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Pattern of skeletal muscle regeneration after reautotransplantation of regenerated muscle

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SUMMARY

Autotransplantation of rat extensor digitorum longus muscle results in initial myofibre degeneration and subsequent regeneration from precursor myosatellite cells. To determine what effect a reinjury would have on the regenerative response, in the present study, once transplanted and regenerated muscles were reinjured by reautotransplantion. In rats, four weeks after initial transplantation, when the regeneration was complete, the extensor digitorum longus muscle was transplanted again and the pattern of regeneration in reautotransplanted and once autotransplanted muscles was compared. Muscles were analysed 2, 4, 7, 14 and 30 days after autotransplantation and reautotransplantation. Both autotransplanted and reautotransplanted muscles underwent degeneration and regeneration; however, the pattern of regeneration in these two transplants was quite different. In autotransplants, a thin myogenic zone, marked by activated myoblasts, was first seen at 4 days. By 7 days the width of myogenic zone increased but still many degenerating myofibres were present in the central region of the muscle. By 14 days the muscle was filled with regenerated myotubes and myofibres. The reautotransplanted muscles underwent similar regenerative events; however, the rate of regeneration was considerably faster. The myogenic zone was apparent as early as 2 days and was much larger at 4 days, and by 7 days the entire muscle was filled with regenerated myotubes and myofibres which matured at later time intervals. Furthermore, the decrease in muscle weight in reautotransplanted muscles was not as much as that seen after autotransplantation. These findings reveal that not only is skeletal muscle capable of regeneration after a second injury, but the rate of this regeneration is much faster. This increased rate and recovery may be due to a conditioning effect of the first injury.

INTRODUCTION

Both physical and vascular injury to mammalian skeletal muscle elicits a regenerative response from the precursor myosatellite cells. A widely used model to study various aspects of this regenerative response is the autotransplantation of extensor digitorum longus muscle in rats (Carlson & Gutmann, 1975; Hall-Craggs & Brand, 1977; Hansen-Smith & Carlson, 1979; Gulati, Reddi & Zalewski, 1982, 1983; Gulati, 1985). After autotransplantation, the majority of myofibres except a thin rim of peripheral myofibres undergoes intrinsic degeneration as a result of devascularization. As the blood supply is slowly restored, reserved precursor myosatellite cells become active, proliferate and differentiate into myoblasts.

Key words: skeletal muscle, regeneration, conditioning effect, autotransplantation, rat, myofibres.

These myoblasts eventually fuse to form multinucleated myotubes, and gradually increase in size, resulting in the formation of myofibres (Allbrook, 1962, 1981; Snow, 1977; Carlson, 1978; Hansen-Smith & Carlson, 1979). The regenerated muscle becomes innervated and by four weeks closely resembles the normal muscle (Carlson, Hansen-Smith & Magon, 1979; Hansen-Smith, 1983; Carlson & Faulkner, 1983). Although morphologically the regenerated muscle resembles the normal muscle, functionally it is quite different as determined by its contractile strength and wet weight which are about half that of the normal muscle (Carlson *et al.* 1979; Faulkner, Niemeyer, Maxwell & White, 1980; Gulati *et al.* 1982).

The present study was designed to examine the effect of reinjury (by reautotransplantation) on once regenerated muscle. The rationale for this study is based on evidence demonstrating a beneficial effect of a prior injury in regenerating nervous tissue (McQuarrie, Grafstein & Gershon, 1977; McQuarrie, 1978; Forman *et al.* 1980). This beneficial effect has been arbitrarily called the conditioning effect, which results in enhanced regeneration. In fact such a conditioning lesion to peripheral nerve has also been shown to facilitate the reinnervation of autotransplantated muscles, resulting in better recovery (Hall-Craggs & Brand, 1977). Since the effect of reinjury to skeletal muscle has not yet been investigated, in this study a comparison of regeneration patterns in autotransplanted and reautotransplanted muscles was made. The results revealed that skeletal muscle is capable of regeneration after reinjury, and that the regeneration process is much enhanced.

MATERIALS AND METHODS

Male Fischer rats weighing 300–325 g were used in this study. Animals were prepared by using a two-phase surgical procedure as described below. For the first phase rats were anaesthetized with chloral hydrate (40 mg 100 g^{-1} body weight, *i.p.*), and the left extensor digitorum longus (EDL) muscle was exposed by cutting the overlying skin and muscles. The EDL muscle was then autotransplanted according to the procedure described in detail earlier (Gulati *et al.* 1982, 1983). Briefly, it consisted of cutting the proximal tendon close to the knee, lifting the muscle from its bed completely, and transplanting it back in the same site. No attempt was made to join any blood vessels or nerves since it was expected that revascularization and reinnervation would occur from the blood vessels and nerves in the graft site (Carlson & Gutmann, 1975; Carlson *et al.* 1979). Finally, the overlying muscle and skin were separately sutured. As shown previously this procedure consistently induces myofibre degeneration and regeneration, and by 30 days the regenerated muscle is mature and reinnervated (Gulati *et al.* 1982, 1983). The second phase was thus carried out at 30 days, when all animals were reanaesthetized and at this time both the left (reautotransplantation) and the right (autotransplantation) EDL muscles were autotransplanted using the same procedure as described above. By following this two-stage surgical protocol the autotransplanted and reautotransplanted muscle were compared in the same animal. At least six such animals were prepared for each time interval and analysed at 2, 4, 7, 14 and 30 days after the second surgery. For histological analysis the left and right EDL muscles were removed by cutting both the proximal and distal tendons. Muscles were quickly weighed and frozen in liquid nitrogen. The autotransplanted and reautotransplanted muscles removed from the same animal were placed on each other, held together with forceps, and then frozen in liquid nitrogen. By following the freezing procedure, adjacent sections could be easily prepared and compared between the two types of transplants. Frozen cross sections of 6 μm thickness were prepared in a cryostat set at −20°C from different regions of the middle two-

thirds of both muscles. Tissue sections were stained with periodic acid–Schiff (PAS)–haematoxylin for histological analysis.

Six unoperated control muscles from rats with the same body weight were also removed, weighed and processed for histological analysis as above. The regeneration pattern in autotransplanted and reautotransplanted muscles was compared at each time interval.

RESULTS

Events involved in skeletal muscle regeneration after a single autotransplantation have been studied in detail (Carlson & Gutmann, 1975; Carlson *et al.* 1979; Gulati *et al.* 1983), thus in the present context they will be briefly described only to compare them with reautotransplanted muscles. Normal rat muscle comprises many myofibres of variable diameter, variable staining intensity, and each possessing peripherally located nuclei (Fig. 1). Regenerated muscle 30 days after autotransplantation resembles normal muscle and comprises myofibres of variable diameter, and the majority of these myofibres possess central nuclei (Fig. 2). After reautotransplantation of the once regenerated muscle (as shown in Fig. 2), the reautotransplanted muscles undergo initial degeneration followed by regeneration. Although the sequence of events after reautotransplantation is similar to that after initial autotransplantation, the timing is much faster in the case of reautotransplants. The progression of myofibre degeneration and regeneration in different regions in individual autotransplanted or reautotransplanted muscles was similar. In addition, the widths of various zones (i.e. surviving myofibre zone, myogenic zone, ischaemic myofibre zone) although not measured appeared similar in each muscle analysed. However, when adjacent sections of autotransplanted and reautotransplanted muscles were compared, the width of various zones was remarkably different between the two muscle transplants. It should be pointed out that in results to follow, the illustrations used to demonstrate differences in the rate and extent of regeneration in autotransplanted and reautotransplanted muscles were taken from adjacent regions.

2-day transplants

As expected in 2-day autotransplants two regions were distinguishable, a thin region of darkly stained peripheral surviving myofibres and the remaining region of faintly stained ischaemic myofibres (Fig. 3). The histological appearance of muscle taken 2 days after reautotransplantation from the contralateral side of the same animal is shown in Fig. 4. Again, regions of surviving and ischaemic myofibres were seen but their boundary was not distinct. In addition in 2-day reautotransplants, clusters of cells were seen. Although the exact nature of these cell clusters cannot be conclusively determined at the light microscopic level, based on our experience and that of others they probably represent activated myoblasts and macrophages. Additional evidence that these cell clusters are primarily activated myoblasts is based on their specific binding ability with lectin, wheat germ

agglutinin, which has been shown to bind specifically in the myogenic zone (Gulati & Zalewski, 1985).

4-day transplants

In the 4-day autotransplants, three distinct regions were visible, a peripheral region of surviving myofibres, a thin myogenic zone comprising primarily activated myoblasts, and the remaining zone of ischaemic myofibres (Fig. 5). In 4-day reautotransplanted muscle from the contralateral side, the myogenic zone was much larger (Fig. 6). Proportionally the zone of ischaemic myofibres was smaller in these grafts (compare Figs 5 and 6). The ischaemic region of the autotransplants had fewer cells as compared to the reautotransplants.

7-day transplants

The histological appearance of autotransplants and reautotransplants was very different, as seen in Figs 7 and 8. In the autotransplants (Fig. 7), the width of myogenic zone had increased but still three distinct zones were visible. In the reautotransplant obtained from the other side, the regeneration process was much advanced and the entire muscle was filled with original myofibres and newly regenerated myofibres and myotubes (Fig. 8). No ischaemic fibres were seen in these grafts.

14- and 30-day transplants

By 14 days the regeneration process was complete in the autotransplants and the entire muscle was filled with regenerated myotubes and myofibres (similar to Fig. 8). In reautotransplants further maturation of myofibres was seen and their histological appearance resembled Fig. 2. At 30 days both types of transplants were similar consisting of mature myofibres. The endomysium in these regenerated muscles was slightly thicker as compared to the unoperated control muscles.

In addition to these histological characteristics, wet weights of normal, autotransplanted and reautotransplanted muscles were compared as a crude measure of muscle recovery. Wet weight determinations were made only at 30-day transplants as regeneration process is known to be complete by this time. The changes observed in the wet weights are shown in Fig. 9. There was about a 50 % decrease

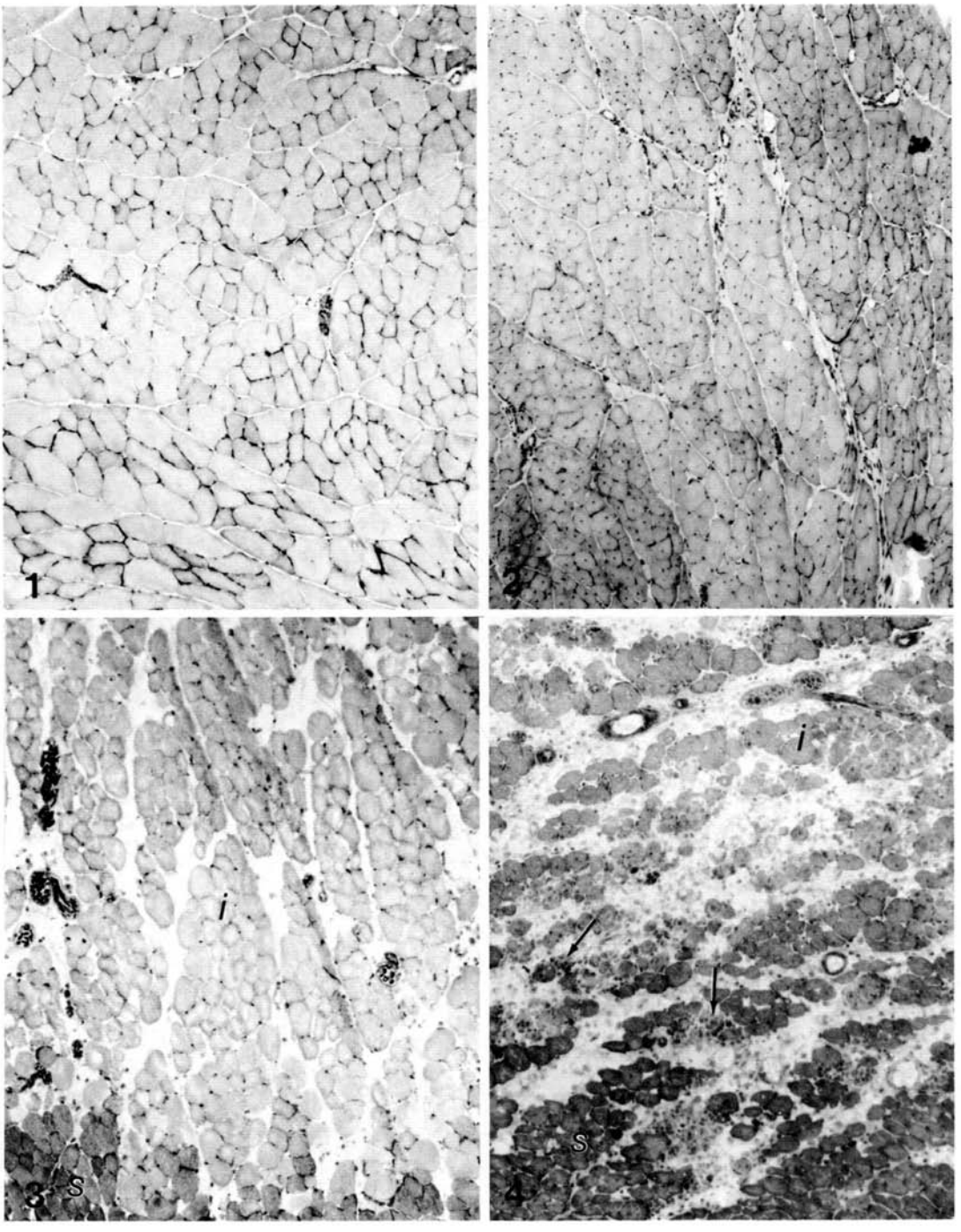
Fig. 1. Cross section of a normal rat EDL muscle. The muscle consists of myofibres of different sizes and staining intensity. PAS–haematoxylin. ×120.

Fig. 2. Cross section of a 30-day regenerated muscle. Myofibres of variable diameter and prominent centrally located nucleus are seen. PAS–haematoxylin. ×120.

Fig. 3. Cross section of a 2-day EDL muscle autotransplant. Two zones are distinguishable: a peripheral zone of surviving myofibres (*s*) and an inner zone of ischaemic myofibres (*i*).

Fig. 4. Cross section of a 2-day EDL muscle reautotransplant. Again a zone of surviving myofibres (*s*) and ischaemic myofibres (*i*) is seen. Also randomly distributed pockets of cells, probably myoblasts and macrophages, are seen throughout the muscle (arrows). Such cells are not seen in 2-day autotransplants (compare Figs 3 and 4). PAS–haematoxylin. ×120.

in the wet weight of once autotransplanted muscle as compared to the normal unoperated controls. This weight loss is consistent with previous findings (Carlson *et al.* 1979; Gulati *et al.* 1982). A decrease of about 20 % in weight was, however, observed between reautotransplanted and autotransplanted muscles.



DISCUSSION

Injured skeletal muscle is known to regenerate and restore its functional activity. By employing the rat EDL muscle autotransplantation model various aspects of this regenerative response have become increasingly clear. These regenerative events are similar to those seen during the course of embryonic development (Carlson, 1973, 1978). Although several earlier studies have reported unsuccessful regeneration after muscle autotransplantation (Peer & Walker, 1951; Roy, 1966), much of the recent work has reversed this belief, and maturation and restoration of normal function have been shown clearly to occur after transplantation. The present results have taken this a step further by showing that skeletal muscle regeneration occurs after a subsequent injury.

The sequence of regenerative events after reautotransplantation is basically similar to that after autotransplantation but the timing and pattern of these events are considerably different. In autotransplants, initiation of regeneration as marked by activation and proliferation of myosatellite cells is closely related to the restoration of blood supply to these grafts and progresses from the peripheral region centrally (Carlson & Gutmann, 1975; Carlson *et al.* 1979; Gulati *et al.* 1982, 1983). In reautotransplanted muscles both activation and proliferation of myosatellite cells occur earlier, and do not appear to follow the peripheral to central direction because the cellular response is seen throughout the muscle. This finding suggests that this activation response may occur independently of the restoration of blood supply, and persisting myosatellite cells become active in response to the injury caused by reautotransplantation. This pattern of cellular response may contribute to the rapid rate of regeneration seen in reautotransplanted muscle.

In addition to enhancement of the cellular response, changes in extracellular glycoconjugates also occurred earlier in reautotransplanted muscles as determined by the binding pattern of lectin wheat germ agglutinin (WGA). In a recent study we reported increased and specific binding of WGA in the myogenic zone of regenerating EDL muscle after an autotransplantation. The binding of WGA was restricted to the myogenic zone and progressed from peripheral to central

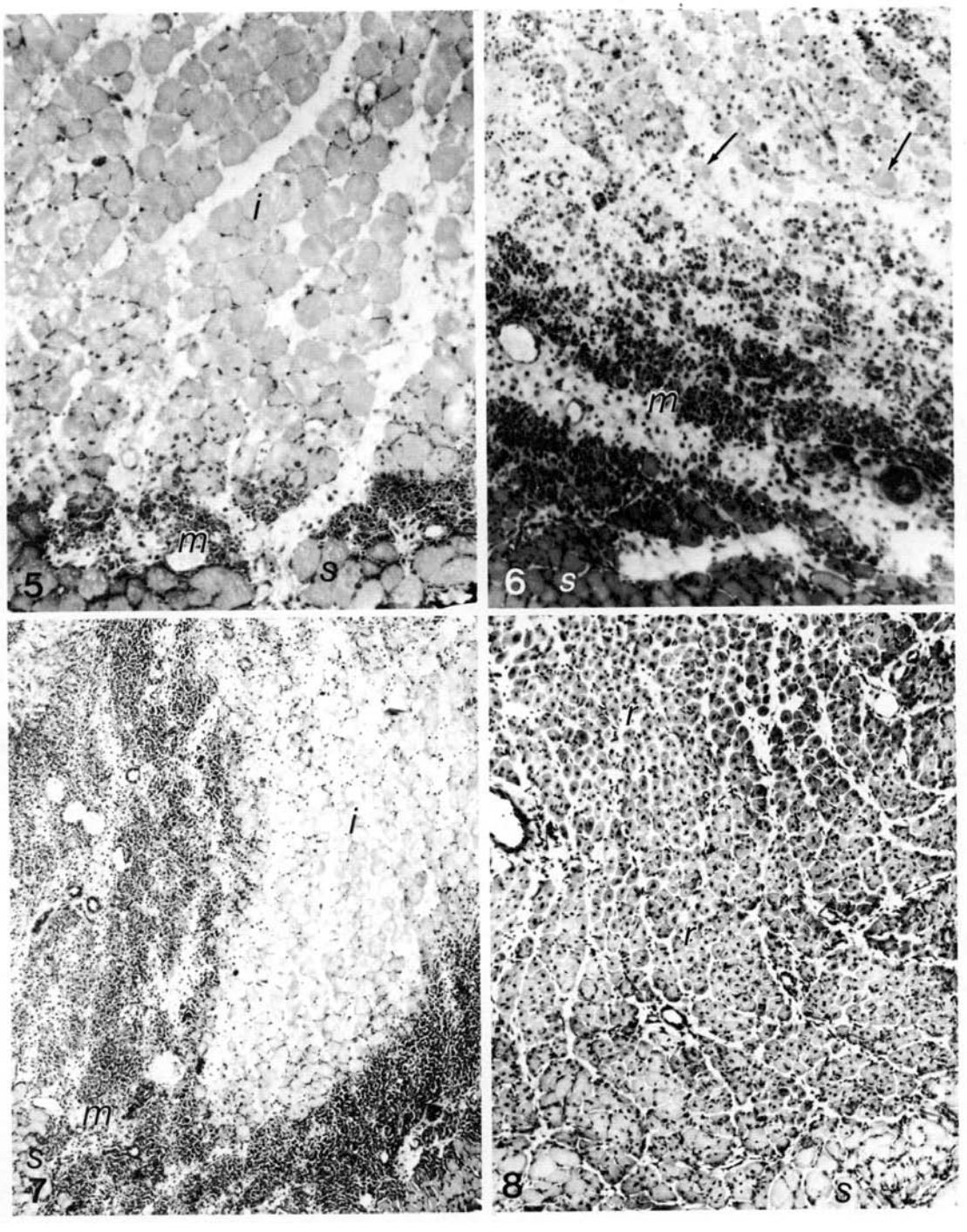
Fig. 5. Cross section of a 4-day EDL muscle autotransplant. Three zones are now distinguishable: a peripheral zone of surviving myofibres (*s*), a small myogenic zone (*m*) consisting of activated and proliferating myoblasts and an inner zone of ischaemic myofibres (*i*). PAS–haematoxylin. ×120.

Fig. 6. Cross section of a 4-day EDL muscle reautotransplant. A zone of surviving myofibres (*s*) is again visible. The myogenic zone (*m*) which is bigger as compared to Fig. 5 and ischaemic myofibres (arrows) are also seen. PAS–haematoxylin. ×120.

Fig. 7. Cross section of a 7-day EDL muscle autotransplant. Three zones are again distinguishable: a peripheral zone of surviving myofibres (*s*), the myogenic zone (*m*) which is much wider, and an inner zone of ischaemic myofibres (*i*). PAS–haematoxylin. ×100.

Fig. 8. Cross section of a 7-day EDL muscle reautotransplant. The regeneration process is almost complete and the entire muscle is filled with regenerated myotubes and myofibres (*r*). Also a thin rim of original surviving myofibres (*s*) is seen but no ischaemic myofibres are present (compare Figs 7 and 8). PAS–haematoxylin. ×100.

direction (Gulati & Zalewski, 1985). Since WGA binds to cell surface glycoconjugates rich in *N*-acetylglucosamine and sialic acid we proposed that extracellular matrix environment rich in these sugars is favourable for myoblast proliferation and fusion (Gulati & Zalewski, 1985). When similar WGA-binding



analysis was done on reautotransplanted muscles (as in the present study), binding of WGA was seen much earlier. The binding did not progress from peripheral to central region, but was seen throughout the muscle and was seen in regions corresponding to the activated myoblast clusters (Gulati, unpublished data). These observations indicate that some of the molecular events involved in muscle regeneration also occur sooner and possibly independently of the restoration of the blood supply.

Another factor contributing to the rapid rate of regeneration in reautotransplanted muscle may be the beneficial effect of the first autotransplantation. Due to this the myosatellite cells in the newly regenerated muscle remain in the ready metabolic state and upon reinjury, proliferate, fuse and lead to a rapid formation of regenerated myotubes and myofibres. Such a ready state has been proposed to play a role in enhanced regeneration of axons in reinjured nervous tissue (McQuarrie, 1978; McQuarrie *et al.* 1977). Thus a similar conditioning effect could be playing a role in enhanced timing and rate of muscle regeneration after reinjury. The enhanced regeneration rate is reflected in the increased recovery of the muscle regenerate as determined by the wet weights of reautotransplanted muscle.

Rate of innervation, although not monitored directly in the present study, could also be responsible for better recovery after a second injury. After free autotransplantation, the muscle is completely denervated because of the severing of the innervating nerves. These severed nerves undergo Wallerian degeneration and

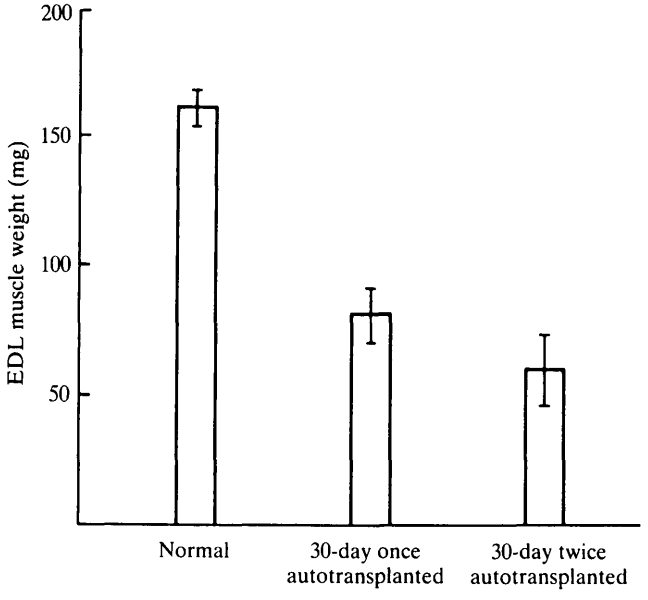


Fig. 9. Comparison of changes in the wet weight of EDL muscle 30 days after autotransplantation and reautotransplantation and normal unoperated controls. Mean weight in mg (±S.E.M.) is plotted for each type of muscle transplants, 'n' is equal to six in all cases.

with time the axons regenerate and re-establish innervation (Carlson *et al.* 1979; Hansen-Smith, 1983). In reautotransplants regenerated axons are severed a second time and undergo degeneration before reinnervating the regenerating muscle. This reinnervation is rapid (due to a conditioning effect on nerves) resulting in improved recovery, as seen in this study. In fact results obtained by Hall-Craggs & Brand (1977) have shown that early innervation of autotransplanted muscle results in improved recovery and this may be another factor contributing to the reduction in weight loss in reautotransplants. Since once regenerated muscle is smaller in size than normal muscle, this difference in size may be another factor responsible for rapid regeneration.

In summary, the present study has shown that skeletal muscle is capable of regeneration after reautotransplantation and the pattern of regeneration after a reinjury is different in several aspects as compared to once injured muscle. Both the rate and extent of regeneration and recovery are improved after reinjury.

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