Stimulation-induced damage in rabbit fast-twitch skeletal muscles: a quantitative morphological study of the influence of pattern and frequency

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Abstract. The aim of this study was to determine whether muscle fibre degeneration brought about by chronic low-frequency electrical stimulation was related to the pattern and frequency of stimulation. Rabbit fast-twitch muscles, tibialis anterior and extensor digitorum longus, were stimulated for 9 days with pulse trains ranging in frequency from 1.25 Hz to 10 Hz. Histological data from these muscles were analysed with multivariate statistical techniques. At the lower stimulation frequencies there was a significantly lower incidence of degenerating muscle fibres. Fibres that reacted positively with an anti-neonatal antibody were most numerous in the sections that revealed the most degeneration. The dependence on frequency was generally similar for the two muscles, but the extensor digitorum longus muscles showed more degeneration than the tibialis anterior at every frequency. Muscles subjected to 10 Hz intermittent stimulation showed significantly less degeneration than muscles stimulated with 5 Hz continuously, although the aggregate number of impulses delivered was the same. The incidence of degeneration in the extensor digitorum longus muscles stimulated at 1.25 Hz was indistinguishable from that in control, unstimulated muscles; for the tibialis anterior muscles, this was also true for stimulation at 2.5 Hz. We conclude that damage is not an inevitable consequence of electrical stimulation. The influence of pattern and frequency on damage should be taken into account when devising neuromuscular stimulation regimes for clinical use.

Key words: Degeneration - Histochemistry - Immunohistochemistry - Morphometry - Muscle - Stimulation, chronic - Rabbit

Introduction

The fibres of adult mammalian skeletal muscle are highly specialised, yet they retain a capacity for altering their properties in response to changes in use. This plasticity of behaviour is most clearly evident in the response of muscles to chronic electrical stimulation. Fast-twitch, fatigue-susceptible fibres subjected to sustained high levels of use for several weeks develop slow-contracting, fatigue-resistant properties. There is much evidence to show that these changes take place within existing muscle fibres (Salmons and Henriksson 1981; Brown et al. 1989). This interpretation has been further corroborated by a recent quantitative morphological study (Lexell et al. 1992), from which it was concluded that damage is not a prerequisite for fibre type transformation.

The damage that does result from electrical stimulation varies considerably between muscles and individual animals, as well as within a muscle cross-section and along the length of a muscle (Lexell et al. 1992). While its contribution to transformation may not be significant, damage could be important in the clinical application of transformed skeletal muscle grafts (Acker et al. 1987; Williams et al. 1989; Hagege et al. 1990; Salmons and Jarvis 1991a). A better understanding of the aetiology of stimulation-induced damage could help in the design of non-damaging stimulation regimes for clinical use.

Muscles stimulated at 10 Hz intermittently showed less damage than those stimulated at 10 Hz continuously (Lexell et al. 1992), which suggests that the amount of damage is related either to the stimulation frequency or to the aggregate number of impulses received by the muscle. Here we examine these possibilities by comparing the effects of stimulating muscles with different patterns and frequencies. Light-microscopical techniques are combined with a multivariate statistical approach that enables us to assess the influence of the stimulation pattern in the presence of variation between individual animals and different muscles.
Materials and Methods

Stimulation

Twenty-four New Zealand White rabbits of either sex and with body weights 2.5–3.5 kg were used in the study. Each was given a preanaesthetic medication, injected subcutaneously, of diazepam (Roche, Hertfordshire, UK; 5 mg/kg) and atropine sulphate (Bimecosa, Merseyside, UK; 3 mg/kg), followed after 30 min by an intramuscular injection of fentanyl citrate/fluanisone (Janssen Pharmaceuticals, Oxfordshire, UK; 0.3 ml/kg). Under aseptic conditions, a low-profile stimulator was implanted under the skin of the left hind flank and anchored to the abdominal wall by sutures. The leads were passed subcutaneously to a lateral incision in the left hind limb and the electrodes secured close to the common peroneal nerve. Skin incisions were closed and the animal was returned to the approved facility for post-operative recovery.

The implantable stimulators were both of established design (Jarvis and Salmons 1991; Salmons and Jarvis 1991b). Each had an integral power source and a circuit that allowed remote switching by a transcutaneous optical link. The 24 rabbits were divided into 6 groups. In the first group (n = 5), the common peroneal nerve was stimulated at 10 Hz for 24 h/day (‘10 Hz continuous’). In the second group (n = 5), the 10 Hz pattern was delivered for one hour ‘on’ and one hour ‘off’ throughout the day, a total of 12 h/day (‘10 Hz intermittent’). In the third (n = 4), fourth (n = 4) and fifth (n = 4) groups, the common peroneal nerve was stimulated at 5 Hz, 2.5 Hz and 1.25 Hz, respectively, for 24 h/day (‘5 Hz continuous’; ‘2.5 Hz continuous’; ‘1.25 Hz continuous’). All stimulating pulses had an amplitude of 3 V and a pulse duration of 200 μs. Stimulation was initiated at least 2 weeks after the operation and lasted 9 days. Two remaining rabbits were operated, but no stimulation was delivered; these served as operated, non-stimulated controls (‘control’).

For the terminal procedure, all rabbits were deeply anaesthetised by intravenous injection of urethane (Sigma, Dorset, UK; 250 g/l; 500 mg/kg) followed by pentobarbitone sodium (Rhône-Mérieux, Essex, UK; to effect). The tibialis anterior (TA) and extensor digitorum longus (EDL) muscles of the left and right hind limbs were removed, after which the animal was killed by an overdose of pentobarbitone sodium. All animal procedures were performed in accordance with the Animals (Scientific Procedures) Act 1986.

Tissue Processing

Each of the 4 muscles was weighed and cut into 10–12 transverse slices by use of a specially made cutter incorporating equally spaced razor blades. The slices were placed on aluminium foil and frozen in liquid nitrogen, or on cork disks and frozen in isopentane chilled in liquid nitrogen. All slices were sealed in plastic bags and stored below −70°C pending use.

Serial cryosections of 5–10 μm thickness were cut and stained as follows (Dubowitz 1985): haematoxylin and eosin (H & E) and modified Gomori trichrome, for general morphological assessment; Verhoef-van Gieson’s stain, for connective tissue; succinate dehydrogenase, for the integrity and distribution of mitochondria; acid phosphatase, as a marker of activated macrophages and lysosomes; acidine orange, for nucleic acids. Sections were also reacted with a monoclonal antibody (WB-MHCn), raised by Dr. W.E. Brown against rabbit neonatal myosin heavy chain isoform (anti-neonatal), as a marker of newly-formed fibres. Binding of the primary antibody was detected by immunofluorescence of a second antibody, rabbit anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (FITC) (Sigma, Dorset, UK) and the sections were viewed with a Leitz Diaplan microscope fitted for epifluorescent illumination.

Sampling Procedure

All sampling and quantification was done on three H & E-stained sections from each muscle. Sections were prepared from the mid-belly of the muscle, and from slices approximately 20 mm proximal and 20 mm distal to it. A square 1 x 1 mm grid was placed on the mounted section and a graticule with a square subdivided into 100 (10 x 10) smaller squares was placed in the eyepiece of the microscope. With the x16 objective selected, a square of size 1 x 1 mm on the specimen grid corresponded approximately to the whole square in the eyepiece. Every fourth mm² throughout a muscle cross-section, referred to in the following as a ‘sample area’, was selected for measurement. For each section, between 7 and 10 sample areas were used, depending on the size of the whole muscle cross-section.

The square graticule in the eyepiece contained 121 intersections. For each of the selected sample areas, the numbers of intersections falling on normal and degenerating muscle fibres were recorded separately. A fibre was recorded as ‘degenerating’ when it showed one or more of the following features: a pronounced shift from a polygonal to a rounded shape; hyper eosinophilia; invasion of mononuclear cells; hyalinization; vacuolation; disruption of the cytoplasm.

Data and Analyses

Each of the 1372 sample areas was characterised by 5 variables: m number of intersections falling on normal muscle fibres; nd number of intersections falling on degenerating muscle fibres; id the individual rabbit; exp the type of experiment (pattern and frequency of stimulation); m the muscle (TA, EDL).

The ‘degeneration index’, i.e. the estimated volume percentage of degenerating muscle fibres in a sample area, 100 nd/(m + nd) was calculated. This index is easy to record accurately and invariant to changes in the number of fibres within a sample area. In the previous study (Lexell et al. 1992), the degeneration index was found not to vary systematically within an animal, with the sole exception of a variation with length of EDL muscles that were subjected to 10 Hz continuous stimulation. Consequently, the level of the cross-section is not considered here.

Because the degeneration index has some outlying values, and because its distribution is skewed, the analyses were repeated four times: for the values themselves, their ranked values, their square and frequency of stimulation and for the control group were tested pairwise by a t-statistic. The unbalanced analyses of variance (ANOVA) were performed with procedure GLM, which carries out the various F-statistic, were investigated using a multiple comparison method. For each type of muscle, the degeneration indices for each pattern and frequency of stimulation and for the control group were tested pairwise by a t-statistic.

The statistical package SAS (SAS Institute Inc., USA) was used throughout. The unbalanced analyses of variance (ANOVA) were performed with procedure GLM, which carries out the various tests and forms the necessary residual plots. The Logistic procedure was used for the additional analyses.

Results

General morphological assessment

Muscles from the controls and from the undisturbed right hind limbs of the stimulated groups showed poly-