Muscle healing and nerve regeneration in a muscle contusion model in the rat

The nervous system is known to be involved in inflammation and repair. We aimed to determine the effect of physical activity on the healing of a muscle injury and to examine the pattern of innervation. Using a drop-ball technique, a contusion was produced in the gastrocnemius in 20 rats. In ten the limb was immobilised in a plaster cast and the remaining ten had mobilisation on a running wheel. The muscle and the corresponding dorsal-root ganglia were studied by histological and immunohistochemical methods.

In the mobilisation group, there was a significant reduction in lymphocytes (p = 0.016), macrophages (p = 0.008) and myotubules (p = 0.008) between three and 21 days. The formation of myotubules and the density of nerve fibres was significantly higher (both p = 0.016) compared with those in the immobilisation group at three days, while the density of CGRP-positive fibres was significantly lower (p = 0.016) after 21 days.

Mobilisation after contusional injury to the muscle resulted in early and increased formation of myotubules, early nerve regeneration and progressive reduction in inflammation, suggesting that it promoted a better healing response.

Muscle injury is common in sport and accounts for 10% to 55% of all sports-related injuries. Contusions are the most common type of injury to muscle. They result from a blunt, non-penetrating impact of a rigid object on the muscle. The latter is capable of healing, but incomplete functional recovery often occurs and chronic pain can persist.

Jarvinen and Jarvinen and Lehto assessed the healing of a crush injury of striated muscle in the rat and compared the effects of mobilisation and immobilisation. The former was found to be better for healing. Various other factors with a potential to improve healing of muscle have been extensively studied including the role of weight-bearing, satellite cells, growth factors and the molecular adaptation of neuromuscular-disease-associated proteins in response to exercise.

The release of neuropeptides and neurogenic inflammation occur in various tissues, and the beneficial effects of sensory neuropeptides on healing have recently been observed in the eye, skin, tendons and ligaments. Recently, we have shown that mobilisation promotes the healing of rupture of the tendon of Achilles and that this effect was possibly linked to accelerated neuronal plasticity. By contrast, immobilisation showed reduced sensory neuropeptide expression and impaired healing of the tendon.

Sensory neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP), are especially implicated in wound healing. However, the neural component of muscle injury has not yet been described. In our study, we have observed the pattern of the neural response of injury to the gastrocnemius muscle in a rat model and analysed the differences in this response in regard to mobilisation and immobilisation of the muscle.

Materials and Methods

The experiments were performed after approval from the institutional Ethics Committee for Research on Animals. A modified custom-made frame was used for muscle injury using a drop-ball technique (Fig. 1). The rats were anaesthetised with intraperitoneal ketamine (Calypsol, Gedeon Richte, Hungary) at a dose of 75 mg/kg of body-weight. They were placed on the platform of the frame, the right hind leg was shaved and stimulating electrodes were applied on the medial and lateral sides. Muscle contraction was induced by tetanic electrical stimulation, synchronised with the dropping of a metal ball weighing 55 g from a constant height of 135 cm on to the mid-belly of gastrocnemius. A pilot study was initially performed on three rats. After two days the muscles were harvested, sectioned and

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stained. Histological examination confirmed the presence of muscle injury.

For the main study, we used 20 female Sprague-Dawley rats aged between 10 and 12 weeks and weighing between 150 g and 200 g. They were divided into two groups of ten each, one for mobilisation and the other for immobilisation. In each group five rats were allocated for analysis after three days, and five after 21 days. Muscle injury was inflicted on the right hind leg as described above. For immobilisation a plaster-of-Paris cast was applied immediately after the injury. Rats which were mobilised were made to run for one hour every day in a custom-made running-wheel cage and allowed normal activity. Immobilisation of the affected limb in a plaster cast was difficult. One rat was able to slip out of a loose cast and another suffered gangrene of the foot. The experiment was repeated on two fresh animals to adhere to the study protocol.

One group of rats was killed at three days and the other group at 21 days after injury by exsanguination under ketamine anaesthesia. After performing a thoracotomy, the right atrium was incised to initiate exsanguination, then 0.01 M phosphate buffered saline (PBS) was perfused through the ascending aorta followed by fixation with Zamboni’s buffered 4% paraformaldehyde solution containing 0.2% picric acid in 0.2M Sörensen phosphate buffered at pH 7.2. The entire gastrocnemius muscle from the injured leg along with the corresponding dorsal-root ganglia (L4-6) were dissected. There was no control group because we expected to find a major difference between the injured and the normal muscles. The samples were immersed in Zamboni’s solution for 24 hours at 4°C, then transferred to 20% sucrose in 0.1M Sörensen phosphate buffer (pH 7.2), containing sodium azide, for two days.

For the immunohistochemical analysis, samples were embedded in Tissue-Tek OCT compound (Miles Inc, Elkhart, Indiana). The tissues were cut on a cryostat (CM 3050 S; Leica Microsystems, Nussloch, Beirsheim, Germany) into sections 14 μm thick. The frozen sections were mounted directly on SuperFrost Plus slides (Menzel-Glaser, Freiberg, Germany) and stained according to the avidin-biotin complex method for indirect immunofluorescence staining. The tissue sections were incubated with 10% normal goat serum in PBS, then with primary antibodies against SP (1:5000) and CGRP (1:5000; Peninsula Laboratories Europe Ltd, St Helens, United Kingdom) as well as a general neuronal marker, protein gene-product (PGP) 9.5 (1:5000; Ultraclone, Cambridge, United Kingdom), overnight in a humid atmosphere at 4°C. They were then incubated at room temperature with biotinylated polyclonal goat anti-rabbit antibodies (1:250; Vector Laboratories Inc., Burlingham, California), then with streptavidin-labelled fluorochrome Cy3 (1:5000; Amersham Pharmacia Biotech Ltd, Little Chalfont, United Kingdom) and mounted. In order to demonstrate specificity, omission of the primary and/or secondary antibodies and preadsorption of the primary antiserum with an excess of homologous antigen were undertaken.

For microscopy, an epifluorescence microscope (Eclipse E800; Nikon Inc., Yokohama, Japan) with a G-2A (EX-510-560) fluorescence filter, 20× objective and a DXM-1200 digital camera (Nikon Inc.) with the supplied ACT-1 software was used.

In order to assess the immunohistochemical staining, one slide containing two tissue sections of each sample was examined under fluorescence microscopy using 20× objectives. The whole section was screened and the three fields showing the strongest staining were selected. The number of nerve fibres positively stained in each of the three fields was counted manually. The mean of all the readings was then calculated for each rat. For the dorsal-root ganglia only one section from the centre of each ganglion was analysed for each rat because of the small size of the ganglia and relative homogeneity in cell density. One of the authors (RR), experienced in immunofluorescence microscopy, carried out the semiquantitative analyses, without being aware of the group allocation.

For the histological examination, serial sections of muscle were taken at a thickness of 7 μm and stained with haematoxylin and eosin. The analysis entailed the scoring of six parameters, namely the presence of leucocytes, lymphocytes, macrophages, fibroblasts, myofibres and connective tissue, on each section. The first four were scored by counting the cell number in a 20× objective using a scale of 0 to 3 in which 1 was equal to a count of 1 to 4, 2 of 5 to 10 and 3, a count of more than 10 cells. Myofibres and connective tissue were scored based on their
morphological appearance, adapted from previous reports. In brief, the myofibres were scored on a scale from 0 to 4 according to the regeneration of myoblasts, the formation of myotubules and the development of mature myofibres, with a lower score indicating mature muscle. Connective tissue was scored according to the amount of scar tissue (none to abundant). One of the authors (SHH), a pathologist, performed the analysis without prior knowledge of the experimental grouping. The mean value from two sections, superficial and deep, was calculated.

Statistical analysis. Data was analysed using the Statistical Package for Social Sciences, version 13 (SPSS Inc., Chicago, Illinois), and summarised as mean ± SEM for positively stained density of nerve fibre and dorsal root ganglion cells per high power field as well as for histological scores. Bar graphs showing mean and standard errors were used to represent group comparisons. Statistical significance of differences between groups was calculated using a non-parametric test (Mann-Whitney) owing to skewed distribution. A p-value of ≤ 0.05 was considered to be significant. Since only one observer performed the microscopic analysis, there was no interobserver variation. Intra-observer reliability was estimated from scoring based on multiple measurements on different days from the same slide, and came out to be 93%.

Results

Histological findings. Light microscopy of stained sections showed distorted architecture, ruptured and oedematous muscle bundles and dense infiltration of inflammatory cells in the injured muscles (Fig. 2). Image analysis showed that at three days, the number of myofibres was significantly higher (p = 0.016) in the mobilised compared with the immobilised group (Fig. 3), indicating a greater regenerative response. Moreover, between three and 21 days there was a significant reduction in the number of lymphocytes (p = 0.016), macrophages (p = 0.008) and myofibres (p = 0.008) in the mobilisation group (Fig. 3), suggesting a more complete response of repair. However, in the immobilisation group, there was no significant difference in the histological parameters between three and 21 days, except for the myofibres which had declined significantly (p = 0.016) at three weeks, although less than in the mobilisation group (Fig. 3).

Immunohistochemical findings. Nerve fibres immunoreactive to SP, CGRP and PGP 9.5 were seen in the connective tissue surrounding the muscle bundles, and alongside blood vessels as well as in the bodies of nerve cell of the dorsal-root ganglion (Fig. 4). Semiquantitative analysis of nerve fibres in the injured muscles showed that in the immobilisation group, there was no significant difference (p = 0.548, p = 0.690 and p = 0.310 for SP, CGRP and PGP 9.5 respectively) in the nerve fibre density between three days and 21 days (Table I). By contrast, in the mobilisation group, the density of nerve fibres containing CGRP was significantly lower (p = 0.008) at 21 days compared with that at three days (Table I). Furthermore, at three days the density of nerve fibres containing the general neuronal marker PGP 9.5 was significantly higher (p = 0.016) (Table I). Although no significant difference was noted, there was an increase of 40% in the density of nerve fibres positive to both SP and CGRP in the mobilised compared with the immobilised group after three days (p = 0.222 and p = 0.095) (Table I). In the dorsal-root ganglia, there was strong staining for both CGRP and SP in the sensory nerve
cell bodies. Although no significant difference was observed between the third and 21st days or between the immobilised and mobilised groups, the number of CGRP-positive nerve cell bodies appeared to be lower at three weeks in the mobilised compared with the immobilised group (Table I, Fig. 4).

### Discussion

The optimal repair of injured tissues requires many interactions between cells, various mediators and the microenvironment. The understanding of these events is improving, but is much more complex than initially imagined. Muscle injury in particular, has been studied extensively. Experimental evidence is available confirming that early mobilisation after muscle trauma leads to better regeneration of muscle fibres in comparison with immobilisation. Although involvement of a neurogenic component in various injured tissues has been increasingly recognised, the neurogenic response associated with muscle injury has not been described. Our study has shown an increased neuronal response in the early reparative phase of muscle contusion in mobilised compared with immobilised rats. The increased density of nerve fibres coincided with the increased number of myotubules in the early phase after injury in the mobilised group, suggesting that peripheral nerves not only participate in nociception, but also have a potential role in the repair of the injured muscle. The sensory neuropeptides may modulate the expression and effects of local growth factors initiating a better reparative response in the mobilised group.

The sensory nerves implicated in neurogenic inflammation and inflammatory arthritis have cell bodies located in dorsal-root ganglia from where afferents project both centrally to the dorsal horn cells of the spinal cord and peripherally to end-organs. Neurones in the dorsal-root ganglia synthesise and secrete sensory neuropeptides, notably SP and CGRP, which are released through nerve terminals to various target organs. The gastrocnemius muscle where the injury was created, receives its innervation from the dorsal-root ganglia at the lumbar level of L4-6. We analysed the sensory neuropeptides in response to muscle injury at both the site of synthesis in the dorsal-root ganglia and at the site of release in the muscle. The intense staining of cell bodies in the ganglia as well as the nerve regeneration in the injured muscles, especially in the mobilised group, supports increased synthesis and release of sensory neuropeptides as a response to injury.

**Table I.** Details (mean, SEM) of the semiquantitative analysis of nerve fibres in the injured muscle and neuronal cell bodies in the dorsal-root ganglia according to the specific neuronal marker at day 3 and day 21

<table>
<thead>
<tr>
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<th>Muscle (nerve fibres per HPF*)</th>
<th>Dorsal-root ganglia (cell bodies)</th>
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<tbody>
<tr>
<td>Day 3</td>
<td>Immobilised</td>
<td>Mobilised</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>3.0 (0.2)</td>
<td>4.5 (0.4)</td>
</tr>
<tr>
<td>CGRP</td>
<td>2.5 (0.4)</td>
<td>3.5 (0.4)</td>
</tr>
<tr>
<td>SP</td>
<td>1.7 (0.2)</td>
<td>2.4 (0.3)</td>
</tr>
<tr>
<td>Day 21</td>
<td>Immobilised</td>
<td>Mobilised</td>
</tr>
<tr>
<td>CGRP</td>
<td>27.9 (7.0)</td>
<td>29.0 (3.5)</td>
</tr>
<tr>
<td>SP</td>
<td>19.1 (3.1)</td>
<td>17.4 (2.2)</td>
</tr>
</tbody>
</table>

* high-power field
† p = 0.016, mobilised vs immobilised group for respective day according to the Mann-Whitney test
‡ p = 0.008, day 3 vs day 21 for the respective mobility group according to the Mann-Whitney test
loose prosthetic implants and in skeletal muscles after eccentric exercise.\textsuperscript{18,38,39} Our study highlighted a neural response in muscle contusions. Such injuries are commonly seen in orthopaedic practice related to sports injuries. The mechanism of injury used in our study simulated the clinical picture since there was a synchronised muscle contraction at the time of injury. Furthermore, histological and neuronal assessment was carried out at three and 21 days, consistent with the healing phases after the injury. Our results suggest that early mobilisation favourably affects the healing of injured muscle. Mobilisation resulted in greater early formation of myotubules and their subsequent conversion to mature muscle cells. In the mobilisation group an increased expression of nerve fibres in general (PGP 9.5) as well as sensory nerve fibres (SP, CGRP) in particular was observed after three days and a significant reduction in CGRP-positive fibres from three to 21 days.

Our results suggest that the improved muscle regeneration with early mobilisation is associated with an initial augmented neuronal response followed by a reduction of inflammation and expression of sensory neuropeptide in the later phase of healing.

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Immunochemical photomicrographs showing CGRP-positive nerve fibres (a, b) in muscles and (c, d) nerve-cell bodies in the dorsal-root ganglia from (a, c) the immobilised and (b, d) mobilised groups. Positively stained nerve fibres and cell bodies are seen (bar = 50 \(\mu\)m, objective \(\times 20\).)
References


