Chapter 3

Molecular and Cellular Markers in Skeletal Muscle Damage after Acute Voluntary Exercise Containing Eccentric Muscle Contractions

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Additional information is available at the end of the chapter

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Abstract

In eccentric muscle contraction, the muscle is lengthening while contracting. For example, in downhill walking, the thigh muscles are contracting eccentrically. It is well known that unaccustomed eccentric exercise causes pain and may lead to inflammation reactions on muscles few days after the exercise. The theme of the present chapter is molecular and cellular markers in skeletal muscle damage after voluntary exercise containing eccentric muscle contractions. The chapter contains three topics: In the first topic, the damaging process followed by regeneration is demonstrated with antibody stainings of connective tissue, plasma membrane, and cytoskeletal proteins. The second topic is infiltration of inflammatory cells in damaged skeletal muscle. Neutrophils are usually the first inflammatory cells mostly present in the injured tissues; however, neutrophils are not present in exercise-induced skeletal muscle damage. Finally, the relationship between skeletal muscle damage and systematic markers, serum creatine kinase and voluntary maximal force production, is described.

Keywords: skeletal muscle damage, eccentric exercise, infiltration of inflammatory cells, neutrophils, monocytes/macrophages, extracellular matrix

1. Introduction

In movements such as walking, running, and jumping, both eccentric (the muscle is actively lengthened/stretched) and concentric (the muscle is actively shortened) muscle contractions are present as the muscles undergo repeated stretching-shortening cycles. [1] Typical physical activities, which contain a great deal of eccentric contractions, are going down stairs, walking

and running downhill, lowering weights, and the downward motion of squats and push-ups. It is fair to say that muscles contracting eccentrically produce "more for less." High mechanical muscle tension produced by eccentric muscle contraction is generated at lower metabolic cost and at a greatly reduced oxygen requirement compared to concentric muscle contraction. It has been reported that the oxygen requirement of submaximal eccentric cycling is only 1/6–1/7 of that for concentric cycling at the same workload [2]. Furthermore, eccentric training can increase the size and strength of muscles with very little demand on the cardiovascular system [3]. Therefore, eccentric training is potential training method for elderly and patients suffering from diseases that limit either the uptake or delivery of oxygen, e.g., chronic obstructive pulmonary disease or chronic heart failure. Any exercise that requires a significant increase in respiration and in cardiac output may not be only uncomfortable but also impossible for fragile individuals. However, it is not only fragile individuals who benefit from eccentric training: It is advantageous for anyone since less time is needed and training feels less strenuous compared to concentric type of training. As a disadvantage, unaccustomed eccentric exercise causes muscle pain and may lead to inflammation reaction on muscles for few days post-exercise. Likely, one bout of eccentric exercise induces protective effect against muscle pain and against skeletal muscle fiber injury for several weeks or even months [4].

Maximal eccentric exercise with the knee extensors or elbow flexors on an isokinetic dynamometer has frequently been used to induce and to study skeletal muscle damage in humans. A typical response to such high-force, single-joint eccentric exercise protocols is on average a 50% reduction of the force-generating capacity immediately post exercise, followed by gradual recovery over the next days or weeks [5]. There are clearly individual differences both in force-generating capacity immediately post exercise and in the length of the force recovery period [6, 7]. It is not known why the same amount of eccentric exercise induces skeletal muscle fiber damage, loss in force-generating capacity, and prolonged force recovery period for some individuals, whereas only short-term decrease in muscle force-generating capacity was observed in other subjects [6]. Prolonged recovery of muscle force is thought to be related to distortion of the myofibrillar structure and disturbed calcium homoeostasis and/or prolonged inflammatory response [5]. Consequently, it has been suggested that reduction in muscle force-generating capacity may be a valuable indicator for monitoring muscle damage following exercise [8]. Physiological adaptation takes place after a single bout of unaccustomed eccentric exercise by making muscles more resistant against structural changes from the second bout of eccentric exercise [9].

In the next sections, few examples are presented for visualizing skeletal muscle fiber injury in muscle biopsies after eccentric exercise. Heat shock protein 27 (HSP27) antibody staining shows abnormal sarcoplasmic staining pattern already during the first hour after the exercise. Plasma membrane protein (dystrophin)-negative skeletal muscle fiber indicates quite severe muscle damage, while basement membrane proteins laminin and type IV collagen are intact and are keeping the muscle fiber together. Counting the number of infiltrated inflammatory cells in a damaged skeletal muscle is often reported after different exercise protocols. However, antibodies used for counting neutrophils and monocytes/macrophages do not always exclusively recognize the studied leukocyte. Finally, the relationship between skeletal

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muscle damage and systematic markers, serum creatine kinase and voluntary maximal force production, is described.

2. Molecular markers for exercise-induced skeletal muscle damage

The first changes in the sarcoplasm of skeletal muscle fibers occur within few hours after the damaging exercise such as myofibrillar loss (**Figure 1D**). Furthermore, accumulation of mitochondria, local loss of myofilaments, Z line streaming (**Figure 1E**), swelling of mitochondria, disconnection of plasma membrane, swelling of sarcoplasmic reticulum, infiltration of inflammatory cells, complete loss of plasma membrane (**Figure 1F**), and disrupted muscle fibers may appear depending on how severe the damage is. In the next subsections, antibody stainings for HSP27, dystrophin, and type IV collagen are given as an example for visualizing changes after eccentric exercise in sarcoplasm, plasma membrane, and extracellular matrix, respectively.

2.1. Sarcoplasm of the skeletal muscle

In general, HSPs are considered to be the cellular protein quality control machinery. They can stabilize proteins during cellular damage, contribute to protein folding during increased protein synthesis, and protect proteins from aggregation. HSP27 can interact with actin and with many actin-binding proteins such as tropomyosin and troponin T [10]. HSP27 immunostainings in longitudinal sections of unexercised skeletal muscle fibers appear as fine lines (**Figure 2A**) indicating that HSP27 is localized to the Z-disks and/or I-band [11]. Immediately and 3 h after the exercise, in this case continuous drop jumping unilaterally on a sledge apparatus with a submaximal height until complete exhaustion, HSP27 immunostainings showed intensively stained and variable-sized clusters in both cross-sectional and longitudinal sections of skeletal muscle fiber HSP27 (**Figure 2C–F**) [12]. These stained clusters were probably formed due to translocation and accumulation of HSP27 on cytoskeletal/myofibrillar structures [11].

2.2. Plasma membrane of the skeletal muscle

Dystrophin is part of dystrophin protein complex, which transmits force laterally from contractile filaments to extracellular matrix through sarcolemma. Dystrophin is located beneath the sarcolemma, and it strengthens muscle fibers and protects them from injury. Immediately after forced lengthening contractions, the immunostaining of antibody against C-terminus of dystrophin fades out or disappeared partially before immunostaining of antibody against rod domain from few fibers in muscle sections from rat tibialis anterior muscle (**Figure 3**). This sequence of structural disturbance after eccentric exercise is interesting in relation to recent finding of new membrane-binding domain in dystrophin C-terminus [13]. In addition, proteinase-resistant regions in the rod domain of dystrophin make it more resistant against degradation.

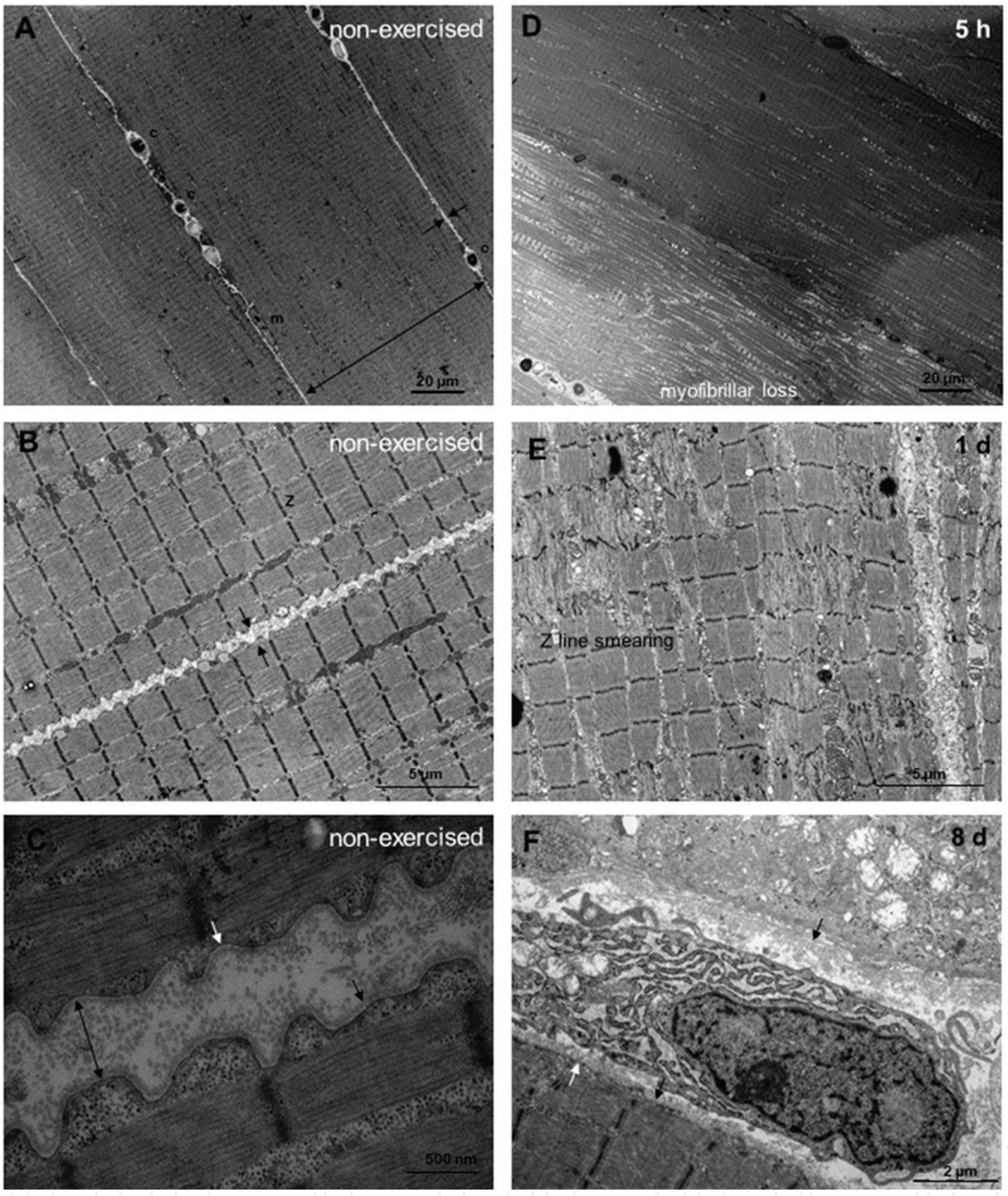

Figure 1. Electron micrograph of longitudinal sections of epoxy-embedded human skeletal muscle biopsy from vastus lateralis. (A–C) Non-exercised muscles. Skeletal muscle fiber (long two-headed arrow), endomysium (space between the two small arrows facing each other), myonuclei/satellite cell (m), capillary (c), plasma membrane is the fine dark line (white small arrow), basement membrane is the thick gray line (black small arrow). (D–F) Eccentric exercised muscle. (D) Five hours after the exercise, some myofibrils are lost in skeletal muscle fiber (lower left corner). (E) One day after the exercise, Z line smearing. (F) Eight days after the exercise, only basement membrane can be seen in damaged skeletal muscle fiber (upper small black arrow). Cell between two muscle fibers is probably fibroblast containing rough endoplasmic reticulum.

2.3. Basement membrane of the skeletal muscle

The extracellular matrix provides mechanical support for skeletal muscle fibers and plays an important role in force transmission. Fibrillar type I and III collagen are present in endomysium

of skeletal muscle fibers (see **Figure 1C**, small gray dots between muscle fibers), whereas non-fibrillar type IV collagen is in basement membranes of skeletal muscle fibers (see **Figure 1C**, small black arrow) and capillaries. Type IV collagen is present in swollen, necrotic (**Figure 4**), and regenerated fibers, similarly as in undamaged skeletal muscle fibers. This suggests that basement membrane including type IV collagen holds on skeletal muscle fiber during the process of fiber damage, when the cytoskeleton is disrupted, contractile proteins are disorganized, and inflammatory cells are infiltrated [14].

2.4. What to remember about molecular markers for exercise-induced skeletal muscle damage

The first structural changes appear within few hours after eccentric exercise in sarcoplasm. This can be visualized using, for example, HSP27 immunostainings, which can be seen as intensively stained and variable-sized clusters (**Figure 2C–F**). During the next few days after the damaging exercise, antibody stainings for dystrophin fade out or disappear partially (**Figure 3**) and in severe damaged skeletal muscle fibers disappear totally (**Figure 4B**), whereas basement membrane including type IV collagen serves as a supportive structure during skeletal muscle fiber injury (**Figure 4A**) and regeneration.

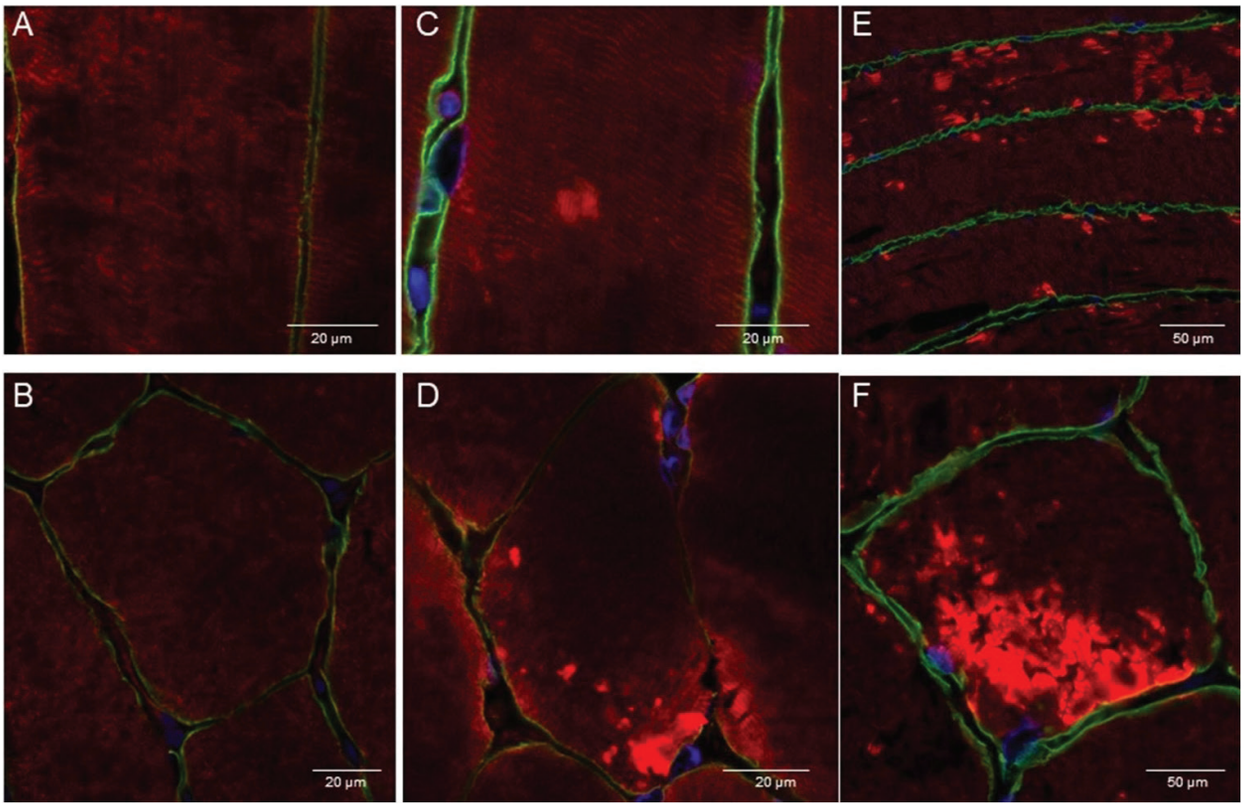

Figure 2. HSP27 (red) immunostaining as a marker for muscle damage. In biopsies obtained from human vastus lateralis before the exercise, no HSP27-stained clusters were observed (A and B). HSP27 was localized to the Z-disks of skeletal muscle fibers. After the exercise, intensively stained and variably sized clusters of HSP27 were observed immediately (C–E) and 3 hours post exercise (F). Dystrophin (green) immunostaining was used to visualize the borders of muscle fibers and DAPI (blue) stained nuclei. Published in Koskinen et al. [12].

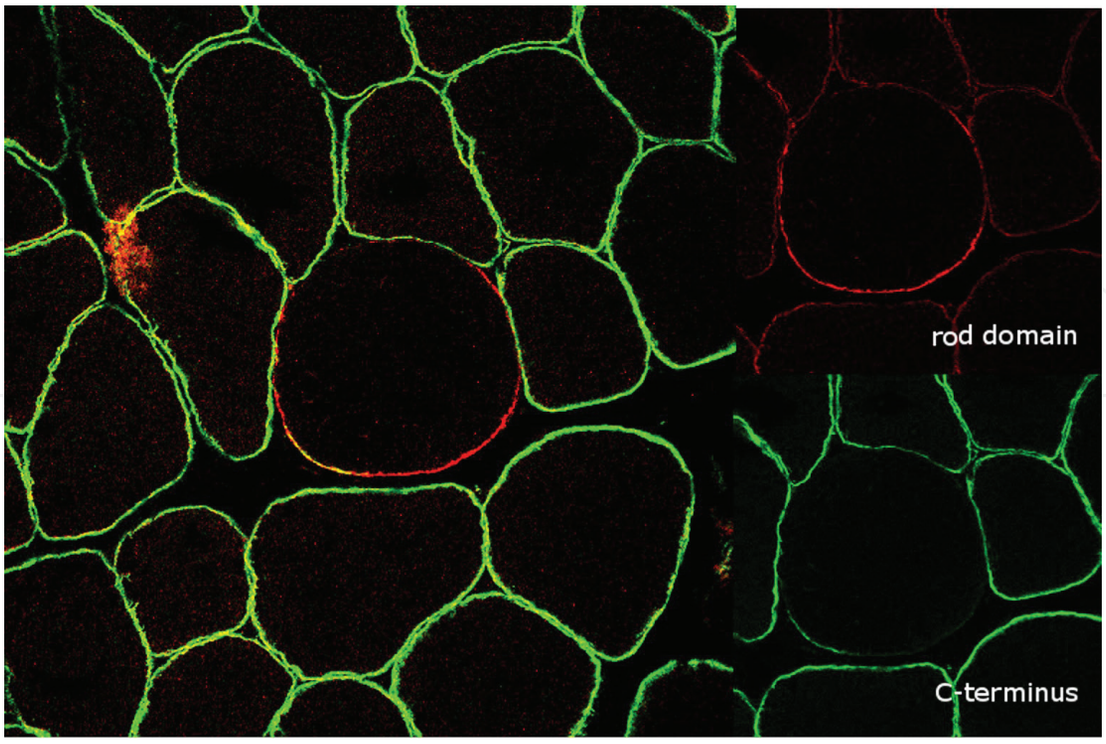

Figure 3. Immunostaining of antibody against C-terminus dystrophin (green) faded out or disappeared partially from individual muscle fiber (rat tibialis anterior muscle immediately after forced lengthening contractions), before than the staining for antibody against rod domain of dystrophin (red) that was still partially as bright as in non-exercised muscles.

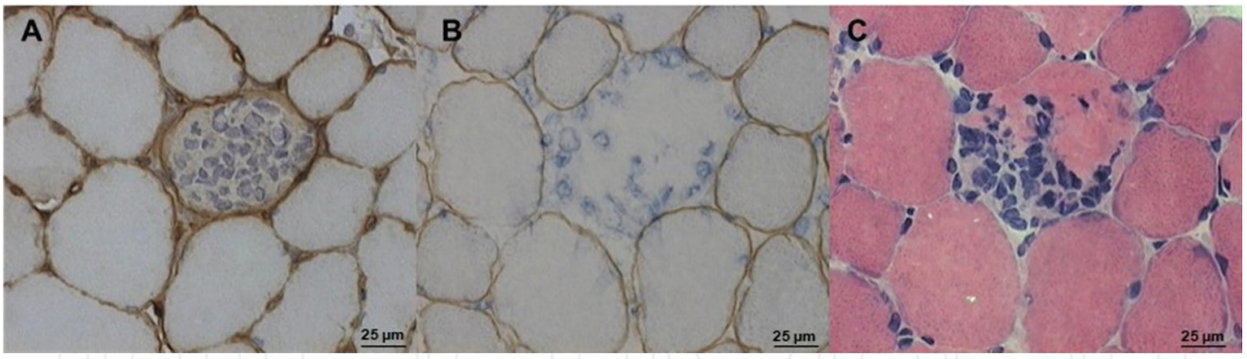

Figure 4. Injured skeletal muscle fiber in rat tibialis anterior muscle 2 days after forced lengthening contractions. Type IV collagen immunohistochemical staining is visible in the borders of basement membranes of skeletal muscle fibers (A), while dystrophin staining is negative (B). Infiltration of inflammatory cells inside the damaged fiber (D, hematoxylin–eosin staining). Bar = 25 µm. Published in Koskinen et al. [14].

3. Inflammatory cell markers in exercise-induced skeletal muscle damage

Immunostainings of leukocyte markers for neutrophils (CD11b, CD16, CD66b, neutrophil elastase, and myeloperoxidase) and for monocytes/macrophages (CD68) have been applied for localizing and counting the number of these inflammatory cells in human skeletal muscles

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after damaging exercise (e.g., see [15–20]). It is often concluded that the inflammatory cell reaction in skeletal muscle fiber injury caused by unaccustomed eccentric exercise is initiated by infiltration of neutrophils. However, the antibodies for CD11b, CD16, neutrophil elastase, and myeloperoxidase recognize also other leukocytes than neutrophils in leukocyte blood smears, whereas antibody for CD66b recognized only neutrophils (**Figure 5**) [6]. Therefore, the CD66b antibody is more suitable for detecting neutrophils in skeletal muscle sections than antibody for CD11b, CD16, myeloperoxidase, and neutrophil elastase. The CD68 antibody, the marker for monocytes/macrophages, recognized monocytes/macrophages and a portion of cells with bilobed nuclei (basophils or eosinophils) on leukocyte blood smears. In skeletal muscle biopsies after eccentric exercise, the CD68 antibody recognized more cell types than monocytes/macrophages [6].

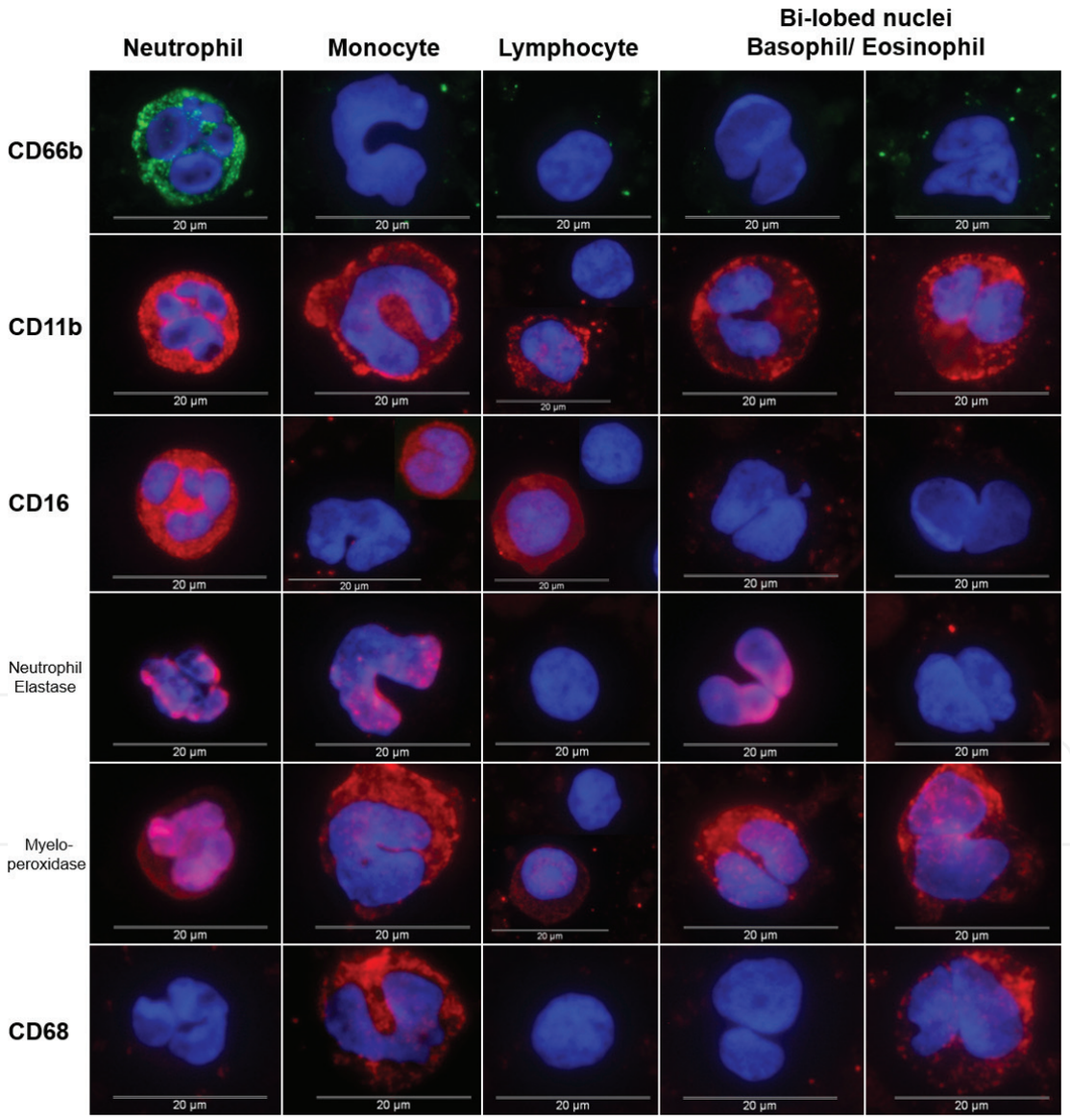

Figure 5. Immunostaining of CD11b, CD16, CD66b, CD68, myeloperoxidase, and neutrophil elastase antibodies on circulating leukocytes extracted from whole blood. Secondary antibody for CD66b Alexa Fluor 488 anti-mouse; for CD11b, CD16, CD68, and neutrophil elastase Alexa Fluor 594 anti-mouse; and for myeloperoxidase Alexa Fluor 594 anti-rabbit. Scale bar of 20 μm. Published in Paulsen et al. [6].

3.1. Neutrophils in damaged skeletal muscle

The number of CD66b-positive cells is very low in both exercised and non-exercised skeletal muscles [6]. As an example, only 34 of 122 biopsies from eccentrically exercised and non-exercised human biceps brachii muscles contained CD66b-positive cells. A closer examination showed that in 23 of these biopsies, the CD66b stained cells (varied from 1 to 4 CD66b-positive cells per 100 muscle fibers) were located inside capillaries or vessels (**Figure 6B**), attached to the wall of the vessels, or were detected in blood clots (**Figure 6A**). In the remaining 11 biopsies, single CD66b-positive cells (varied from 1 to 30 CD66b-positive cells per 100 muscle fibers) were observed in the endomysium of affected muscle fibers or in the sarcoplasm of damaged fibers. There was no consistent pattern regarding how CD66b-positive cells were distributed between exercised and non-exercised samples or between different time points. These results indicated that neutrophils were not involved in exercise-induced skeletal muscle fiber injury. Unusual high numbers of CD66b stained cells located in the endomysium or inside fibers may indicate trauma from the previous biopsy.

3.2. Monocytes/macrophages in damaged skeletal muscle

CD68-positive cell counts are widely used for indication of monocyte/macrophage infiltration in skeletal muscle biopsies after single bout of eccentric exercise (e.g., see [19]). However, it has been shown that the CD68 antibody recognized more cell types than monocytes/macrophages in human skeletal muscle biopsies after eccentric exercise [6]. The highest individual CD68-positive cell counts were related to skeletal muscle fiber injury, which was observed in exercised biopsies at 4 and 7 days after acute eccentric exercise. In these biopsies, CD68-positive cells typically occupied the entire sarcoplasm of damaged skeletal muscle fibers (**Figure 7A**). Therefore, monocytes/macrophages were probably the most prominent CD68-positive cell type in these biopsies. In addition, CD68-positive cells inside capillaries (**Figure 7C**) and vessels (**Figure 7D**) were most likely monocytes/macrophages. In the exercised biopsies without damaged skeletal muscle fibers, the determination of the monocytes/macrophages proportion of CD68-positive cells is not straightforward. Comparison of cells with similar appearance and location between light microscopy pictures of CD68-positive cells and transmission electron microscopy pictures

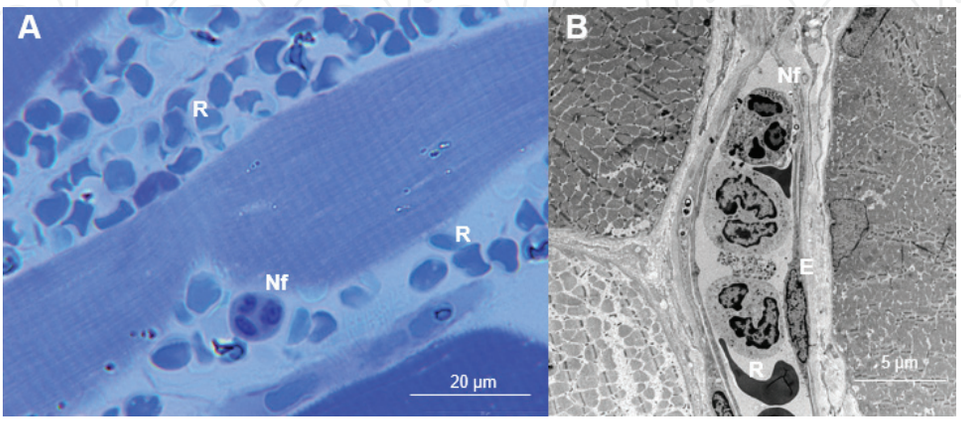

Figure 6. The nuclei of neutrophils are divided in few parts, which makes them easily recognizable. (A) Semi-thin toluidine blue stained section contains a cluster of red blood cells and single neutrophil (non-exercised skeletal muscle biopsy from human biceps brachii muscle). (B) Leukocytes inside a blood vessel (electron micrograph of epoxy-embedded exercised skeletal muscle biopsy from biceps brachii muscle). Endothelial cell (E), neutrophil (Nf), red blood cell (R).

showed that the sarcoplasmic CD68 immunostaining observed in single cells aligned next to the laminin (**Figure 7B** arrow) was most likely satellite cells (in electron microscopy pictures, these cells were located between the plasma membrane and the basement membrane of skeletal muscle fibers). Furthermore, CD68-positive cells with long extensions located in endomysium (**Figure 7B** arrowhead) were probably fibroblasts or myofibroblasts. According to the observation from electron microcopy pictures, these types of cells contained prominent rough endoplasmic reticulum indicating cells with high capacity for protein synthesis such as fibroblasts/myofibroblasts. Both satellite cells and fibroblasts were most likely involved in the skeletal muscle adaptation to increased mechanical loading. The number of CD68-positive cells is not optimal to be applied as a quantitative value for monocytes/macrophages in human skeletal muscles.

3.3. What to remember about inflammatory cells in damaged skeletal muscle

Neutrophils are not involved in exercise-induced skeletal muscle fiber injury, although in other injured tissues neutrophils are usually the first inflammatory cells present. CD66b antibody seems to exclusively recognize neutrophils both in leukocyte blood smears and skeletal muscle biopsies. Numerous monocytes/macrophages are present in damaged skeletal muscle fibers, typically totally occupying the entire sarcoplasm of damaged fibers. CD68 antibody is not optimal to be applied as a quantitative value for monocytes/macrophages, because it recognizes also satellite cells and fibroblasts in skeletal muscle biopsies.

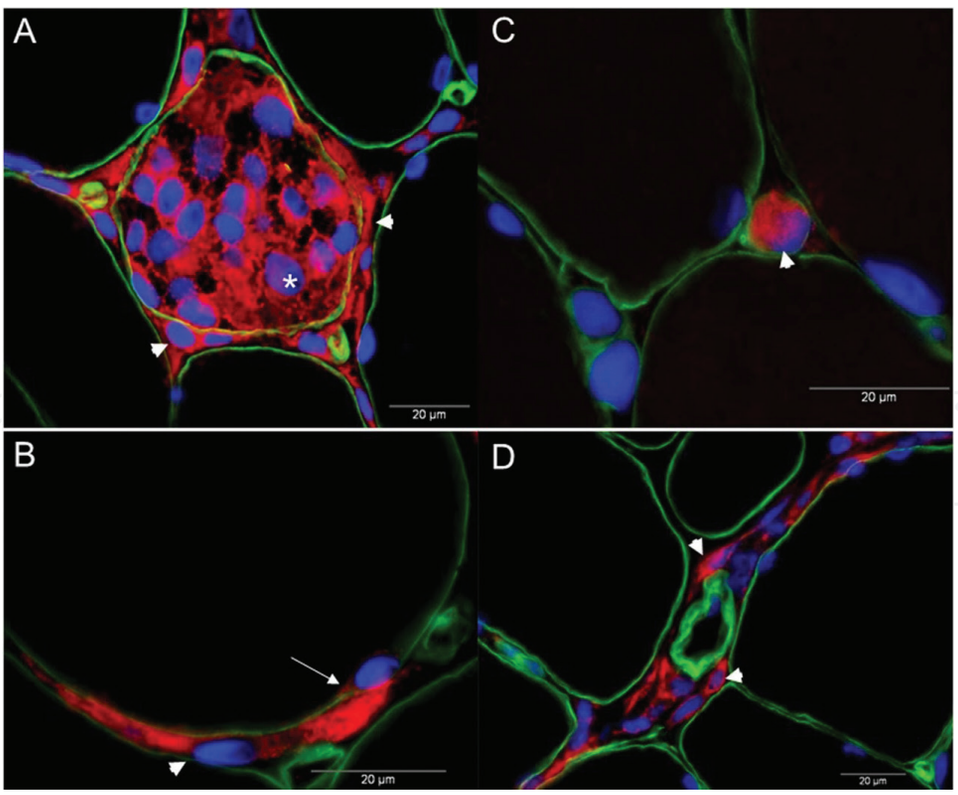

Figure 7. CD68 (red), laminin (basement membrane, green), and DAPI (nuclei, blue) staining on eccentric exercised human skeletal muscle. (A) Damaged skeletal muscle fiber infiltrated by CD68 stained cells (asterisk). In the endomysium, CD68 stained cells seemed to form a chain around necrotic fiber (arrow heads). (B) CD68 stained cells with long extensions in sarcoplasm (arrow) and in the endomysium (arrow head). (C) CD68 stained cell (arrow heads) inside capillary. (D) Vessel surrounded by CD68 stained cells. Scale bar of 20 µm. Published in Paulsen et al. [6].

4. Serum creatine kinase and voluntary maximal force production as indirect indicator for severity of skeletal muscle damage

Decrease in muscle force-generating capacity after eccentric exercise is a valuable tool as an indirect indicator for severity of skeletal muscle damage together with serum creatine kinase. As an example, 23 subjects were divided into 3 categories, mild (n = 6), moderate (n = 10), and severe (n = 7) effect of eccentric exercise, depending on the muscle force loss immediately after performing 70 maximal eccentric actions with elbow flexors on an isokinetic dynamometer and how fast muscle force was recovered during the following 7 days [6]. Muscle force loss immediately after the exercise was 64, 53, and 50% of pre-exercise in mild, moderate, and severe categories, respectively (**Figure 8**). After 7 days post exercise, muscle force was totally recovered in the category mild effect of eccentric exercise, whereas in the categories moderate and severe effect of eccentric exercise, force was still clearly below the baseline level (**Figure 8**).

The average values of serum creatine kinase (207, 228, and 140 U/l; mild, moderate, and severe, respectively) before eccentric exercise were similar in all three categories (**Figure 9**). The average values of serum creatine kinase changed the most in categories moderate and severe effects of eccentric exercise. The highest average creatine kinase values, 2929 and 10,266 U/l, in these categories were observed 4 days after eccentric exercise, whereas in the mild effects of eccentric exercise category, the highest average value, 538 U/l, was observed 7 days after the exercise.

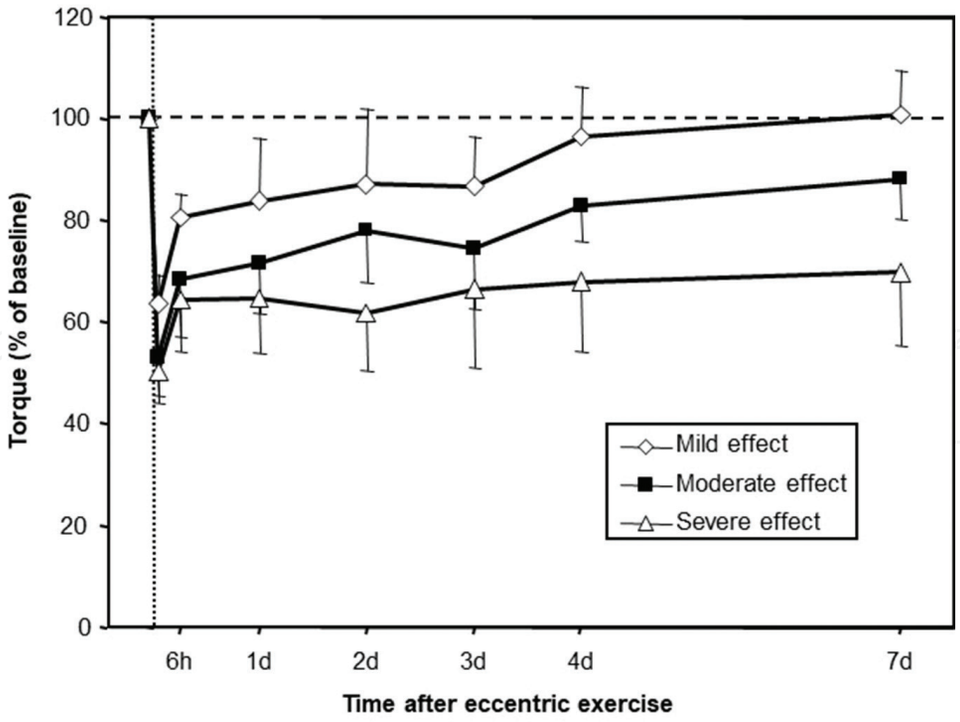

Figure 8. Post-exercise muscle force-generating capacity of the exercised arm. Subjects were divided into three categories, mild (n = 6), moderate (n = 10), and severe (n = 7) effect of eccentric exercise, based on the loss and the recovery of muscle force. Vertical dashed line indicates the time of eccentric exercise bout was performed and horizontal dashed line is the baseline for muscle force. Error bars are standard deviation. Published in Paulsen et al. [6].

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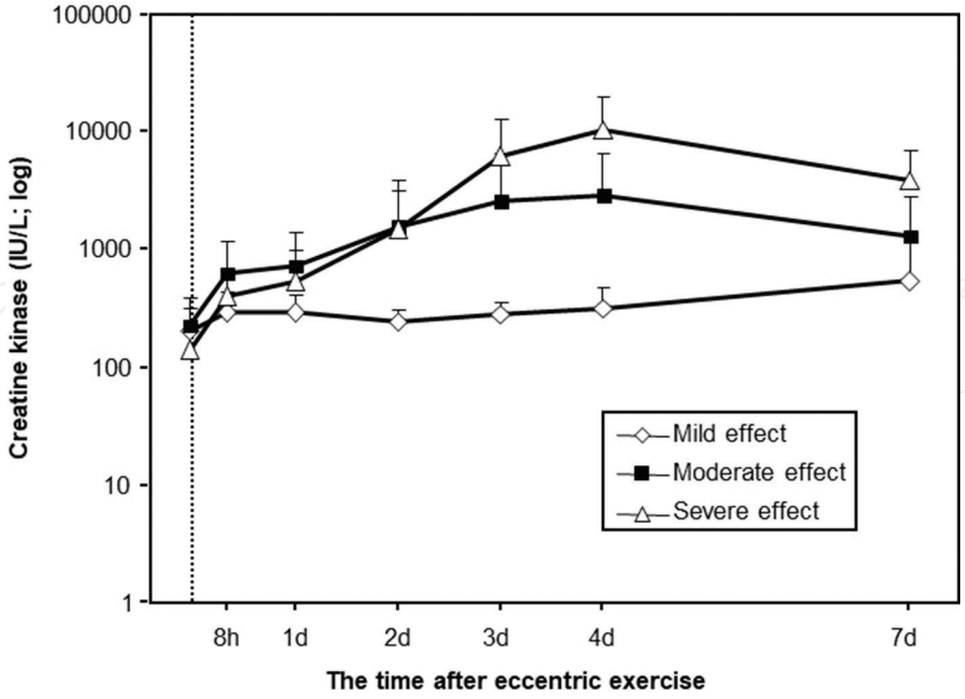

Figure 9. Serum creatine kinase. Subjects were divided into three categories, mild (n = 6), moderate (n = 10), and severe (n = 7) effect of eccentric exercise, based on the loss and the recovery of muscle force. Vertical dashed line indicates the time of eccentric exercise. Y-axis is logarithmic. Error bars are standard deviation. Published in Paulsen et al. [6].

Eight of total 23 subjects showed skeletal muscle fiber injury detected at light microscopy level as dystrophin-negative fibers and infiltration of CD68-positive cells at 4 or 7 days after the exercise. These subjects belonged to the categories moderate (five subjects of ten showed dystrophin-negative fibers) and severe (three subjects of seven showed dystrophin-negative fibers) effects of eccentric exercise. If the muscle force-generating capacity and serum creatine kinase have not totally recovered to the baseline level, it is most likely that the muscle is undergoing regeneration.

4.1. What to remember about indirect indicators for severity of skeletal muscle damage

Skeletal muscle fiber injury and prolonged regeneration process are probably the reasons for impaired peripheral muscle function after high-force eccentric exercise. Muscle force-generating capacity after single bout of eccentric exercise is a good indirect indicator of muscle damage in humans together with serum creatine kinase analysis. In the future studies, more attention should be paid for making sure that subjects' muscle force has recovered to the baseline level after the experiment is over.

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Conflict of interest

The authors declare they have no conflict of interest.

Abbreviations

HSP	Heat shock protein

Author details

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