THE ANATOMICAL RECORD 235:374–380 (1993)

Promotion of Muscle Regeneration in the Toad (*Bufo viridis*) Gastrocnemius Muscle by Low-Energy Laser Irradiation

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ABSTRACT **The effect of low-energy laser (He-Ne) irradiation on the process of skeletal muscle regeneration after cold injury to the gastrocnemius muscle of the toad (*Bufo viridis*) was studied using quantitative histological and morphometric methods. The injured zones in the experimental toads were subjected to five direct He-Ne laser (632.8 nm wavelength) irradiations (6.0 mW for 2.3 min) every alternate day starting on the fourth day postinjury. Muscles that were injured as above, and subjected to red-light irradiation, served as a control group. Morphometric analysis was performed on histological sections of injured areas at 9, 14, and 30 days postinjury. At 9 days postinjury, mononucleated cells populated 69.3% ± 16.8% of the total area of injury. Thereafter, their volume fraction (percent of total injured zone) decreased gradually but more rapidly in the laser-irradiated muscle than in the control. The volume fraction of the myotubes in the laser-irradiated muscles at 9 days of muscle regeneration was significantly higher (7.0% ± 2.2%) than in the control muscle (1.2% ± 0.4%). Young myofibers in the laser-irradiated muscles populated 15.5% ± 7.9% and 65.0% ± 9.5% of the injured area at 9 and 14 days of muscle regeneration, respectively, while in control muscles these structures were not evident at 9 days and made up only 5.3% ± 2.9% of the traumatized area at 14 days postinjury. The volume fraction of the young myofibers further increased by 30 days of muscle regeneration making up 75.7% ± 13.2% of the traumatized area, while in the laser-irradiated muscles most of the injured zone was filled with mature muscle fibers. It is concluded that He-Ne laser irradiation during the regeneration process markedly promotes muscle maturation in the injured zone following cold injury to the toad gastrocnemius muscle.**

Key words: Amphibia, Injury, Morphometry, Promotion, Skeletal muscle

The process of skeletal muscle regeneration following injury in mammals has been well-documented and reviewed (Allbrook, 1981; Carlson and Faulkner, 1983; Plaghki, 1985). Skeletal muscle regeneration has been studied to a much lesser extent in amphibians. While in mammals proliferating satellite cells give rise to newly formed regenerating muscle fibers at the injured site, for amphibians it has been suggested that satellite cells are absent in normal muscles (Hay, 1970). However, it was proposed that the precursor cells for regeneration are derived, at least in part, from dedifferentiating muscle fibers by a process of syncytium fragmentation. These stem cells further fuse to form myotubes with long nuclear chains at ~2 weeks after mincing injury and then further differentiate to form young myofibers with cross striation and peripheral nuclei at ~1 month after injury (Carlson, 1970a,b; Trupin, 1979). The possible regulatory mechanisms associated with the process of muscle regeneration, and the stimulation of the dormant satellite cells following injury, have, to some extent, been investigated in mammals (Bischoff, 1986a,b, 1990; White and Esser, 1989), while in amphibians factors that may affect the regeneration process have not yet been studied.

Low-energy laser irradiation has recently been found to modulate various processes in different biological systems (Belkin et al., 1988; Karu, 1989). For example, in isolated mitochondria, He-Ne laser irradiation (5 J/cm^2) caused the elevation of membrane potential and production of ATP (Passarella et al., 1984), while, in isolated fibroblasts, with the same irradiation, an increase in collagen production was observed (Kovacs et al., 1974). The effect of low-energy laser irradiation on regeneration processes following trauma has, thus far, been investigated in the skin and central and peripheral nervous systems. He-Ne laser irradiation has been shown to accelerate the process of cutaneous wound healing and growth of blood vessels in the wound re-

Received February 18, 1992; accepted June 10, 1992.

gion (Mester et al., 1973; Belkin et al., 1988). Laser irradiation of a crushed sciatic nerve in rats caused a significant increase in the amplitude of the compound action potential of the corresponding gastrocnemius nerve and diminished neural scar tissue formation (Rochkind et al., 1987). Following crushing of the optic nerve of rabbits, He-Ne laser irradiation maintained its morphological integrity and viability (Schwartz et al., 1987). In recent experiments, it was found that irradiation (10.5 mW) of He-Ne laser for 2 weeks slowed down Wallerian degeneration of the optic nerve following crush injury as was manifested morphologically and physiologically (Schwartz et al., 1987; Assia et al., 1989). It was concluded that, in the case of trauma to the nervous system, laser irradiation probably acts to prevent or delay the degeneration processes rather than to promote regeneration.

The regulatory mechanism of low-energy laser is not yet clearly understood. It is photochemical in nature; the energy is probably absorbed in intracellular chromophores and converted to metabolic energy, most likely by involving the respiratory (cytochrome) chain (Belkin et al., 1988; Karu, 1989).

It was previously proposed that photosensitivity of cells is not an all-or-nothing phenomenon but depends on the physiological state of the cells before radiation to various degrees. It was, for example, impossible to stimulate growth of microorganisms by laser irradiation in the summer, while during the winter the irradiation had an enhancing effect on the growth rate of the microorganisms. In the case of mammalian cellular culture after irradiation with an He-Ne laser, the growth rate of slow-growing subpopulations was also increased but not the growth of fast-growing ones (Karu, 1989).

The possible effects of low-energy laser on muscular tissue or regenerative processes in this tissue in amphibians have not been investigated previously. It was, therefore, of interest to follow-up the effect of laser irradiation on the process of muscle regeneration in particular in amphibians that have a low metabolism relative to mammals. Part of this work was previously published as an abstract (Bibikova and Oron, 1991).

MATERIALS AND METHODS

Experimental Procedure

Forty-nine male toads (*Bufo viridis*), weighing 30–35 g, were used for the experiments. The toads were maintained for at least 1 month in captivity before the experiments. They were kept in large containers (80 × 40 × 50 cm) with flowing water; temperature range was 20–25°C under a 12 hr light-12 hr dark lighting regime, and they were fed mealworms twice weekly. At least 1 week before the injury, and throughout the experiment, they were kept in small containers (30 × 20 × 20 cm) at 19°C ± 1°C under the same light-dark conditions.

The toads were anesthetized for surgery and laser irradiations by immersing them in a 0.15% w/v solution of Tricain methan sulphonate (Sigma, St. Louis, MO) in tap water for ~ 5 min. Surgery was performed under sterile conditions. The gastrocnemius muscle was exposed after a longitudinal incision was made in the skin, and the cold injury was performed by placing the end of a copper rod (1.8 mm diameter), prechilled in liquid nitrogen, against the muscle for 10 sec. All injuries were made at the same location (lateral and central) of the gastrocnemius muscle. In preliminary studies, it was found that all muscle fibers in the injured zone were in an initial degenerative state at the second day postinjury. The skin was then sutured, and the toads were thoroughly washed in tap water and were awake within 1 hr after surgery. They were then immersed in tap water containing 1.4 mg/liter Gentamycin (Teva, Israel) for 1 hr. Five or six injured experimental (laser-irradiated) and control (injured and irradiated with red light) toads were used for each time interval (9, 14 and 30 days) postinjury. In the experimental group, the injured zone was irradiated five times every alternate day for 2.3 min (31.2 J/cm^2) starting on day 4 postinjury. Laser irradiation was performed directly on the injured area of the exposed gastrocnemius muscle, following removal of sutures in the skin. Since the injured zone was larger than the laser beam diameter, two adjacent irradiations (for 2.3 min each) were applied to ensure irradiation of the total traumatized area. The irradiation did not cause elevation of the temperature in the injured zone, as measured by a special probe (0.3 mm diameter), which had been implanted in the muscle prior to irradiation. Following each laser irradiation, the toads were treated with antibiotics as described above. The control group underwent the same experimental procedure as that of the experimental toads but were subjected to red-light irradiation (660 nm) instead of laser irradiation. The laser used was an Ealing Electro-Optics (Holliston, MA) He-Ne laser (632.8 nm, 6.0 mW) with a 1.9 mm beam diameter.

Light Microscopy

At 9, 14, and 30 days postinjury, the toads were lightly anesthetized with chloroform, and the gastrocnemius muscle was exposed and removed in cross sections at ~ 3 mm from its proximal and distal ends. The toads were then sacrificed by an overdose of chloroform. The muscles were fixed in Bouin's fixative for 3 days, dehydrated in alcohol, and embedded in paraffin. Eight-micrometer serial longitudinal sections were prepared and stained with hematoxylin-eosin and Masson's trichrome stain.

Morphometric Analysis and Statistics

In preliminary experiments, it was observed that the various histological structures to be analyzed (mononucleated cells, myotubes, young and degenerated myofibers) were not randomly distributed throughout the entire injured zone (Fig. 1a). It was, therefore, decided that the sample units for morphometric measurements in this particular case would be the *entire* injured zone, rather than random-area samples within the injured area. The borderline of the injured area could be clearly identified by comparison between the low density of myotubes and young myofibers structures with central nuclei relative to mature packed muscle fibers (see Fig. 1). The morphometric analysis was carried out on photographs of the entire injured zone at a final magnification of ×120, as demonstrated in Figure 1, using the point-counting method (Weibel, 1979, 1980; Mathieu et al., 1981). The volume fraction (percentage of the total volume of injured zone) was calculated for each of the

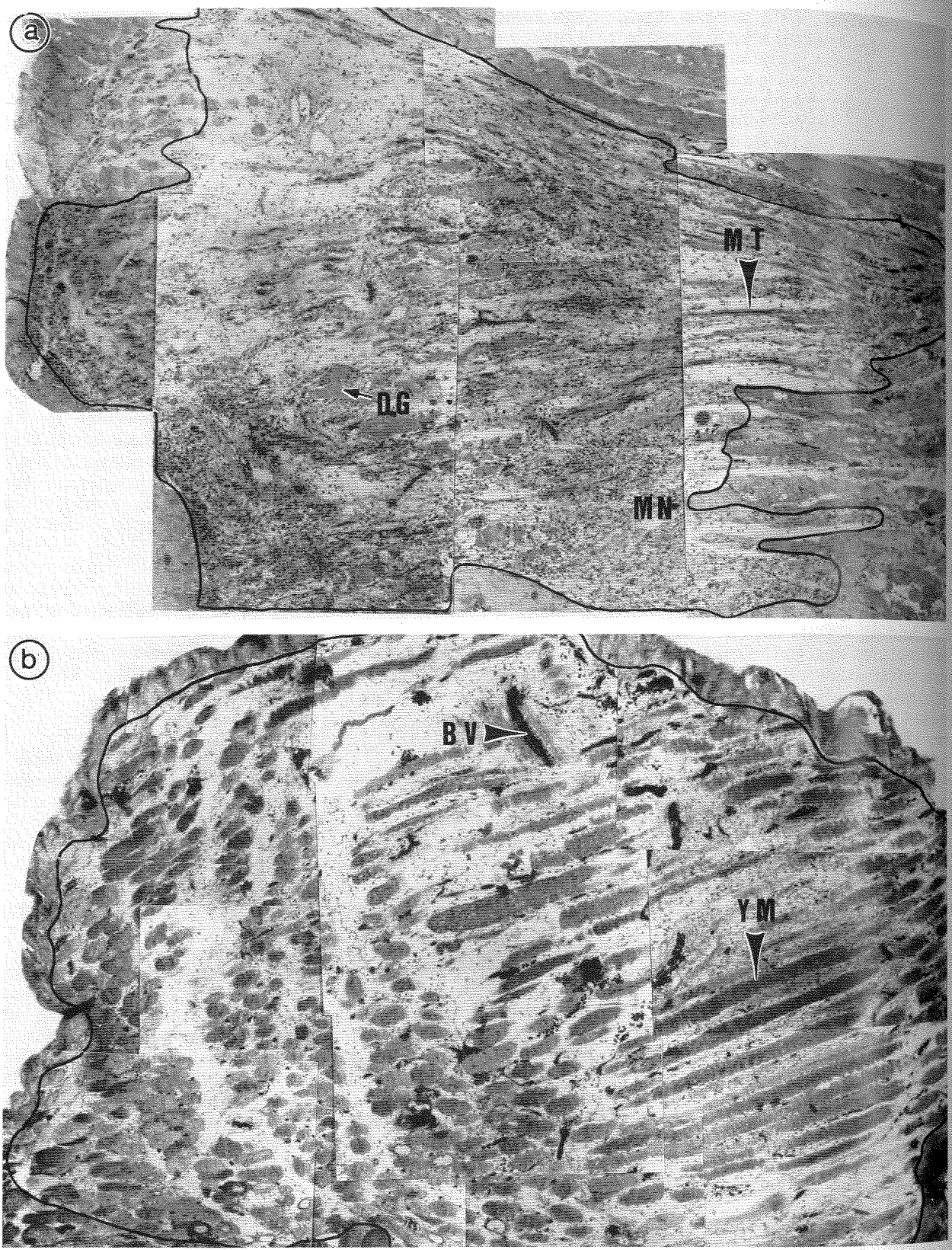

Fig. 1. Light micrographs of the entire injured zone of control (**a**) and experimental (**b**) gastrocnemius muscle of toads 14 days after cold injury. Each figure is a combination (panorama) of several zones in the injured area as described in Materials and Methods. Note degenerated muscle fibers (DG), mononucleated cells (MN), and myotubes (MT) in injured area of the control muscle. The injured zone in the experimental muscle is characterized by few myotubes and numerous young myofibers (YM) that were sectioned in longitudinal and oblique direction. The solid line represents the border of the injured zone. BV, blood vessels. a was stained with H&E and b with Masson's trichrome. ×60.

structures analyzed. The structures were usually further identified by correlation between the photographs and viewing of the same section on the microscopic slide in the microscope at a high magnification ($\times 400$) (see Results).

Fifteen to 20 microscopic slides containing ~ 10 serial sections on each slide were obtained from the entire injured zone of each muscle. Three-level nested ANOVAs (Sokal and Rohlf, 1981) were carried out on morphometric results. This was done to determine the number of slides, and the number of sections for each slide, to be taken from each muscle according to the magnitude of variability of the structures within the sections on each slide, the slides, the animals, and the control/treated group. According to this test, six injured zones (three slides and two sections per each slide) were randomly chosen and photographed for each muscle. Morphometric analysis was performed on three control muscles (toads) and three laser-irradiated muscles. The results of the three-level nested ANOVA revealed variance components (in percentage) as follows: 7% between sections within the same microscopic slide, 6% among slides taken from the same muscle, 6% among the muscles (toads) of the same group (control or experimental), and 81% between control and experimental muscle. According to this test, four injured zones (from two microscopic slides, two sections per slide) were randomly chosen, photographed and analyzed for each muscle. The results were finally statistically analyzed using the three-level nested ANOVA (Sokal and Rohlf, 1981).

RESULTS

The process of muscle regeneration in control muscles following cold injury was characterized by sequential changes in the volume fraction of typical structures such as mononucleated cells, myotubes, and young myofibers in the injured area (Figs. 1–3). At 9 days postinjury, mononucleated cells filled 69.3% ± 16.8% (mean ± SE throughout) of the injured zone. Macrophages and leukocytes were also evident and were included within the category of mononucleated cells. The rest of the traumatized area was occupied by degenerated muscle fibers (10.2% ± 3.2%), blood cells and vessels, and a minimal amount of myotubes (Figs. 2a, 3). Two weeks following injury, there was a further reduction in the volume fraction of the mononucleated cells. Macrophages and leukocytes were still evident in the traumatized area associated with the appearance of degenerated fibers that populated 14.5% ± 3.3% of the area (Fig. 1a). Progressive and significant ($P < 0.05$) increase with time after injury was noticed in the area populated by myotubes and young myofibers. The myotubes, which were characterized by long nuclear chains that occupied almost the entire width, made up 1.2% ± 0.4% and 9.1% ± 2.2% at 9 and 14 days, respectively, and were absent in 30 day muscle regenerate (Figs. 2a,c,e, 3). The young myofibers, characterized by several parallel nuclear chains and a more conspicuous cytoplasm, were not evident in the regenerated zones at 9 days postinjury, but after cold injury populated 5.3% ± 2.9% and 75.7% ± 13.2% of the traumatized area at 14 and 30 days, respectively.

The laser irradiation during the process of muscle regeneration caused a significant change in the volume fraction of the various structures in the traumatized area at all time intervals following injury (Figs. 1–3). The volume fraction occupied by the mononucleated cells was not significantly different from control in laser-irradiated muscles 9 days after injury, but at 14 days made up 21.8% ± 6.2% in the experimental muscles. This value was significantly ($P < 0.01$) lower than in control muscles. At 30 days of muscle regeneration in control muscles the mononucleated cells still occupied 15.6% ± 3.1% of the traumatized area, while in the laser-treated muscle they were absent.

At 9 and 14 days of muscle regeneration, there was a three- and a tenfold reduction, respectively, in the volume fraction of the degenerated fibers in the injured area of the laser-irradiated muscles compared to controls (Figs. 2a–d, 3). While there was a gradual increase with time after injury in the area occupied by the myotubes in the control muscle, this structure was conspicuous in the experimental muscles only at 9 days postinjury, making up 7.0% ± 2.2% of the traumatized area, and thereafter myotubes were not detected (Figs. 1–3). Young myofibers populated 15.5% ± 7.9% and 65.0% ± 9.5% of the injured zone in the laser-treated muscles at 9 and 14 days of muscle regeneration, respectively, while in control muscle regenerates they were not evident at 9 days postinjury, and made up only 5.3% ± 2.9% of the area at 14 days. The process of muscle regeneration was almost completed after 30 days in the laser-treated muscles (Figs. 2f, 3), while in control muscles young myofibers still populated a large part (75.7% ± 13.2%) of the injured zone (Figs. 2e, 3).

DISCUSSION

The quantitative morphometric data on the sequential histological events during skeletal muscle regeneration presented in this paper provide some additional information on the process of muscle regeneration in amphibians. Degeneration of injured muscle fibers proceeds for at least 2 weeks after cold injury. Indeed, these fibers made up ~ 12% of the total injured area during this period, and macrophages and leukocytes, which are associated with inflammatory processes, were evident in the injured zone even at 14 days posttrauma. The inflammatory response after injury to skeletal muscles in amphibians (at 19°C) is thus far more prolonged than during regeneration in mammals. Furthermore, myotube formation commences while the inflammation process and degeneration are still in progress, unlike regeneration in mammals. The mononucleated cells continue to populate about one-half of the injured area even at 14 days postinjury, although with time there is a gradual decrease in their content. The fusion process of these cells to form myotubes is not confined to a certain period after excision but continues throughout the first 2 weeks of the regeneration process; their volume fraction was found to increase significantly and gradually with time, exceeding 9.1% of the total area of the injured zone at 14 days of muscle regeneration. Some of the myotubes further matured to form young myofibers, a process that took place mainly during the third and fourth weeks of the regeneration process. Regarding the overall kinetics of regeneration, this study corroborates previous studies on muscle regeneration in amphibians (Carlson, 1970a,b; Trupin, 1979).

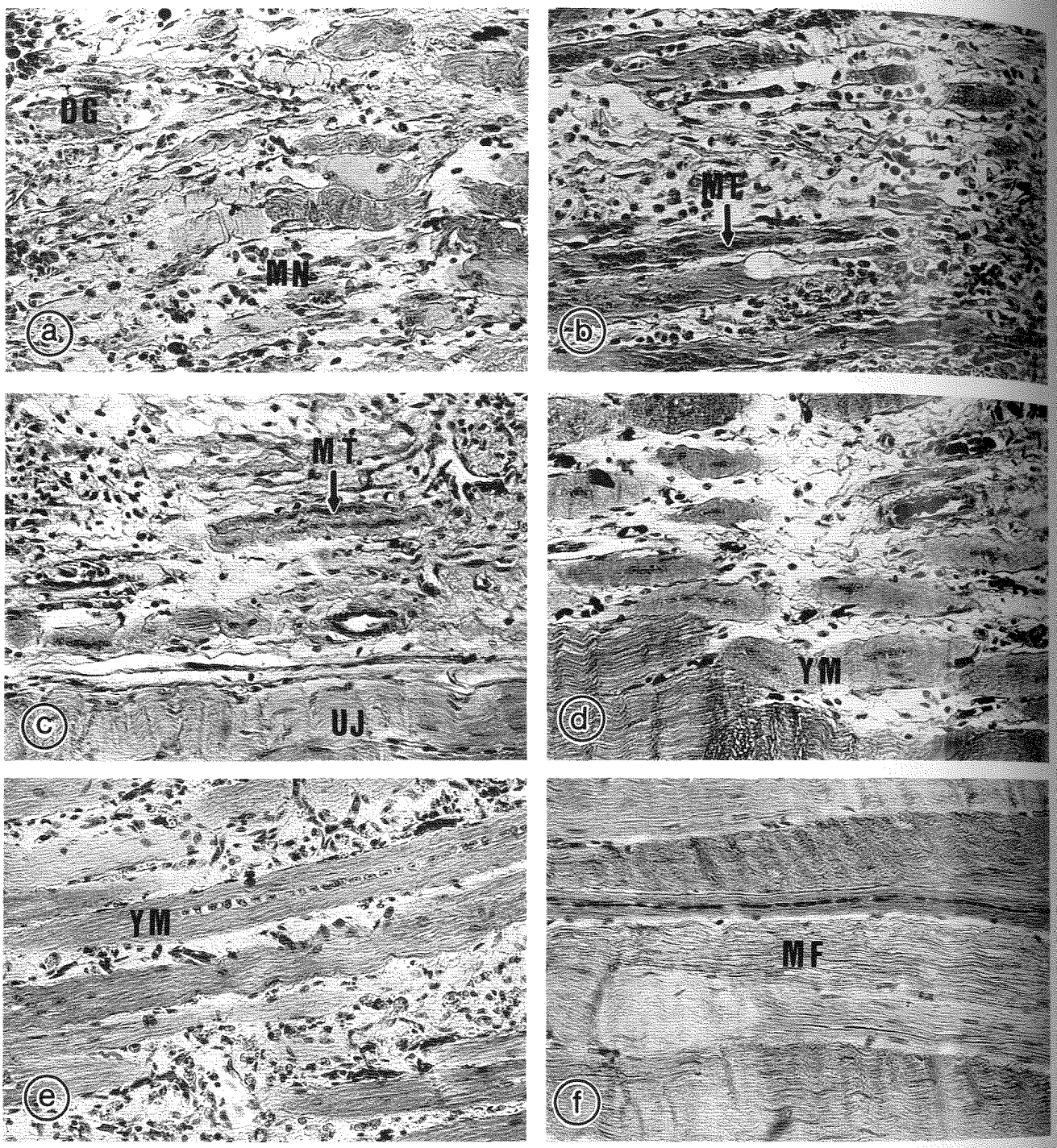

Fig. 2. Light micrographs of typical injured zones at 9 days **(a,b),** 14 days **(c,d),** and 30 days **(e,f)** after cold injury of control (a,c,e) and experimental (b,d,f) muscles. In a, the area is populated mainly by mononucleated cells (MN) and degenerated fibers (DG), while at the same time in the laser-irradiated muscle (b) myotubes (MT) are already conspicuous in the injured zone. At 14 days postinjury (c), myotubes (MT) populate the injured zone, while young myofibers (YM), some with parallel central nuclear chains, fill to a large extent the traumatized area of the experimental muscle (d). Note mononucleated cells and young myofibers (YM) in the 30 days control muscle regenerate, while in the experimental muscle at the same time (f) the fibers have a complete "mature" appearance (MF). The myofibers with the central nuclear chain in f show that this micrograph is from a previously injured zone and not an uninjured zone in the muscle. UJ, uninjured mature fibers. a and b were stained with H&E and c–f with Masson's trichrome. ×200.

The results of this study clearly indicate that degenerative and regenerative processes follow cold injury to the toad's gastrocnemius muscle and are affected by low-energy irradiation. The fact that the degenerative muscle fibers made up only 1.5% of the injured area in the laser-irradiated muscles, compared to 14.5% in the control at 14 days postinjury, indicated a marked promotion of the degeneration processes by laser irradia-

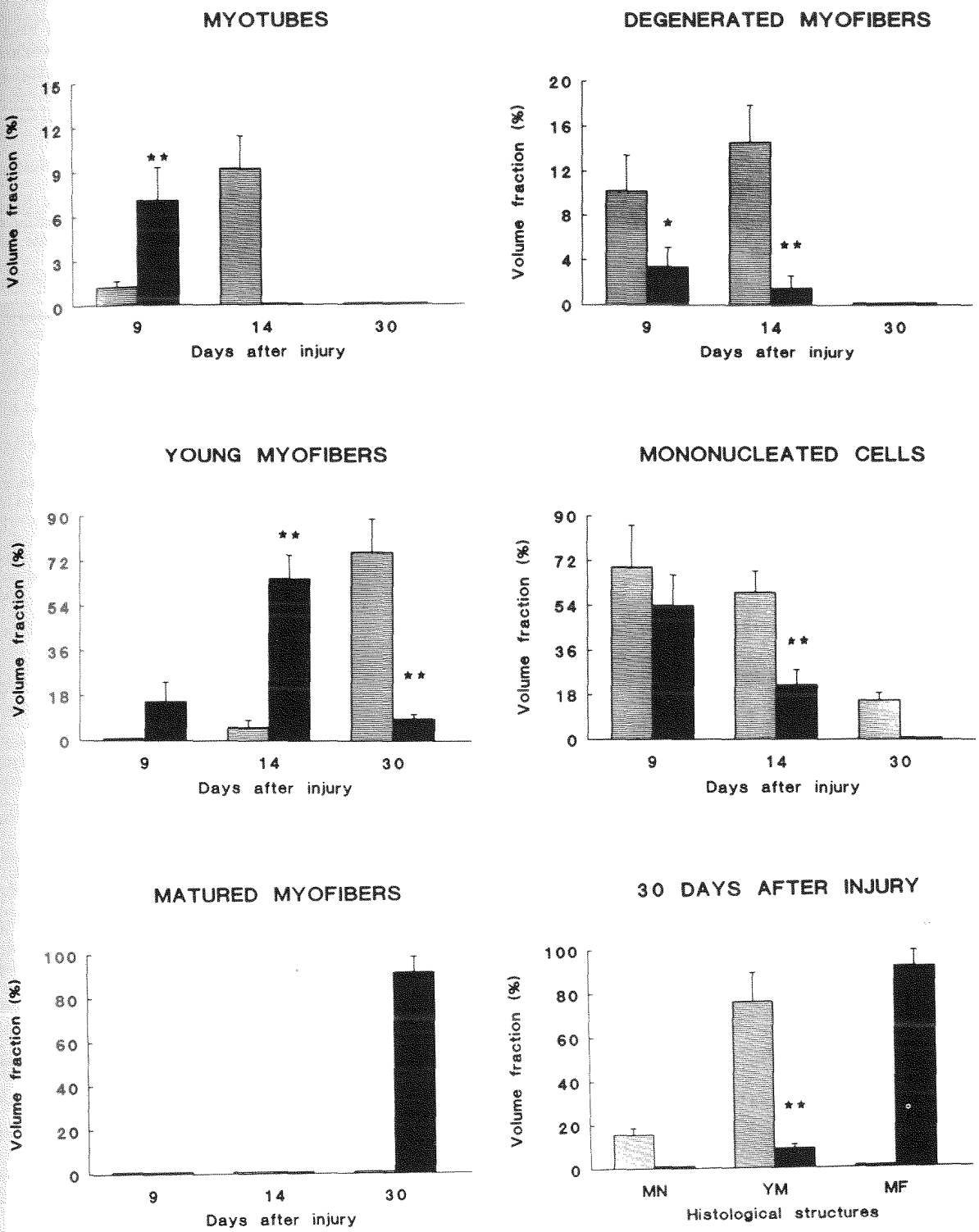

Fig. 3. Histograms of the volume fraction of the various histological structures in the injured zones of control (shaded columns) and laser-irradiated (solid columns) muscles as a function of time after cold injury. The results are mean ± SE from five or six toads at 9 and 30 days. At 14 days postinjury, they are mean ± SE of 10–14 toads in two experiments performed in the winter and the summer. The histogram at the right bottom corner summarizes the 30-day muscle regeneration in control and experimental muscles. MN, mononucleated cells; YM, young myofibers; MF, mature fibers. The levels of statistical difference from control group were $^{*}P < 0.05$ or $^{**}P < 0.01$.

tion. It may be postulated that the laser irradiation that took place during the existence of macrophages and leukocytes in the injured zone may have activated them and/or promoted the division of these cells, which in turn attenuated the degenerative processes. It was previously shown by Assia et al. (1989) that laser irradiation acts to delay degenerative processes following crush injury to the rat optic nerve. Those results do not necessarily contradict the results of the present study, since tissue reaction and the characteristics of cells as-

sociated with degeneration in the nervous system are probably entirely different from those in muscular tissue. In addition, the differences in energy, schedule, and number of laser irradiations between the present study and that of Assia et al. (1989) may show different attenuation of the degenerative processes by the laser irradiation (Belkin et al., 1988; Karu, 1989).

The process of muscle regeneration is also markedly promoted when the injured area is exposed to He-Ne laser irradiation during the regeneration process. Throughout this process, the volume fraction of the structures that are indicative of muscle maturation in the traumatized area, were higher in the laser-treated toads than in the control. Already at 9 days postinjury, the volume fraction of the myotubes was sixfold higher in the experimental toads than in the controls, and young myofibers made up 15.5% of the area of injury in laser-irradiated muscles as opposed to none in the control muscles. It is possible that the laser irradiation stimulated differentiation and/or proliferation of stem cells to myoblasts and/or the fusion of the latter to form myotubes. Furthermore, the laser irradiation markedly augmented the maturation of the myotubes to young myofibers, as indicated by the fact that they populated 15.5% of the injured area in experimental muscles at 9 days postinjury as opposed to zero in the controls. This trend of marked promotion of muscle regeneration proceeded further at 2 weeks postinjury, when the volume fraction of young myofibers was 4.3-fold higher in the experimental muscles than in the controls. Furthermore, after 1 month of muscle regeneration, regeneration is almost completed in experimental muscles, while in control muscles young myofibers made up 75.7% of the regenerated area.

To the best of our knowledge, the present study is the first to report on the effect of low-energy laser irradiation in lower vertebrates. Since the precise mode of action of the low-energy irradiation on cells and tissues is not yet clearly understood, it is difficult to interpret its dramatic effect on the process of muscle regeneration in amphibians solely in the light of the low metabolism of their cells, which may be more permissive to the laser effect (Karu, 1989). However, it may be postulated that if the light energy is converted to metabolic energy, as was previously suggested (Belkin et al., 1988; Karu, 1989), this energy may promote various processes (i.e., fusion, contractive protein synthesis, neovascularization) associated with the regeneration process. Furthermore, it is tempting to propose that the laser irradiation may also act as a trigger for intracellular processes that attenuate the physiology of the cells, even far beyond the period of actual irradiation, as shown in the present study. This mode of action differs from the temporary effect of laser irradiation in the case of posttraumatic processes in the optic nerve (Assia et al., 1989). The mode of action of low-energy laser irradiation in regulating degenerative and regenerative processes, in general, and in skeletal muscle, in particular, should be a subject of further study.

ACKNOWLEDGMENTS

We thank Mrs. L. Maltz for technical assistance, Prof. D. Wool for help with the statistical analysis, Prof. E. Kochva for valuable discussion, and Mrs. C. Shapiro for typing the manuscript. This work was supported in part by the Ministry of Absorption Research Fund, Israel.

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