Injury and Repair of the Soleus Muscle after Electrical Stimulation of the Sciatic Nerve in the Rat*

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Summary. To study injury and subsequent changes in skeletal muscles, the rat sciatic nerve was electrically stimulated at 50 Hz and muscle contraction was induced for 30 min. Muscle damage was classified into five types (hypercontraction, hyperstretching, Z band disorders, misalignment of myofilament and regions of scarce myofilaments) by electron microscopy and quantified by ultrastructural assessment. After electrical nerve stimulation, the percentages of the injured areas of the soleus muscle were $18.8 \pm 15.8\%$ (mean $\pm$ SD) at 0 h, $9.7 \pm 1.0\%$ at 6 h, $22.0 \pm 23.6\%$ at 12 h, $13.1 \pm 3.2\%$ at 24 h, $4.9 \pm 6.0\%$ at 3 days and $0.5 \pm 0.4\%$ at 7 days. At 0 h, the vast majority of ultrastructural alterations were sarcomere hypercontraction. At 6 h, hypercontraction was not recognizable and sarcomere hyperstretching and Z band disarrangement constituted the major findings. At 12 h, when the injury reached its maximum, myofilament disorganization and hyperstretching were predominant. At 24 h or afterwards, the injury began to decrease and recovered to almost normal conditions by 7 days. There were very few necrotic muscle fibers in all specimens. It is considered that the muscle lesions in the present study were reversible, and recovered through changes in various types of sarcomere alterations. Z band streaming and free ribosomes were frequently found at 12 and 24 h, which may indicate repair processes rather than newly formed lesions.

It is well known that muscle injury is induced by overexercise. Damaged muscle exhibits various ultrastructural, functional, and biochemical alterations, and these alterations differ depending on the sampling time after overexercise. Treadmill running resulted in sarcomere lesions (ARMSTRONG et al., 1983; OGILVIE et al., 1988; KOMULAINEN et al., 1994) and an efflux of intramuscular enzymes (ARMSTRONG et al., 1983; KOMULAINEN et al., 1994) such as creatine kinase, glucose-6-phosphatase, and β-glucuronidase in rats. Prolonged swimming induced a functional decrease in contractions in rats (FITTS et al., 1982). Sarcomere hypercontraction with alterations of Z bands was demonstrated in reloaded adductor longus muscle after microgravity unloading by space flight in rats (KRIPPENDORF and RILEY, 1994; VIJAYAN et al., 1998). In human subjects, eccentric contractions of upper (GIBALA et al., 1995) and lower limbs (HORTOBÁGYI et al., 1998) resulted in ultrastructural and functional alterations of muscles. After eccentric contractions of rat skeletal muscle, various sarcomere lesions (THOMPSON et al., 1999), the disappearance of intermediate filament desmin (LIEBER et al., 1996; KOMULAINEN et al., 1998), hypofunction of muscle (MCCULLY and FAULKNER, 1985) as well as subsequent necrosis and regeneration of muscle fibers (MCCULLY and FAULKNER, 1985; KOMULAINEN et al., 1998) were demonstrated. It is reported that chronic overloading due to ablation of synergists caused the streaming of Z bands in rats (SNOW, 1990).

Sarcomere lesions have been classified into either three (OGILVIE et al., 1988) or five types (THOMPSON et al., 1999). THOMPSON et al. (1999) suspected that these sarcomere lesions may change from one type to another. However, there have been few electron microscopic studies of the time course of sarcomere lesions, and the mechanisms of the generation and repair of muscle injury are not fully understood. To study these problems, we induced muscle contraction by nerve stimulation instead of loading rats with overexercise and examined the fine structural alterations of the soleus muscle at various intervals after treatment.

MATERIALS AND METHODS

Eight-week-old female Wistar rats (18 animals; B. W. 179.9 ± 6.0 g) were used in this study. Experimental procedures were approved by the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University. Pilot studies showed that electrical stimulation at 0.5 Hz had little effect on the muscle fine structure even after long treatment, whereas stimulation at 50 Hz resulted in varying degrees of sarcomere alterations depending on the duration of stimulation. Stimulation at 50 Hz for 15 min induced few sarcomere lesions. Stimulation for 30 or 60 min caused some injuries of similar degree. Stimulation for 120 min produced extensive and severe damage. We therefore employed a 30-min stimulation. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/ kg B.W.) with subsequent ether inhalation when required. In nerve-stimulated rats, the skin covering the buttock was cut on one side. The sciatic nerve was exposed and separated from the surrounding tissues. The nerve was connected to plus and minus wire electrodes, returned to its original *in situ* position and covered with cotton that had been soaked in saline solution. The hindlimbs of these rats were not restrained. First, we stimulated the nerve by a 1-Hz, monophasic 1-ms square-wave pulse (Nihonkohden SEN-2201, Tokyo, Japan) and then gradually increased the voltage from 0 V until twitch threshold response (plantar flexion) occurred. After determining the twitch threshold voltage, we doubled the voltage to 3.8–6.0 V and stimulated the nerve. After the start of electrical nerve stimulation, the ankle joint immediately began to plantar flex and the digits were extended. Several minutes later, the extension of digits somewhat weakened. However, the legs and digits maintained this posture until termination of the stimulation. All rats were laid on a bag containing water at about 37℃ during treatment to prevent a drop in body temperature.

Rats were stimulated for 30 min. Electrodes were removed immediately after the cessation of stimulation, and the rats were sacrificed at 0, 6, 12, or 24 h, or 3 or 7 days after treatment. In groups of rats that were sacrificed at 6 h or afterwards, the incisions were sutured, and the animals were placed back in their cages in the animal quarters with free access to food and water until they were killed for tissue sampling. No rats suffered nerve paralysis after awakening.

Rats were anesthetized with either sodium pentobarbital (at 0 h after treatment) or ether (at 6 h or afterwards)

and an incision was made through the ribs to expose the heart. The inferior vena cava was cut for blood removal. An 18-gauge needle was inserted through the apex of the heart into the left ventricle. The animal was perfused by gravity-mediated flow first with 50 ml of saline to clear the blood and then with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The soleus muscles of the treated and control sides (the contralateral side of nerve stimulation at 0 h) were removed, and the midbelly portions were excised in rectangular pieces to facilitate orientation during embedding and sectioning. Specimens were fixed in the same fixative for 2 h–1 day, postfixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) overnight at 4℃, rinsed in 10% saccharose three times (10 min each) and stained *en bloc* in 3% aqueous uranyl acetate for 1 h at room temperature. Samples were then dehydrated in an ascending series of ethanol, longitudinally oriented, and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed using a JEM-1200 electron microscope.

For quantitative analysis of muscle injuries, three blocks were selected at random from each soleus muscle. Ultrathin sections were cut, collected on grids, and stained. Three holes of each grid were chosen following a protocol to prevent the investigator's bias. Electron micrographs of the centers of these three holes were taken at a magnification of 2,500 and printed at a final magnification of 5,600. Each film covered an area of approximately 680 μm^2. A total of 324 micrographs were inspected by two investigators on a blinded basis. Alterations in sarcomere length and the disarrangement of sarcomere components enabled the identification of abnormal muscle areas, and margins of the lesions were delineated on micrographs. Sarcomere lesions were classified into five types. Using a scanner and a computer, the micrographs were analyzed and the percentages of injured areas of each lesion type were separately calculated using NIH Image software. This method of analysis was adopted since it was impossible to quantify the numbers of damaged sarcomeres.

RESULTS

The soleus muscles after treatment contained various types of small focal lesions among normal portions. The type and severity of injuries varied depending on the interval after treatment. Few lysosomes and necrotic fibers appeared in all specimens.
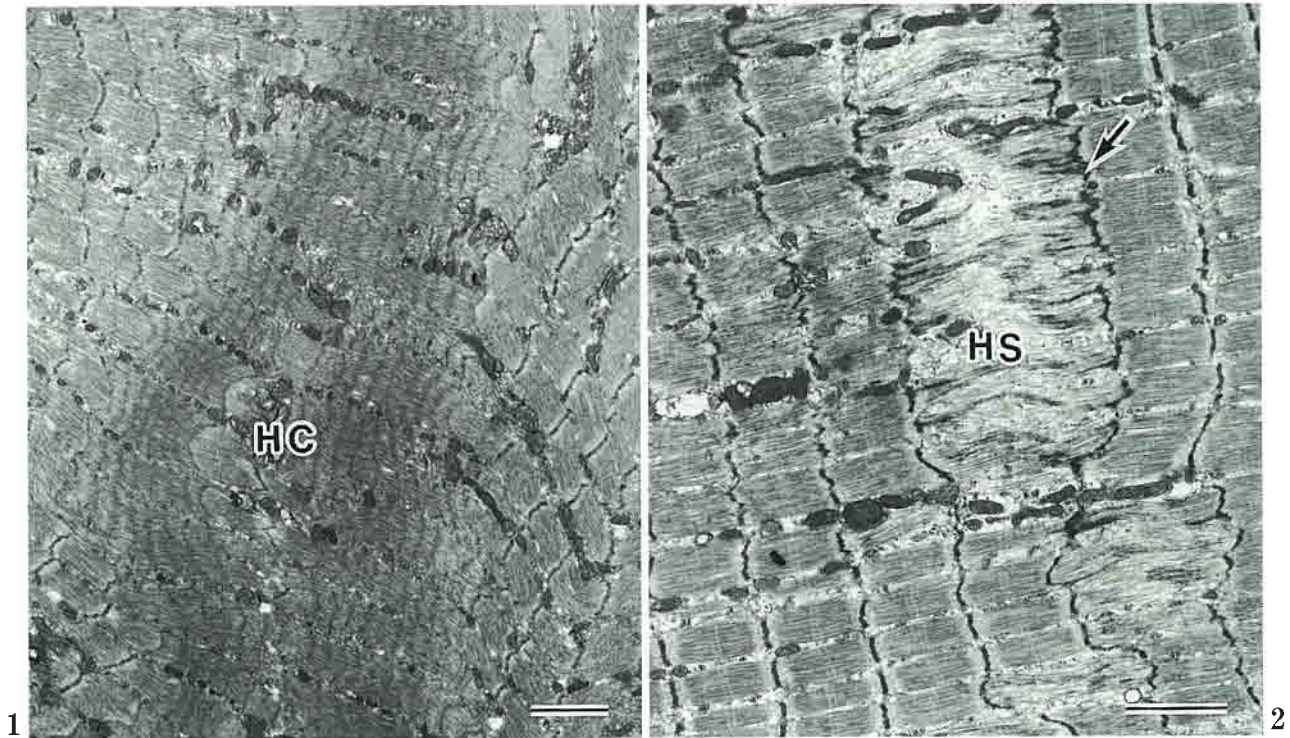
Fig. 1. Electron micrograph of sarcomere hypercontraction (*HC*) seen in the soleus muscle at 0 h after nerve stimulation for 30 min. Sarcomeres are significantly shortened. A and I bands and M lines are not discernible. Bar = 2 μm, ×5,100

Fig. 2. Hyperstretching (*HS*) at 24 h after nerve stimulation. Sarcomeres are considerably lengthened. Z bands are wavy or slightly curved. Z band streaming (*arrow*) is seen. Bar = 2 μm, ×6,700

Types of sarcomere lesions

Sarcomere lesions showed various types of alterations. Some lesions contained more than one type of alteration; however, we were able to classify these lesions into five types based on the following characteristics: 1) the length of the sarcomeres, i.e., shortened or lengthened, 2) the alignment of Z bands, and 3) the organization of myofilaments.

Sarcomere hypercontraction

Sarcomeres were obviously shortened (less than 70% in length compared with surrounding normal-looking sarcomeres). A lesion that showed hypercontraction in combination with other kinds of alterations was also included in this group. Sarcomeres were segmentally hypercontracted and formed bands which usually contained from several to a dozen sarcomeres longitudinally and extended transversely far across muscle fibers (Fig. 1). Myosin filaments invaded the I bands and reached the Z bands. The A and I bands were indistinguishable. The Z bands were wavy or curved and less electron dense. Sarcomere hyper-

contraction was found at 0 h after nerve stimulation.

Sarcomere hyperstretching

Sarcomeres were lengthened (more than 130% compared with surrounding sarcomeres). A lesion that showed hyperstretching in combination with other kinds of alterations was also included in this group. Myofilaments of these sarcomeres were displaced laterally or dispersed. Due to the lateral slippage of myofilaments, the A and I bands were not discernible. The Z bands were wavy or curved to varying degrees, sometimes showing "streaming (wavy, blurred and irregularly broadened Z bands)" (Fig. 2).

Z band disarrangement

Sarcomeres were within the normal range in length (between 70 and 130% of the length of surrounding sarcomeres). However, the Z bands were wavy, often blurred, widened, and sometimes broken down into fragments. They had slipped laterally along the long axis of myofibrils. As a result, mild lateral sliding of myofilaments was observed. The A and I bands were indistinct (Fig. 3). Z bands were usually preserved in
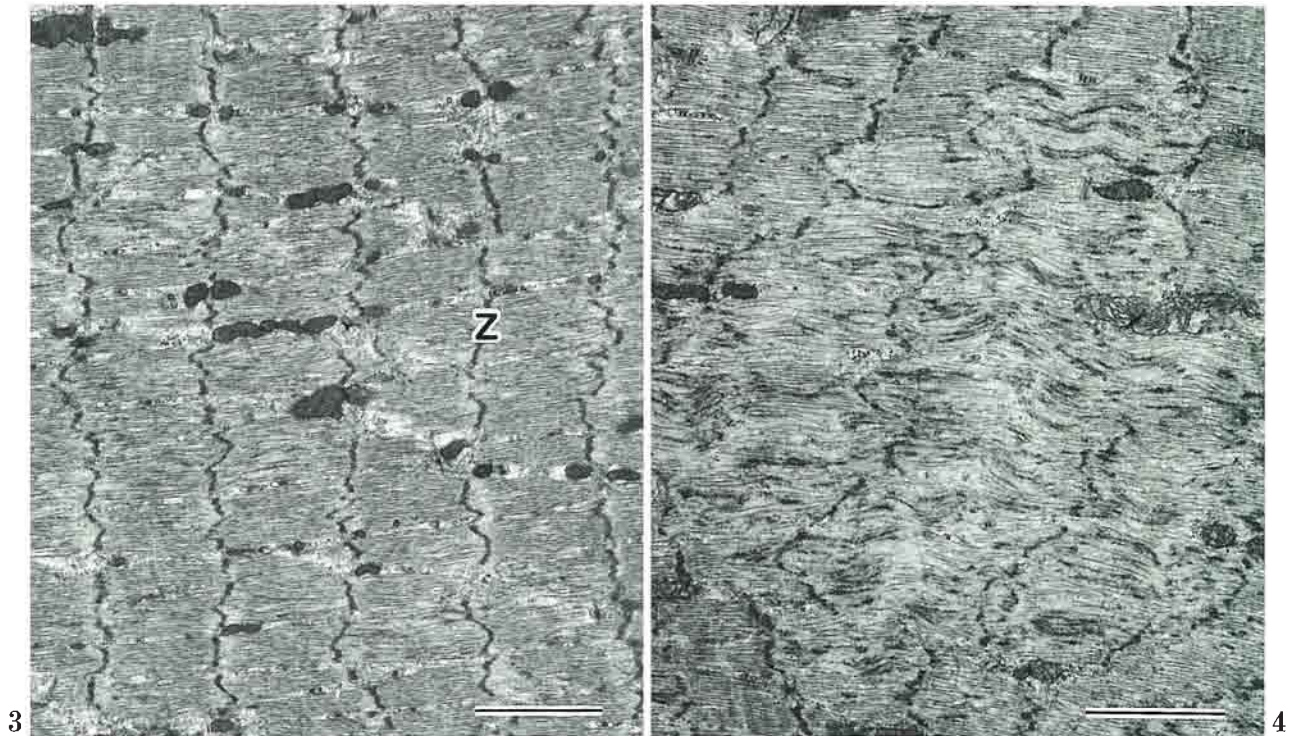
Fig. 3. Z band disarrangement at 6 h after nerve stimulation. The lengths of sarcomeres are within the normal range. Z bands (Z) are wavy and irregular. Myofilaments have slid laterally and are not in register. Bar= 2 μm, ×8,400

Fig. 4. Myofilament disorganization at 12 h after nerve stimulation. Sarcomere structure is severely disrupted. Z bands are frequently interrupted. Bar=2 μm, ×9,100

this type of lesion, whereas they were not in the lesions with myofilament disorganization described below.

Myofilament disorganization

The structure of the sarcomeres was so severely disrupted that it was often difficult to identify each sarcomere. Actin and myosin filaments were highly displaced and the A and I bands were not observable. Myofilaments were irregularly arranged and undulating. The Z bands were usually broken down into fragments or had disappeared (Fig. 4).
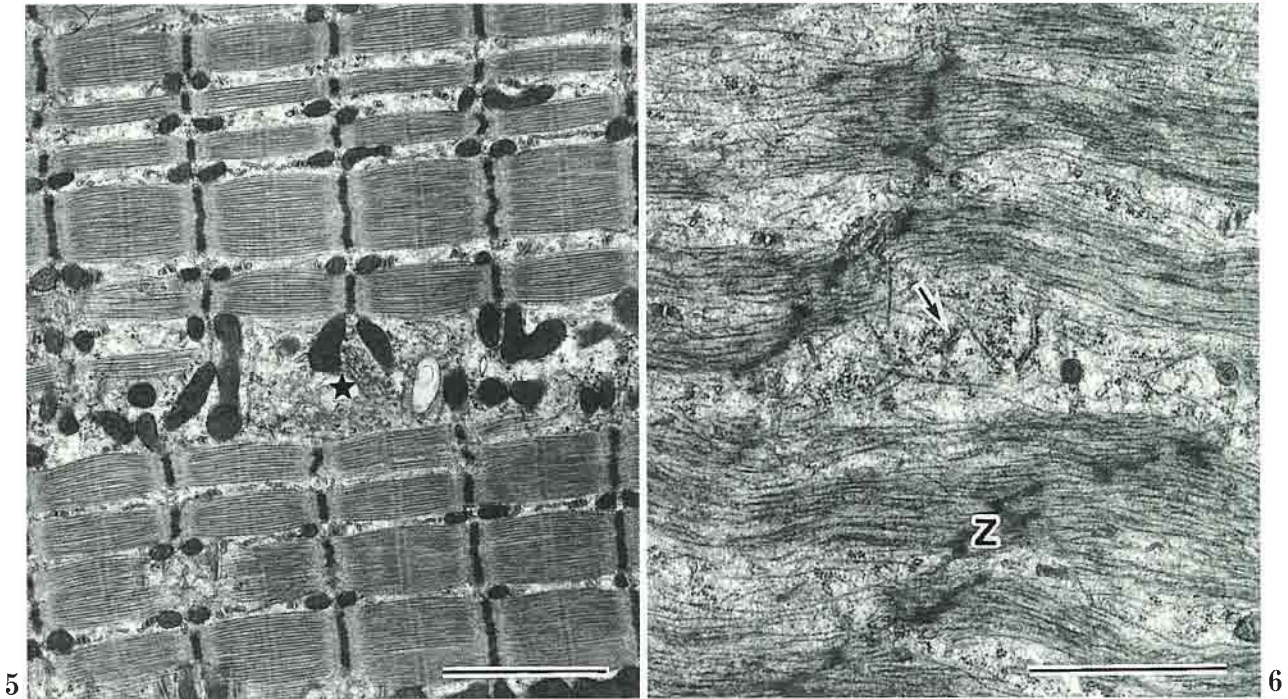
Regions of scarce myofilaments

Myofilaments and Z bands were missing in these regions. Due to the scarcity of myofilaments, mitochondria were easily seen (Fig. 5). These regions were infrequent and sporadic but found in almost all specimens. These regions were also found after eccentric contraction in human quadriceps femoris muscles (HORTOBÁGYI et al., 1998).

Findings after treatments

Immediately after the cessation of stimulation (0 h), $18.8 \pm 15.8\%$ of the soleus muscle contained lesions, most of which were classified as sarcomere hypercontraction ($16.8 \pm 17.8\%$). Z band disarrangement ($1.3 \pm 2.3\%$) and regions of scarce myofilaments ($0.7 \pm 1.2\%$) were also found.

At 6 h, sarcomere lesions constituted $9.7 \pm 1.0\%$ of the muscle sectional areas. The ultrastructural appearance of muscle lesions was markedly different from those at 0 h. Sarcomere hypercontraction was not seen. Sarcomere hyperstretching ($5.2 \pm 0.7\%$) and Z band disarrangement ($4.5 \pm 0.9\%$) were predominant. Hyperstretched sarcomeres were often one sarcomere in length (Fig. 2). They extended transversely across muscle fibers, as in the case of sarcomere hypercontraction.

At 12 h, the percentage of the lesioned area reached a maximum of $22.0 \pm 23.6\%$. Myofilament disorganization was predominant ($20.1 \pm 22.2\%$). Sarcomeres were severely deformed and disassembled. Z bands were undulating or broken down into fragments and

Fig. 5. Regions of scarce myofilaments at 24 h after nerve stimulation. Contractile components of sarcomeres are scarce in some portions (★). Bar=2 μm, ×11,000

Fig. 6. Sarcomere alterations at 12 h after nerve stimulation. Clusters of free ribosomes and rough endoplasmic reticulum (*arrow*) are seen among myofilaments. Z Z bands. Bar=2 μm, ×18,000

dots. Myofilaments were scarce in some regions. Sarcomere hyperstretching was seen in 1.3 ± 1.8% of the muscle area, and sarcomeres were somewhat longer than those at 6 h. In sarcomeres showing hyperstretching and myofilament disorganization, numerous small granules − presumably ribosomes − were found among myofilaments (Fig. 6). Regions nearly lacking myofilaments were also observed 0.6 ± 1.0%.

At 24 h, lesions occupied 13.1 ± 3.2% of the muscle area, with sarcomere hyperstretching in 6.6 ± 5.8%, myofilament disorganization in 4.0 ± 4.2%, Z band disarrangement in 1.9 ± 1.7%, and regions of scarce myofilaments in 0.6 ± 1.0% of the muscle area. The degree of disarrangement of Z bands and myofilaments was less severe than that at 12 h. Numerous ribosomes were found among myofilaments. Myofilaments showed a tendency to align longitudinally.

At 3 days, lesions occupied 4.9 ± 6.0% of the muscle area, with sarcomere hyperstretching in 2.5 ± 4.3%, myofilaments disorganization in 1.5 ± 2.6%, and regions with scarce myofilaments in 0.9 ± 0.8% of the muscle area.

At 7 days after nerve stimulation, regions of scarce myofilaments (0.5 ± 0.4%), but no other types of

lesions, were observed.

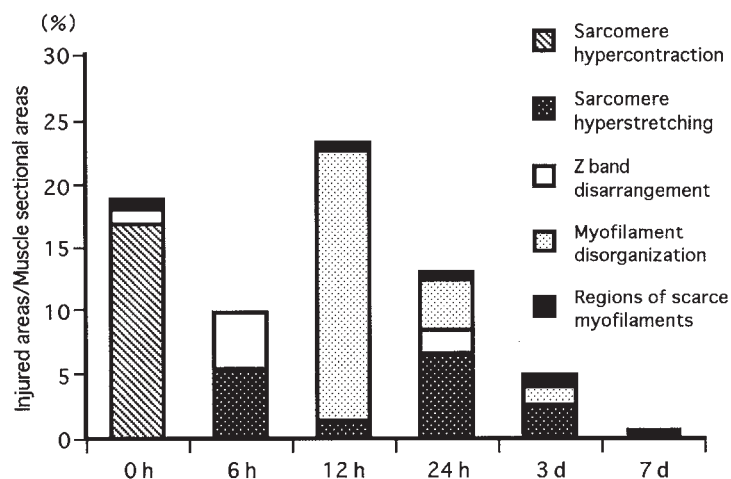The time course of muscle injuries after nerve stimulation is summarized in Figure 7.

Untreated controls

We employed the contralateral soleus muscles of the nerve stimulation group at 0 h as controls (3 rats). These specimens showed a few regions of scarce myofilaments (0.9 ± 0.5%). Other types of lesions were not seen (Fig. 7).

DISCUSSION

Comparison of lesions induced by overexercise and nerve stimulation

The sarcomere lesions demonstrated in our study resembled those seen after rat treadmill exercise (ARMSTRONG et al., 1983; OGILVIE et al., 1988; KOMULAINEN et al., 1994), the reloading of atrophied muscles (RILEY et al., 1992, 1996; KRIPPENDORF and RILEY, 1994) or eccentric contraction (THOMPSON et al., 1999) in rats, or after running down stairs in

Fig. 7. Time course of muscle injuries after nerve stimulation. Types of sarcomere alterations differ significantly depending on the time after treatment. Two peaks are seen at 0 and 12 h after treament. Muscle injuries recovered within 7 days.

human subjects (FRIDZÉN et al., 1981).

OGILVIE et al. (1988) removed rat soleus muscles within 30 min after treadmill running and classified the sarcomere lesions into three types: 1) focal disruptions of the A-band, 2) Z-line dissolution, and 3) clotted fibers. They also found sarcomere hypercontraction. In our study, lesions were classified into five types, including sarcomere hypercontraction. The A-band lesions and Z-line lesions reported by OGILVIE et al. (1988) are considered to correspond to sarcomere hyperstretching, and Z band disarrangement and/or myofilament disorganization in our study, respectively. Clotted fiber lesions (OGILVIE et al., 1988), which seem to consist of necrotic myofibers, were not recognized here, probably because there were few degenerated myofibers in our study. Regions of scarce myofilaments were found after eccentric contraction in human quadriceps femoris muscles (HOR-TOBÁGYI et al., 1998). The agreement of our findings with those of previous studies indicates that the methods we employed are appropriate for studying muscle injury. Furthermore, compared with exercise, electrical nerve stimulation has the advantage of allowing muscle contraction to be accurately controlled.

Time course of sarcomere lesions

In the present study, muscle lesions after nerve stimulation showed two peaks at 0 and 12 h, with a larger affected area at 12 h, but almost disappeared within 7

days. Lesions after overloading are generally reported to increase with time (NEWHAM et al., 1983; FAULKNER et al., 1993; KOMULAINEN et al., 1994, 1998). FRIDÉN et al. (1981) observed delayed muscle soreness during the first 2-3 days after exercise. ARMSTRONG et al. (1983) reported that marker enzymes of muscle injury such as serum creatine kinase and lactic dehydrogenase showed two peaks at 0 h and 1.5-2 days postexercise. Necrotic muscle fibers were demonstrated in other studies (ARM-STRONG et al., 1983; KOMULAINEN et al., 1994, 1998; FRIDÉN and LIEBER, 1998) but not in ours. Recovery from muscle injury may require 7-30 days depending on the severity of the injury (FAULKNER et al., 1993) Thus, the injury was probably mild in our study, resulting in an earlier recovery.

Cause and mechanism of muscle injury

There are several hypotheses about the causes of muscle injury after overexercise. High tensions on muscle fibers during muscle contraction were reported to correlate with muscle injury (FRIDÉN et al., 1981; NEWHAM et al., 1983; GIBALA et al., 1995). On the other hand, FRIDÉN and LIEBER (1992) and LIEBER and FRIDÉN (1999) thought that active strain (strain during active lengthening; LIEBER and FRIDÉN, 1993) is important for inducing muscle injury and stated that passive strain alone does not induce muscle injury. In fact, the high tensions alone resulted in minor muscle injury (unpublished data). ARMSTRONG

et al. (1991) suggested that the degree of injury is proportional to the amount of work done on the muscle.

FRIDÉN and LIEBER (1998) observed fibronectin within muscle fibers after eccentric contractions in rabbits and concluded that fibronectin and extracellular calcium ions entered the muscle fibers. KOMULAINEN et al. (1994) thought that the plasma membranes of muscle fibers in rats ruptured due to an increase of intracellular water in the fibers after treadmill running. KOMULAINEN et al. (1998) suggested that the mechanical damage to the sarcolemma might occur after the destruction of dystrophin and desmin cytoskeleton, which results in an elevation of intracellular calcium ions. The use of a calcium chelating agent such as EDTA which suppresses an elevation of calcium ions attenuates the magnitude of the muscle damage in rats (DUAN et al., 1990).

In the present study, it is unknown whether or not the sarcolemma was damaged. However, the muscle lesions we observed at 0 h after nerve stimulation (sarcomere hypercontraction) resembled the alterations found after the elevation of calcium ions (DUNCAN and JACKSON, 1987). Therefore an elevation of intracellular calcium possibly due to muscle contraction followed by membrane damage may occur at least locally, and trigger the cascade of sarcomere alterations.

Changes in lesions with time

KRIPPENDORF and RILEY (1994) observed different types of lesions at various intervals after loading on atrophied rat adductor longus muscles. THOMPSON et al. (1999) concluded that hypercontraction is transformed into other types of lesions after eccentric contraction. In the present study, the sarcomere lesions differed drastically at 0 and 6 h after nerve stimulation. The possibility that sarcomere hypercontraction at 0 h changes into other types of lesions at 6 h seems more reasonable than the suggestion that hypercontraction disappears and new lesions appear *de novo* from formerly normal-looking areas.

Significance of lesions

The significances of sarcomere hypercontraction, sarcomere hyperstretching, Z band disarrangement and myofilament disorganization remain unclear at present. The last two types of sarcomere alterations in particular have been reported not only after overexercise but also in denervated muscles (SAKAKIMA et al., 2000), muscles of low-protein-fed rats (OUMI et al., 2000) and tenotomized muscles (ABOU SALEM and

ISHIKAWA, 2001; ABOU SALEM et al., 2001). NEWHAM et al. (1983) considered that lesions seen immediately after exercise are the precursors of the more severe damage of myofibers. FRIDÉN and LIEBER (1992) concluded that the disarrangement of sarcomeres is a step on the way to regeneration. RILEY et al. (1992) reported that there are numerous ribosomes in lesions 8-11 h after loading on atrophied rat adductor longus muscles. KRIPPENDORF and RILEY (1994) concluded that Z band-like material is increased at 48 h compared to 0 h after the onset of reloading. They claimed that sarcomere lesions, especially Z band streaming, act as scaffolds for ordering regenerated myofilaments. ABOU SALEM et al. (2001) clearly demonstrated a decrease and recovery of contractile proteins in the tenotomized soleus muscle, which correlates well with ultrastructural alterations. In the present study, total muscle injuries reached a maximum at 12 h and began to diminish thereafter. Numerous free ribosomes and Z band streaming were often recognized at 12 and 24 h, which may reflect repair processes rather than newly formed lesions, but this possibility remains to be proven.

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