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THE EXPRESSION OF MMP-2, MMP-9, AND SOD ON DIFFERENT MUSCLE FIBERS IN TRAINED MUSCLES IN YOUNG RATS

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Abstract. Treadmill running provides a model to investigate the mechanism involved in muscle use and over use. Female rats (8 months old) were randomly assigned to 2 groups: Running group I, at a slow speed (18 m/min; ~ 50% VO_2), and Running Group II, at a very Fast speed (32 m/min; ~ 75% VO2), for 2 weeks. Matrix metalloproteinase (MMP) type 2 and 9, and total activity of superoxide dismutase (SOD) assessed in gastrocnemius, quadriceps and soleus muscles by western blotting and by reverse transcriptase- polymerase chain reaction. The expression of MMP-2 and SOD was shown particular in the fast running group. MMP-9 and SOD were not expressed in the fast and slow running group. Fast twitch muscle fibers (type IIB) were more affected by the fast speed running than slow twitch muscle fibers (Type I).

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Key words: Matrix metalloproteinases - Superoxide dismutase – Running - Skeletal muscle

Introduction

The extracellualr matrix (ECM) outside the myofiber supports, protects and maintains the structural integrity and composition of the cell [4,17]. Activation of matrix metalloproteinase type 2 and 9 (MMP-2, 9) has been implicated in various myopathic and inflammatory conditions [2,7,14,15]. MMP-2 (72-kDa, gelatinase A) and 9 (94-kDa, gelatinase B) belong to a group of calcium and zinc endoproteinases that play a pivotal role in maintaining the ECM during morphogenesis, proliferation, and cell apoptosis [8,12,18,19,24].

Skeletal muscle is responding and adapting to changing in stimulation's such as immobility [22], overloading [6,25], electro stimulation [15] in which MMPs have

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a critical role. Strenuous exercise, especially fast speed running, is known to cause intra and extra myofiber damage [9,16,26].

Muscle over loading increased of reactive oxygen species (ROS), and changes in the antioxidant (AO) scavengers system is well known and previously reported in human and animal species [10]. Prooxidant and antioxidant balance is critical for the survival of aerobic organisms. As a defensive strategy, muscle tissues in human and animals are capable of inducing antioxidant enzymes like superoxide dismutase (SOD) which is one of the major cellular antioxidant enzyme, represents a family of metallo enzymes that catalyzes a common one-electron dismutation of superoxide anion ($\cdot O2^-$) to hydrogen peroxide (H2O2). Two types of SOD exist in human: Copper and zinc-containing SOD (CuZn SOD) found primarily in the sarcoplsma and Manganase-containg SOD (Mn SOD) that presents in the mitochondrial matrix.

The aim of the present study was to investigate the expression of MMP-2, and MMP-9 in type I fibers (slow twitch, high oxidative) and type II fibers (fast twitch, high glycolytic) in young rats following a very fast and slow speed running in order to elucidate their role in the change of ECM composition.

Materials and Methods

Animals: The Pathogen-free Wistar female rats were used (8 month-old, body weight 250-280 g at the beginning of the experiment) and maintained at room under constant temperature (22°C), humidity (40%) with a natural night-day cycle (12/12 h light cycle) and fed with standard rat chow and water ad libitum. Rats were randomized into three main different experimental groups, eight animals in each group. All animals were kept according the principles of laboratory animal care formulated by Florida University (USA) and Tel Aviv University (Israel).

Experimental procedure: Treadmill Running: All animals were adapted to treadmill running during the first 3 days for 10 min at a 15 m per mi. Habituation was followed by 2 weeks, 5 consecutive days per week of continuous running at the following speed: Running Group I - Slow Speed (SS), n=8, of the treadmill exercise started with 20 min of running at a speed of 18 meters per min (18 m/min) and 0% grade, ~ 50% VO_2, with daily increases of 10 min until 50 min were achieved. And Running Group II – Very Fast Speed (FS), n=8, started with 20 min at a speed of 32 meters per min (32 m/min), and 0% grade, ~ 75%VO_2 and each time 10 min were added until 50 min were achieved.

Immediately after the last running session an intraperitoneal injection of pentobarbitol sodium (150mg/kg) was administrated to the animals, followed by a

surgical procedure of carefully removing the right and left Medial Gastrocnemius (M-Gast), Soleus (Sol), Rectus Femoris (RF), and Vastus Intermedius (VI). Than muscles weighted and first immersed in liquid nitrogen (-192°C) for 10 min and then stored at -85°C until assay.

RT – PCR: Total RNA was isolated from 100mg muscle tissue using EZ-RNA isolation kit (Biological Industries co. Beit Haemek, 20-400-100). The RNA was used as a template for RT-PCR reaction (Access Quick ^{TM} RT-PCR system, Promega A1702) using MMP-2 primers: sense:
CCACATTCTGGCCTGAGCTCCC, and anti-sense:
GATTTGATGCTTCCAAACTTCAC, and alpha tubulin primers (as a reference):
sense: ATCACAGGCAAGGAAGATGC, and anti-sense:
ATTGACATCTTTGGGGACCA (Sigma).
MMP-9 sense: ACCTCAAGTGGCACCATCAT, and anti-sense:
CCCTCGAAGATGAATGGAAA. Alpha tubulin primers as indicated above
The reaction products were run on 1.2 % agaros gel.

SDS-PAGE 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 (Sigma, P-8340), and centrifuged (14000 X g for 10 min at 4°C). The supernatants were collected, and total protein concentration was measured using Bradford reagent (Bio-Rad, CA, cat. #500-0006).

Equal amounts of supernatants were suspended in protein sample buffer containing 5% beta-mercapto-ethanol, vortexed, boiled and centrifuged. The supernatant were subjected to 10% SDS-PAGE. Proteins from polyacrylamide gels were transferred onto nitrocellulose membranes.

Blots were blocked with 2.5% skim milk (Bio-Rad, CA, cat. # 170-6404) in PBST (PBS containing 0.05% Tween 20) for 1 hr, reacted with MMP-2 specific goat polyclonal antibody (Santa Cruz Biotechnology, Inc., sc-6838) and alpha tubulin specific mouse monoclonal IgG2a antibody (Santa Cruz Biotechnology, Inc., sc-5286) for 1 h, washed three times with 2.5% skim milk in PBST for 30 min (3 x 10 min), reacted with bovine anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc., sc-2350) or donkey anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc., sc-2314) respectively, for 30 min, and washed once with 2.5% skim milk in PBST for 10 min and three times with PBST for 9 min (3x 3 min). The membranes were developed using Super Signal (West Pico Chemiluminescent substract (Pierce 34080) followed by exposure to X-ray films (Fuji). Quantifications were performed using the Scion Image Version 4.0.2 beta, Scion Cooperation.

SOD assay: This assay utilizes the reduction of cytochrome c via superoxide anions produced via xanthine oxidase reaction. SOD catalyses the reaction below by producing hydrogen peroxide and molecular oxygen from superoxide and hydrogen ions:

$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

Xanthine oxidase (XOD) produces superoxide through the following reaction:
Xanthine + $O_2 \xrightarrow{XOD}$ urate + O_2^-

Superoxide will reduce cytochrome c producing a change in absorbance at 550nm. SOD will the slow the reduction of cytochrome c. One unit (U) of SOD activity is defined as the amount of SOD required for a 50% decrease in cytochrome c reduction rate. Activity is expressed in U/gww or U/mg protein.

Statistical analysis: For the comparison of MMP-2, 9 and SOD levels among experimental groups, and to determine the statistical significances a one-way analysis of variance was used. Where appropriate, group differences were determined using t tests employing a Bonferroni correction for multiple tests. Significance was established at $p<0.05$.

Results

Table 1
Body and muscle weights following two weeks of slow and fast treadmill running*

	Control group (n=6)	2 weeks after SSR (n=8)	2 weeks after FSR (n=8)	% change	P-value
Body weight (g)	298	295	244	-18	0.005
Muscle weight		SSR	FSR	% change	P-value
RF (mg)	710	707	702	-	NS
VI (mg)	806	800	802	-	NS
Sol (mg)	129	127	125	-	NS
Gast (mg)	1.432	1.429	1.433	-	NS

*values represent mean
RF - Rectus Femoris; VI - Vastus Intermedius; Gast - Gastrocnemius; Sol - Soleus; SSR - slow speed running; FSR - fast speed running; NS - not significant

Body and muscle weights: The effects of running on body and muscle weights are demonstrated in Table 1. After 2 weeks of fast speed running the body weight of the rats significantly decreased in 18%. No changes observed in body weight in any of the rats underwent slow speed running. As shown in Table 1 the muscle weights of the slow and fast speed running rats remained unchanged with slight decrease following 2 weeks of fast running.

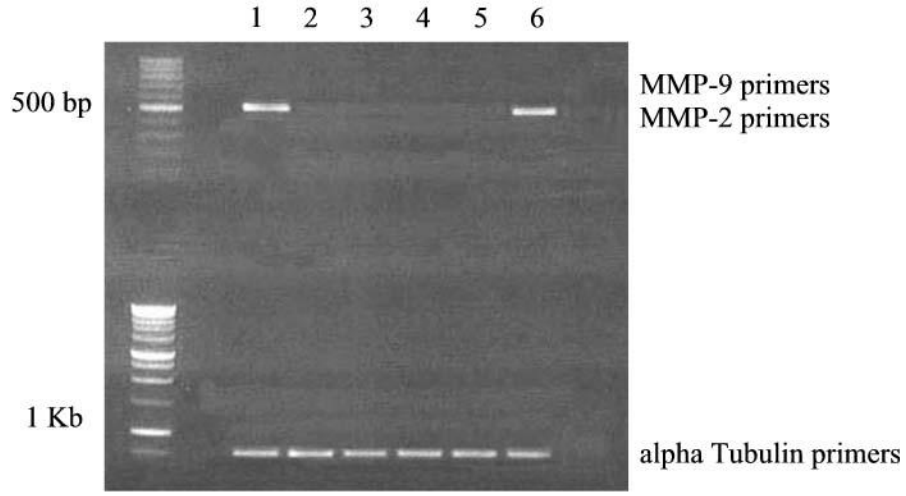

Fig 1: RT-PCR for Medial Gastrocnemius and Recuts Femoris
Left Lane: Marker, Lane 1: Gast. following Fast speed running, Lane 2: Gast. following Slow speed running, Lane 3: Gast. Control, Lane 4: RF control, Lane 5: RF following Slow speed running, Lane 6: RF following Fast speed running

To address the question whether increased or decreased MMP-2, and 9 expression at the mRNA level is associated with protein levels, western immunoblotting and RT-PCR were performed. In agreement with the PCR results, a signal at the molecular size of 72 kDa (latent MMP-2 pro form) was detected in muscles following fast speed running (Figs. 1 and 1a). These signals were more pronounced in type II muscle fibers i.e. medial gastrocnemius and rectus femoris. The proteolytic activity at 94 kDa (MMP-9) was not visible following 2 weeks of slow or fast running neither in 'fast' and 'slow' twitch muscle fibers (Figs. 1, 1a, 2, 2a).

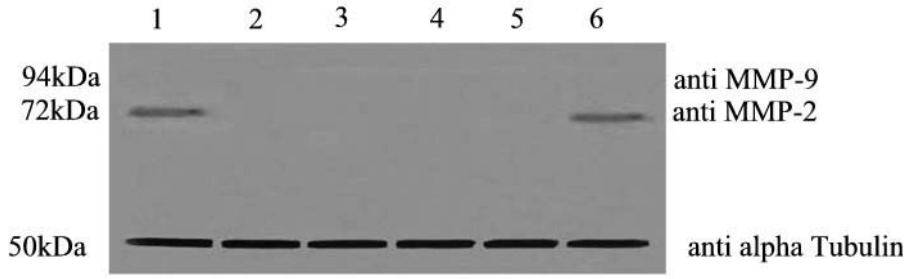

Fig 1a: Western Blot for Medial Gastrconemius and Rectus Femoris
Lane 1: Gast. following Fast speed running, Lane 2: Gast. following Slow speed running, Lane 3: Gast. Control, Lane 4: RF control, Lane 5: RF following Slow speed running, Lane 6: RF following Fast speed running

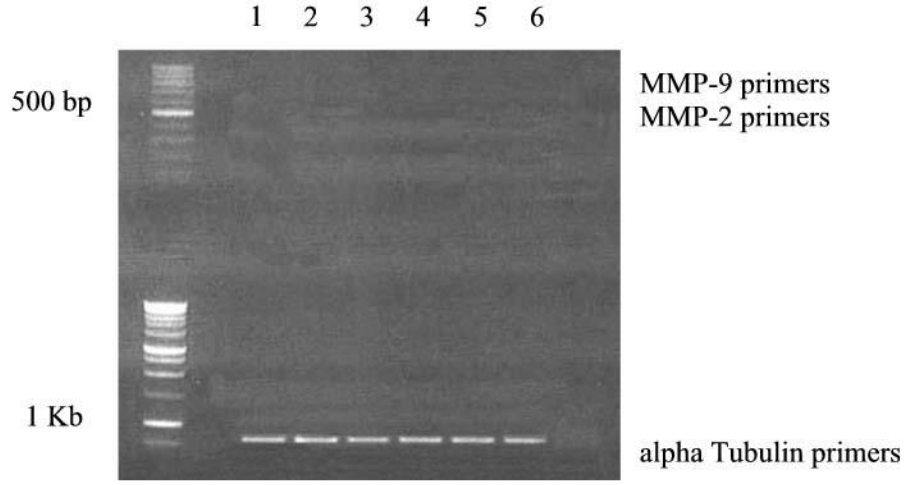

Fig 2: RT-PCR for Soleus and Vastus Intermedius
Left Lane: Marker, Lane 1: Sol following Fast speed running, Lane 2: Sol following Slow speed running, Lane 3: Sol Control, Lane 4: VI control, Lane 5: VI following Slow speed running, Lane 6: VI following Fast speed running

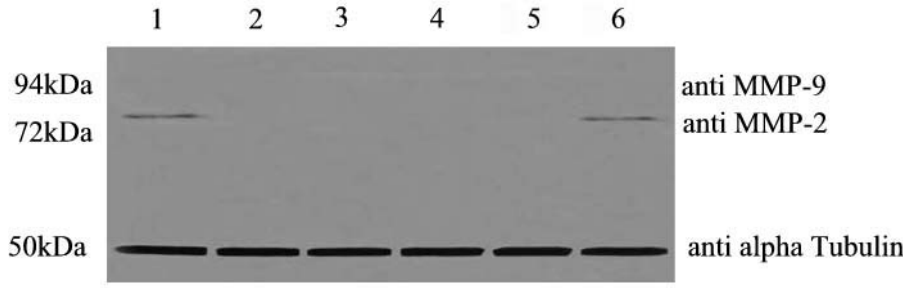

Fig 2a: Western Blot Soleus and Vastus Intermedius
Lane 1: Sol following Fast speed running, Lane 2: Sol following Slow speed running, Lane 3: Sol Control, Lane 4: VI control, Lane 5: VI following Slow speed running, Lane 6: VI following Fast speed running

Table 2 contains mean and ±SD activities for total SOD in four muscles. Following two weeks of fast running the total SOD activity was higher only in Gast and RF muscles.

Table 2
Total Activity of SOD (unit per mg protein)

Muscles	Control	SSR	p	FSR	p
Gastrocnemius	10.2±2.0	10.7±2.7	NS	13.1±1.4	0.05
Soleus	12.7±1.7	13.0±1.8	NS	13.3±2.0	NS
Rectus Femoris	9.7±2.1	10.4±1.9	NS	12.7±1.8	0.05
Vastus Intermedius	11.8 ±2.3	12.2±1.7	NS	12.4±2.4	NS

*$p<0.05$

Discussion

It is well known that MMP-2 is present at low levels in normal ECM muscle tissues and its expression is tightly regulated by cytokines and growth factors. MMP-9 on the other hand is present in acute inflammatory conditions. Although production of proteolytic enzymes is known to be associated with myopathies and inflammatory conditions, their involvement in different fiber types under different loading stimulation had been little investigated. Over use feature protein degradation leading to muscle atrophy and might resulted in muscle malfunction.

The results of this study showed that fast speed running leads to the expression of the inactive precursor, or zymogen, (pro MMP-2, 72 kDa), suggesting accelerated activity of the active form of MMP-2 and therefore increase in the capacity of ECM degradation, whereas up-regulation of MMP-9 was not noticed.
It has been reported that slow twitch muscles contain more collagen than fast twitch muscles and that the concentration of ECM is higher around slow than around fast skeletal muscle fibers in rats [1,26].

In contrast with Koskinen et al 2001, following high speed running the relative increase in gelatinolytic activity of MMP-2 was higher in the fast twitch muscle fibers than in the slow twitch fibers. We were unable to detect MMP-9 either at the mRNA or protein level in fast and slow speed running muscles. These results are in agreement with the argument that only under extreme or abnormal condition of muscle use, when the muscle tissue is inflamed or chronically dennervated only than the expression of MMP-9 might be occurred, by present of leucocytes and macrophages. We recently published that both MMP-2 and MMP-9 can be inhibited by tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1,2), which are secreted by the same myofiber as MMP-2,9. We confirmed that TIMPs level of activity changed in parallel with activity changes demonstrated for MMP-2 and MMP-9 as also previously reported [19,23].

In the current study, every muscle fiber type showed a unique response pattern to running. We could observe that type II muscle fibers were more susceptible to overuse than type I muscle fibers. The degree of muscle tissue change is fiber type specific and appearing to be more pronounced in type II fibers. From previous reports, overuse leads to muscle tissue damage which follow by functional decline. The significant change in type II fibers was observed only following 2 weeks of fast speed running. Both in RF and Gast, net degradation was observed when related to soluble protein concentration, suggesting a higher protein degradation in fast twitch muscle fibers (type II) than in slow twitch muscle fibers (type I).

There are potentially two explanations of the MMP-2 expression differences among the muscle fibers. First, in rat skeletal muscles, type IIB fibers are at least twice as big and stronger as type I fibers, which probably explains the higher volume of collagen therefore, 'white' fibers (fast twitch) require more MMP-2 to maintain its integrity than 'red' fibers (slow twitch). Second, 'white' fibers demonstrate a better muscle plasticity than 'red' fibers and show faster adaptation to exercise training [19]. Under intensified training fast fibers may undergo transition to slow fiber types with corresponding changes in ECM composition [11].

Our findings show that high speed running induced SOD activity only in type II muscle fibers. Increased SOD activity following fast running provides protection

against oxidative stress. Our data agree with previous findings that strenuous exercise promotes production of reactive oxygen species (ROS), which in turn serve as a trigger for SOD elevation which is an essential defense mechanism [21]. In previous reports, fast twitch fibers contain lower SOD than the slow-twitch fibers, possibly indicating that the AO enzyme system is more related to the oxidative capacity of skeletal muscle cell [10]. Following fast (over 70% intensity) and prolong running (50 min for 10 days) the anaerobic type II muscles fibers are thought to be more susceptible to oxidative stress in order to produce a greater aerobic energy. 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.

The results of the present study suggest, that the effect of fast running on the overall balance of protein turnover, i.e. the degradation and synthesis of ECM, is more distinct in type II muscle fibers than in type I fibers.

In summary, the treadmill can serve as a model to demonstrating the damaging exercise due to fast running. The integrity and the composition of the ECM were affected inducing the activity of ECM- through expression of degrading enzymes and changing the collagen synthesis.

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