolds into rat *m. latissimus dorsi* changes the muscle fibre composition of this muscle over time. In this study, changes from fast to slow muscle fibres occurred within six weeks and persisted up to twelve weeks. This transition indicates improved energy utilisation and shows a potential for possible muscle regeneration, stating that there is a synergistic effect between PHB scaffolds and the surrounding muscle tissues. This synergistic could possibly benefit ectopic bone formation and should be further elucidated.

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Conflicts of interest statement: None declared.

REFERENCES

1. Mai R, Hagedorn MG, Gelinsky M et al. Ectopic bone formation in nude rats using human osteoblasts seeded poly(3)hydroxybutyrate embroidery and hydroxyapatite-collagen tapes constructs. J Craniomaxillofac Surg 2006; 34: 101-109.
2. Warnke P, Springer I, Wiltfang J et al. Growth and transplantation of a custom vascularised bone graft in a man. *Lancet* 2004; 364: 766-770.
3. Suwatong O, Waleetorncheepsawats S, Sanchavanakit N et al. In vitro biocompatibility of electrospun poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) fibre mats. *Int J Biol Macromol* 2007; 40: 217-223.
4. Gogolewski S, Javonic M, Perren SM, Dillon JG, Hughes MK. Tissue response and in vivo degradation of selected polyhydroxyacids: Polyactides (PLA), poly(3-hydroxybutyrate) (PHB), and poly(3-hydroxybutyrate-co-3hydroxyvalerate) (PHB/VA). *J Biomed Mater Res* 1993; 27: 1135-1148.
5. Schmack G, Jehnichen D, Vogel R, Tandler B. Biodegradable fibres of Poly (3-hydroxybutyrate) produced by high-speed melt spinning and spindrawing. *Polymer Physics* 2000; 38: 2841-2850.
6. Kjaer M. Role of extracellular matrix in adaption of tendon and skeletal muscle to mechanical loading. *Physiol Rev* 2004; 84: 649-698.
7. Krutki P, Pogrzebna M, Drzymala H, Raikova R, Celichowski J. Force generated by fast motor units of the rat medial gastrocnemius muscle during stimulation with pulses at variable intervals. *J Physiol Pharmacol* 2008; 59: 85-100.
8. Talmadge RJ. Myosin heavy chain isoform expression following reduced neuromuscular activity: potential regulatory mechanisms. Muscle Nerv 2000; 23: 661-679.
9. Andersen JL, Gruschy-Knudsen T, Sandri C, Larsson L, Schiaffino S. Bed rest increases the amount of mismatches fibres in human skeletal muscle. *J Appl Physiol* 1999; 86: 455-460.
10. Staron RS, Pette D. The continuum of pure and hybrid myosin heavy chain-based fiber types in rat skeletal muscle. *Histochemistry* 1993; 100: 149-153.
11. Pette D, Vrbova G. What does chronical electrical stimulation teach us about muscle plasticity? *Muscle Nerv* 1999; 22: 666-677.
12. Pette D. The adaptive potential of skeletal muscle fibres. *J Appl Physiol* 2002; 27: 423-448.
13. Sargeant AJ, de Haan A. Human muscle fatigue: the significance of muscle fibre type variability studied using a micro-dissection approach. *J Physiol Pharmacol* 2006; 57: 5-16.
14. Lauer G, Haim D, Proff P et al. Plate osteosynthesis of the mandibular condyle. *Ann Anat* 2007; 189: 412-417.

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