J Musculoskelet Neuronal Interact 2009; 9(2):81-88

Original Article

Hylonome

Tetracycline therapy for muscle atrophy due to immobilization

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Abstract

Certain proteins such as matrix metalloproteinase -2(MMP-2) and heat shock protein 70(HSP-70) play a role during the degradation process. We hypothesized that tetracycline can be used to reduce tissue degradation in skeletal muscles exposed to immobilization. The right knee of old rats (20-months-old) was immobilized by a rigid external fixator (EF) device for 1, 2, 3 and 4 weeks. Aqueous Tetracycline solution was administrated 3 times a week, following 2 days after the EF was constructed. Control group I was immobilized for 3 weeks, did not receive tetracycline but did received saline injection, and control group II only received tetracycline for 3 weeks. MMP-2 and HSP-70 protein and mRNA levels in the gastrocnemius and soleus muscles were analyzed at the molecular level by RT-PCR and the protein level using SDS-PAGE gels and western blots. We have shown that rats treated by Tetracycline reduce the MMP-2 expression and HSP-70. Theses changes mainly occurred in type IIb and type IIa muscle fibers. Tetracycline administration has beneficial effect on expression of enzymes involved in protein degradation. This may suggest a protective effect on protein degradation during immobilization.

Keywords: Skeletal Muscle, Immobilization, Tetracycline, Atrophy

Introduction

Muscle atrophy occurs in numerous pathologies such as cancer, sepsis, and prolonged periods of muscle inactivity[1,2]. It is well established that muscle atrophy occurs in prolonged bed rest, limb immobilization or unloading the diaphragm via mechanical ventilation[3,4]. Among the mechanisms suggested to contribute to local catabolism and progressive skeletal muscle atrophy is activation of systemic and local markers of inflammation (e.g. TNF-α, IL-1 and IL-6)[5], increased levels of oxidative stress 6,7 and matrix remodeling[8-12]. As a defense strategy, muscle tissues of human and animals, were shown to induce enzymes like matrix metalloproteinases (MMPs)[13,14], and the heat shock protein -70 (HSP-70)[15].

MMPs are a family of about 24 proteolytic enzymes that belong to a large group of zinc enzymes, and are crucially involved in the turnover of extracellular matrix (ECM) components[16]. MMPs play an important role in the homeostasis of the extracellular matrix (ECM) in skeletal muscle[17]. The ECM surrounding muscle fibers provides structural support and protection, and is important in maintaining functional integrity of the fibers[18]. In particular, type IV collagen is a major component in the basement membrane and plays an important role in the cellular arrangement of skeletal muscle fibers. Changing demands in skeletal muscle (e.g. increased or decreased contractile activity) promotes remodeling of the extracellular matrix. Although several MMPs are expressed in muscle, two MMPs are thought to play an important role in skeletal muscle adaptation to changing contractile demands and to response to injury are MMP-2 (also known as Gelatinase A) and MMP-9 (also known as Gelatinase B). MMP-2, for example, was shown to play a key role in maintaining the structure and activity of basement membrane components of atrophied muscles such as in inflammatory myopathies[19,20] and immobilization[21,22]. Both of these MMPs degrade type IV collagen and belong to a group of calcium and zinc endoproteinases that have important functions in homeostasis of the extracellular matrix during morphogenesis, proliferation, and cell apoptosis in a wide range of tissues[23,24]. Activation of MMP-2, MMP-9 has also been shown to be involved in various myopathic and inflammatory-induced changes in skeletal muscle[20,25]. Expression of MMP-2 in skeletal muscle was increased following administration of chronic elec-

The authors have no conflict of interest.

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Accepted 27 February 2009

trical stimulation[26], and expression of MMP-9 exposed to alpha1-adrenergic blocker prazosin induce chronic vasodilation and increase in blood flow[27].

The expression of Heat shock proteins (HSP) is increased when cells are exposed to elevated temperatures or other type of stress. This increased expression is transcriptionally regulated. The HSPs are named according to their molecular weights. HSPs include Hsp90, Hsp70, and Hsp25[28], and play essential roles in refolding, as chaperones, in regulation of protein degradation, and in protection against oxidative stress[29-32]. Impaired protection of muscle cells against atrophy and reloading-induced damage may be related to lower levels of heat shock proteins (HSPs), an important family of protective stress proteins[33].

Severity of muscle atrophy is related to the duration and magnitude of the activity limitation. Extended periods of disuse induce the specific loss of proteins associated with contraction and cytoskeletal structure[34,35]. If left untreated this muscle wasting can lead to long-term sequelae, including impaired functional capacity and permanent muscle damage[36]. Therefore there is a need to find ways not only to improve but also to accelerate muscle recovery. Tetracycline may hold promise, unrelated to their antimicrobial properties, in this regard.

Tetracycline was previously proposed as a therapeutic approach to combat deleterious effects of inflammation processes of connective tissues containing collagen[37]. Moreover, it was also shown to inhibit, by several non-antimicrobial mechanisms, the activity of several host-derived matrix metalloproteinases (MMPs) responsible for connective tissue breakdown[38,39] and was thus used to reduce tissue degradation in maladies such as periodontal disease and arthritis[40,41].

Little is known about changes of MMP-2 and HSP-70 level in the different hindlimb muscles following immobilization and tetracycline supplementation.

As previously reported[42] there are no immobilization-related changes in fiber type distribution in aged rats. In normal skeletal muscle MMP-2 levels are low in the ECM and its expression is tightly regulated by cytokines and growth factors such as vascular capillary endothelial growth factor (VEGF)[43]. Generally, immobilization-induced MMP-2 and HSP-70 in skeletal muscles occurs in situation with myopathies and congenital dystrophies[43].

Although production of proteolytic enzymes is known to be associated with various myopathies and inflammatory conditions[44], their involvement in changes in different fiber types under various conditions of loading stimulation has not been closely examined. Excessive acute or chronic muscle use, such as in intensive sporting activities, may lead to structural damage involving protein degradation, myopathy, and muscle dysfunction.

The purpose of this study was to determine the effects of tetracycline on heat shock proteins and MMPs in slow and fast muscles of rats with external fixation during the early onset of muscle disuse. It was hypothesized that tetracycline administration would orchestrate downregulation of MMP-2 levels and upregulation of heat shock proteins and that this will result in faster and better muscle recovery.

Material and methods

Animals

Wistar rats (20-months-old, body weight ranged 260-320g) were maintained under constant conditions of room temperature (22°C) and humidity (40%) with a 12/12 hour light-dark cycle. Rats were fed on standard rat chow and water ad libitum. All animals were maintained according the principles of laboratory animal care formulated by Tel Aviv University, (no 11-04-031).

Animals were randomly assigned to either of the 6 following groups (n=5 each): four immobilization groups that each was immobilized for 1,2,3, and 4 weeks. Tetracycline was administered 3 times a week. Two control groups (n=5 each) comprised of: C1. rats that were immobilized for 3 weeks and injected with saline without tetracycline; and C2 a group that was injected with tetracycline only for 3 weeks.

Immobilization

Rats were anesthetized by intra muscular injection of 60 mg/kg Ketamine HCl, and 70 mg/kg Cefamizine (70 mg/kg) 40 mg/kg Nembutal intra peritoneal (IP) injection. Immobilization was done as previously described (Zarzhevsky et al., 2001). Briefly, rigid external fixation (EF) was achieved by inserting two 0.8 mm diameter Kirschner wires through the lateral plane of the femur and tibia. Wires were then connected by two threaded brass rods to a rigid frame. The rods were 4.8 mm in diameter and 33 mm in length and had a 13 mm slot cut longitudinally from both ends to contain the wires. The overall fixation device weighed approximately 12 g. The right knee was immobilized in 45° flexion position.

Rats were anesthetized by IP injection of pentobarbital sodium (200 mg/kg), 1 wk, 2 wks, 3 wks, 4 weeks post-fixation. After reaching a surgical plane of anesthesia (stage III, plane 3, resulting in paralysis of muscles and absence of lid, corneal, and skin reflex) the surgical procedure of carefully removing the EF and the right and left hindlimb muscles i.e.,Gastrocnemius (Gast.), Soleus (Sol.), and Quadriceps (Quad.) was carry out. Then, animals were sacrificed with an over dose of pentobarbital. Muscles were weighed and frozen in isopentane chilled by liquid nitrogen (-192°C).

Tetracycline treatment

Two days following external fixation, aqueous Tetracycline solution (Engemycine 10% Oxythetrcybclinum LA) was injected intra peritonealy, 3 times a week, at a dose of 1 mL/Kg which is the minimal effective dose to abolish MMP and HSP activity[51].

Molecular analysis

Total RNA was isolated from 30 mg muscle tissue using SV total RNA isolation kit. (Promega Z3100). RNA was used as a template for RT-PCR reaction (Access Quick™ RT-PCR system, Promega A1702). 50 ng of cDNA was used as

	3 wk of EF		1 and 3 wk of EF + Tetracycline	
	Before	**After**	**Before**	**After**
Body-weight (g)	268±57	250±65	261±62 269±59	252±23 248±20
Mean difference (g)		-18		-9 -21
95% confidence		-2.571 to 4.029		-2.445 to 3.807 -2.360 to 3.500
Interval of difference change (%)		-3.8		-2 -4.5
p value		NS		NS

Table 1. The body-weight mean (±SD) of immobilized leg, and after administration of tetracycline.

template for PCR amplification using touch-down program: 94 C for 1 min, 30 cycles of 94 C for 5 sec, and 72-68 C for 3 min. Annealing and primer extension were done at 72, 70, and 68 C during 1-5, 6-10, and 11-30 cycles, respectively. The PCR products were separated on 1.2% agarose gel electrophoresis and ethidium bromide staining for visualization.

Specific primers were used for PCR:
MMP-2:
Forward CCATCAAACGGGTATCCATC
Reverse GTCGGACCTCTCAGGGTTCT.
HSP-70:
Forward: TCGGGAACCATGAATAGAGG
Reverse: TTTGGAGAAAGGAGCAGCAT.
Alpha (α) Tubulin:
Forward: ATTGACATCTTTGGGGACCA
Reverse: ATCACAGGCAAGGAAGATGC (Sigma).

For negative control, RT reactions using 1 μg of total RNA from each tissue with no reverse transcriptase (No-RT control).

Biochemical Studies

SDS-PAGE gels and Western blot analysis

100 mg muscle tissue was homogenized (20 sec homogenization and 10 sec pause x 3 times) in cold buffer containing 42mM Trizma base, 0.3M KCl, 2.5mM MgCl, 0.1% Triton x-100 and protease inhibitor cocktail (P-8340, Sigma, Israel) and centrifuged (10,000 x g for 10 min at 4°C) and supernatant was collected. Total protein concentration was measured using Bradford reagent (cat. 500-0006, Bio-Rad, Hercules, CA). Samples were then vortexed, boiled and centrifuged. Proteins were separated on 10% SDS-PAGE, and then transferred to nitrocellulose membranes. Blots were blocked with 2.5% skim milk (cat.170-6404, Bio-Rad, Hercules, CA) in PBST (PBS containing 0.05% Tween 20) for 1 hr. the following primary antibodies were added for 1 hour in room temperature: MMP-2 goat polyclonal antibody (sc-6838, Santa Cruz Biotechnology, CA), alpha-tubulin specific mouse monoclonal IgG2a antibody (sc-5286, Santa Cruz Biotechnology, CA), mouse anti-HSP-70 monoclonal antibody (Stressgen, Victoria BC Canada). Secondary antibodies were bovine anti-goat IgG-HRP (sc-2350, Santa Cruz Biotechnology, CA) or donkey anti-mouse IgG-HRP (sc-2314, Santa Cruz Biotechnology, CA). Autoradiographs were developed using Super Signal West Pico chemiluminescent substrate (cat. 34080, Pierce Chemical Co., Santiago, Chile) followed by exposure to X-ray films (Fuji).

The resulting labeled bands were quantified by using a PC computer. The scans were created by using an image scanner (600 dpi transparency module, Mirror Technologies) connected to the computer. The scans were subsequently digitized and imported into an image analysis software program (Scion Image Beta 2, Scion Cooperation), and the quantification of MMP-2 and HSP-70 were performed by calculating the density of each individual band sample.

Protein Standards and Gel Electrophoresis

Purified lyophilized proteins (Sigma) were used as known standards to calibrate the amount of protein in determining concentrations. We carried out one-dimensional SDS-polyacrylamide gel electrophoresis with large format gels (10 x 10 cm) for optimum protein separation. Gels consisted of a 15% separating gel and 3.5% stack gel (200:1 and 20:1 acrylamide to bis ratio, respectively; pH 9.3 or 8.8; 0.75 M Tris. Sample buffer contained 62.5 mM Tris (pH 6.8), 1% (w/v) SDS, 0.01% (w/v) bromphenol blue, 15% (v/v) glycerol, and 15 mM dithiothreitol. Densitometry of bands of purified proteins of equal loads indicated that all had similar stain affinities.

Protein isolation

Frozen muscles were powdered, and total cellular protein was isolated by homogenization of the muscles samples in lysis buffer (120 mMTris, HCL and 5% glycerol, no protease inhibitor). After homogenization, Triton X 100 was added to a final concentration of 0.01%.

	Contralateral	Control 1	Control 2	EF + tetracycline			
	Control			1 wk	2 wks	3 wks	4 wks
Gastrocnemius	***2998***±261	2343±301	2295±288	2303±271	2385±205	***2380***±299	2388±273
*Mean difference						-630	
*95% change of difference						-1105 to -627	
*Change (%)		-22				-2.6	
Soleus	***435***±41	357±54	369±50	341±38	340±45	***323***±42	309±37
*Mean difference						-122	
*95% change of difference						-92 to-51	
*Change (%)		-18				-9.5*	
Quadriceps	***3995***±330	3021±360	2993±311	3200±346	3009±337	***3027***±362	2995±328
*Mean difference						-74	
*95% change of difference						-1393 to -826	
*Change (%)		-24				-2.4	

* values are between contralateral leg of group 1 and 3 weeks of EF + tetracycline, * $p>0.05$

Table 2. The mean (±SD) change in the muscle weight (mg) of three hindlimb muscles after 3 weeks of external fixation (EF) (control 1), 3 weeks of tetracycline administration without EF (control 2), and in comparing to1,2,3,4 weeks of EF plus administration of tetracycline.

Statistical Analysis

t-test for dependent samples (paired *t*-test) was used unless noted otherwise. When multiple t-test comparisons were used, a Bonferroni correction was applied. *P* values less than 0.05 were considered significant.

Results

Body and muscle weights

Body weights of all animals are shown in Table 1. Body weight did not significantly differ between groups at all times. Weights of the three studied muscles are depicted in Table 2. Following three weeks of immobilization, there was a decrease in Gast. Sol. And Quad muscle weights compared to control group 1 (-22%, -18% and -24%, respectively). However, there was no difference in Gast. and Quad. muscle weight between immobilized with tetracycline to non-immobilized controls . Sol. muscle weight, however, was significantly reduced despite the administration of tetracycline ($p<0.05$).

Molecular and Biochemical analysis

The content of MMP-2 and HSP-70 evaluated by RT-PCR was measured in three muscles throughout 4 weeks of immobilization and non immobilization condition. There was a significant increase in the MMP-2 and HSP-70 (Figures 1 and 2), protein and mRNA levels, in type IIb fibers (fast glycolytic, FG) in the immobilized animals (Figure 3). In contrast, levels of the aforementioned factors did not change in the immobilized animals that were treated with tetracycline. The levels of MMP-2 and HSP-70 in type I (Sol.), IIa (Quad.) and IIb (Gast.) in the contra lateral leg in the immobilized group was equivalent and comparable to sedentary control levels as well as to control-2 group. All three muscles in control group 2 were not affected by tetracycline treatment.

Discussion

In this study we used tetracycline in skeletal muscle subjected to immobilization in order to investigate its effect. Our findings suggest that tetracycline participates in minimizing the damage occurred to the muscle fibers due to the immobilization and thus protects muscle protein degradation.

The protein content of MMP-2 and HSP-70 were increased after immobilization but their level remain low and constant following administration of tetracycline without appreciable changes in mRNA abundance, suggesting that post-transcriptional regulation plays an important role in these proteins adaptation[45]. There are differences in the composition of the extracellular matrix surrounding myofibers of differing functional types. Slow-twitch muscles of rats contain more collagen in the ECM than fast-twitch muscles[46]. It is conceivable that the adaptation responses of muscles with predominantly slow-twitch fibers will differ from those of fast-twitch fibers and this may be reflected in changes in expression of MMPs.

The current study was therefore designed to investigate the possible effect of tetracycline on different skeletal muscles (i.e., Gastrocnemius in 20 months old rats is well accepted as primarily type IIa muscle fibers, Soleus is mainly type I muscle fibers, and Quadriceps is composed of a mixed fiber composition (type IIb and IIa)[47], that immobilized for short and longer period of time. The repairing potential of tetracycline due to immobilization was successfully demonstrated rather than its potential to prevent muscle atrophy. The results of our study

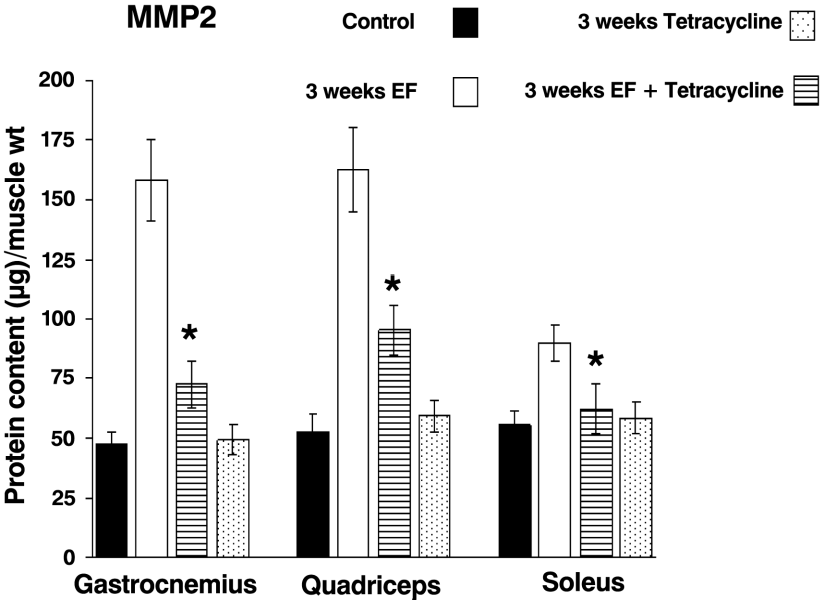

Figure 1. Content of MMP-2 protein (µg) relative to muscle weight (mg) for three different muscles following immobilization and tetracycline administration.

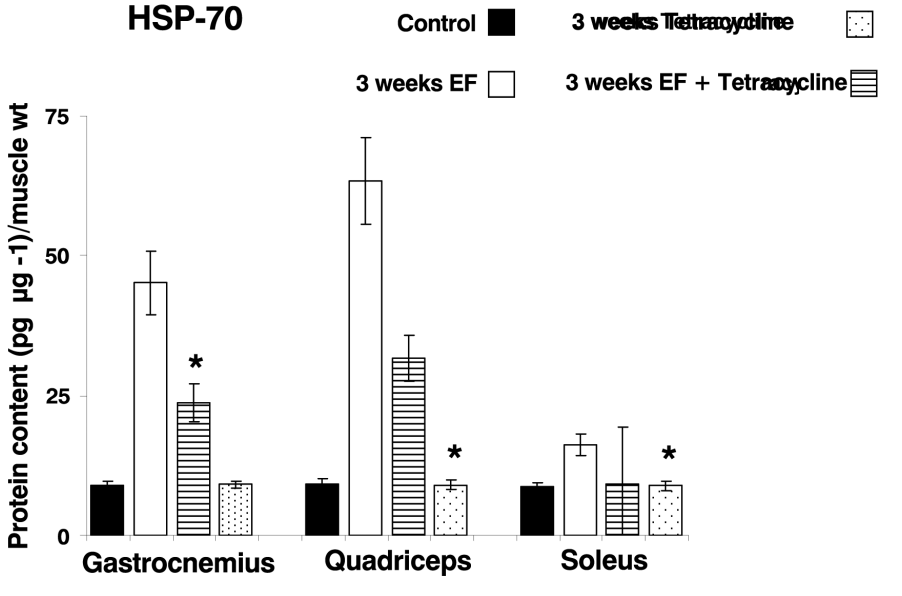

Figure 2. Content of HSP-70 protein (pg μg^{-1}) relative to muscle weight (mg) for three different muscles following immobilization and tetracycline administration.

suggest that disuse muscle atrophy related change due to immobilization effects differently the three muscles studied.

Our novel study is of interest both from a basic science perspective as well as from a clinical angle. Investigating the kinetic of protein metabolism in skeletal muscles following a time period after the EF was removed is important to understand how to minimize the disuse muscle atrophy and the 'sarcopenia' phenomenon. It is possible to look at protein metabolism (i.e. synthesis and degradation) systems within the myofiber (i.e., HSP-70) and out the myocell (i.e., MMP-2), and to study their inter-relationships. Additionally, this experiment was performed to test the therapeutic efficacy by using

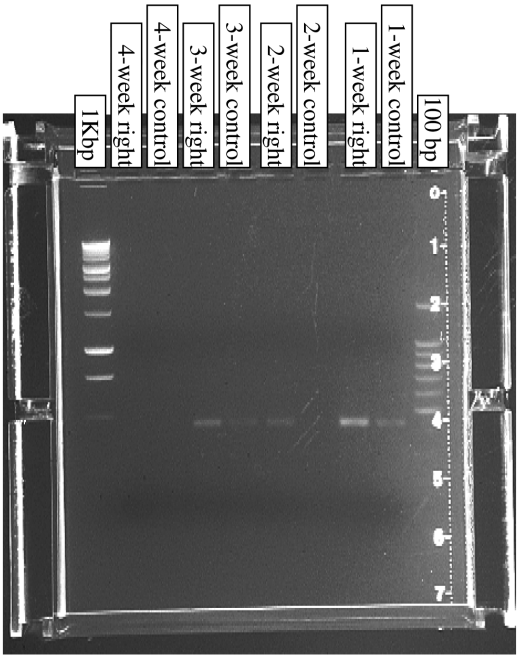

Figure 3. mRNA MMP-2 levels, in type IIb fibers (fast glycolytic, FG) in the immobilized animals.(The volume of the loaded samples was 110 ng following RT-PCR. All samples had the same concentration of RNA during the beginning of the RT-PCR reactions. MMP-2 was running in agrose gel of 2% at 120 voltage for 90 minutes).

tetracycline in reducing the pathological effects of immobilization in MMP-2 and HSP-70 levels.

The increase of HSP-70 mRNA levels correlates with previous investigations indicating elevation of HSP-70 mRNA content against fatigue-induced injury[45].

The results of the present study showed that knee immobilization resulted with muscle atrophy and higher expression of the inactive precursor, or zymogen, (pro MMP-2, 72 kDa), indicating accelerated activities of the active form of MMP-2 and increased capacity for ECM degradation. Following EF the relative increase in gelatinolytic activity of MMP-2 was higher in the fast-twitch muscle fibers than in the slow-twitch fibers. These results agree with the viewpoint that only under extreme or abnormal conditions of muscle use or disuse, possibly following injury-related over use or inflammation-related disuse, MMP-2 is expressed conceivably by leucocytes and macrophages. We recently showed that MMP-2 may be inhibited by tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1, -2), which are secreted by the same myofibers as MMP-2[48].

Moreover, in the current study, we demonstrated that different muscle fiber types show differing response patterns to EF and to administration of tetracycline. We found that type IIb muscle fibers were more susceptible both to disuse and tetracycline than type I muscle fibers. From previous reports, it has been shown that disuse and overuse lead to muscle tissue damage followed by functional decline[49]. The significant changes in type IIb fibers were observed particularly following 3 weeks of immobilization. Both in Gast. and Quad. muscles, net tissue degradation was observed when related to soluble protein concentration, suggesting a higher rate of protein degradation in slow and fast oxidative glycolytic (IIa and IIb) muscle fibers than in (type I) slow-twitch muscle fibers. Therefore, our findings certainly cause us to believe that fast fibers are more responsive to disuse and to tetracycline. However, the positive effect of tetracycline was only demonstrated in muscles underwent atrophy due to immobilization, whereas it had no effect on muscles which were not immobilized. Therefore, we can claim that administration of tetracycline to skeletal muscles did not acquire a "preventing" effect but more as maintaining ECM effect.

There are two possible explanations regarding the differing proteins expression in the various muscle fiber types. In rat skeletal muscles, type IIB fibers are markedly larger and stronger than type I fibers and appear to be associated with larger amounts of extracellular collagen[50]. It is possible that fast-twitch fibers require a different molecular mechanism to maintain their structural integrity than slow-twitch fibers[51]. Moreover, "white fibers" demonstrate better muscle plasticity than "red fibers" and adapt faster to mechanical disuse or overuse[52]. It is also possible that as a result of immobilization fast fibers (low oxidative, type IIb) may undergo a transition to slow-fiber types (high oxidative, type IIa) with corresponding changes in the composition of the extracellular matrix. The results of the present study suggest that immobilization may affect the overall balance of protein turnover in skeletal muscle fibers, and that changes also involving degradation and synthesis of extracellular matrix, are more distinct in type II muscle fibers than in type I fiber. Therefore, it is safe to conclude that the anaerobic type II muscles fibers may be more susceptible to oxidative stress due to immobilization, and less able to cope with the increased energy and oxidative demands. Our study supports the hypothesis that in order to maintain efficiency with elevated energy requirements for long periods, type II fibers show both intracellular and extracellular adaptation responses.

From our study it seems that in order to maintain their efficiency requires producing energy for long period of time, type II fibers underwent some intra and extra cellular adaptation.

Despite of believable results the major limitation in our animal-based study is the exist differences between human and animals skeletal muscle biology. The differences are both in the molecular profile and histo-morphology components i.e., fiber types and fiber type percentage. Therefore, our outcomes should be translated to human with some degree of caution and critics.

Conclusions

Tetracycline, found to abolish the increase in MMP and HSP protein levels, and therefore participates in minimizing the damage occurred to the muscle fibers due to the immobilization and thus prevents degradation or sustain tissue quality at or near the control values.

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