The use of anabolic steroids as a strategy in reversing denervation atrophy after delayed nerve repair

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Abstract

Background Denervation atrophy is one factor contributing to suboptimal motor recovery following major nerve repair. The hypertrophic effects of anabolic steroids may have a potential role in improving reinnervated muscle strength after delayed repair.

Methods Forty-five immature female Sprague–Dawley rats underwent three surgeries and final testing. The tibial nerve was transected in the hind limb of the experimental ($n=13$) and control ($n=14$) animals and exposed, but not transected in the sham ($n=15$) group animals. Three months later, once denervation atrophy was established, all transected nerves underwent repair using an autograft from the contralateral limb. After waiting an additional month to allow axonal regeneration to the gastrocnemius muscles, the rodents were implanted with a subcutaneous infusion pump. For the experimental group, nandrolone was administered over the next 30 days via this pump, while the control and sham group pumps were filled with carrier only.

Results Final testing, 6 weeks later, showed improved muscle contraction strength in the steroid-treated animals (72% of sham group strength) compared to control animals (57% of sham group strength, $p<0.5$). A trend towards increased weight and muscle belly diameter in the steroid-treated group was not statistically significant.

Conclusions These findings support the potential role of anabolic steroids in improving recovery of atrophic muscle after delayed reinnervation.

Keywords Anabolic steroids · Denervation atrophy · Nerve repair

Introduction

Functional motor recovery following repair of major peripheral nerve injuries is often suboptimal [2, 26, 29, 36, 42]. The explanation for this is multifactorial, but delays in muscle reinnervation associated with long regeneration lengths or delayed surgical intervention negatively affects both muscle and nerve recovery potential [17]. Temporally related reductions in the number of regenerating motor units as well as the gradual formation of obstructive scar tissue around motor end plates drastically reduce the number of muscle cells that even achieve reinnervation after enough time has passed [18, 21, 35, 51]. Though larger motor unit sizes (each axon innervates three to five more muscle fibers than normal) can partially compensate for the lost axons in these situations, the reestablished connections are made to atrophied and compromised muscle fibers [17]. This condition of progressively irreversible loss of muscle mass, fiber size, and contractile composition is known as “denervation atrophy” [17, 52].

Most strategies for improving nerve repair clinical outcomes have focused on limiting this denervation atrophy by expediting axonal regeneration and limiting the denervation period as much as possible. Little attention, however, has been directed towards reversing this denervation atrophy once it has occurred.

Anabolic steroids may have a role in this application. These synthetic forms of testosterone designed to maximize anabolic effects while minimizing androgenic side effects [22] have been shown to cause normal muscle hypertrophy, delay atrophy in immobilized muscle, and even slow the

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progression of chronic denervation atrophy [11, 43, 59]. The purpose of this study is to evaluate if anabolic steroids can improve motor recovery following the reinnervation of an atrophic, chronically denervated muscle.

Materials and Methods

Forty-five immature (3 months old) female Sprague–Dawley rats were used for study after obtaining necessary approval from our institution's animal review board in accordance with national guidelines. All animals were housed in a temperature- and humidity-controlled environment with a 12:12 day–night cycle and were provided food and water ad libitum. The rats were divided into a sham group (no denervation atrophy), control group (denervation atrophy established, reversed, no steroid given), and experimental group (denervation atrophy established, reversed, steroid given; $N=15$ per group). Three rats were euthanized for persistent wound dehiscence during the experiment, leaving $N=13$ for the experimental group, $N=14$ for the control group, and $N=15$ for the sham group for final testing and analysis. For all surgeries and final testing, anesthesia was induced with 5% isoflurane in a closed chamber and maintained using 2–3% isoflurane via nose cone inhalation. For all nerve exposures, a standard biceps femoris–semitendinosus muscle-splitting approach was utilized while maintaining proper sterile technique. Postoperative analgesia was consistently accomplished with subcutaneous administration of buprenorphine 0.5 mg/kg augmented by oral consumption of acetaminophen 272 mg/100 cc added to the drinking water.

Initial surgery for the experimental and control groups consisted of transection of the left tibial nerve just distal to the sciatic nerve bifurcation. The sham group underwent exposure without transection. The rats recovered and were maintained for 3 months to establish denervation atrophy of the involved muscles.

A second surgery was performed on all rats at 3 months. The control and experimental groups underwent repair of the left tibial nerve using a 1-cm autograft from the contralateral leg. All repairs were done under microscope magnification with four epineural stitches using a 10-0 nylon suture. The sham group underwent exposure of bilateral sciatic nerves without manipulation of the nerves. All anesthesia, exposure, and closure of wounds were carried out as for the first surgery. Thirty days were then allowed for nerve regeneration and recovery.

A third surgery (at 4 months) consisted of sterile placement of a commercially available osmotic pump systemic drug delivery system (Alzet, Cupertino, CA, USA) in the subcutaneous tissue between the shoulder blades of all rats (to discourage self-mutilating behavior such as chewing at the surgical site). The pumps for the

experimental group contained nandrolone (in cottonseed oil carrier) to be delivered at a concentration of 0.11 mg/kg/day for 30 days. The pumps implanted into the control and sham groups contained cottonseed oil only. An additional 6 weeks was then allowed for muscle recovery.

Final testing consisted of reexposure of the left sciatic nerve and isolation of the medial gastrocnemius muscle and tendon (for all groups). The hind limb was secured to a platform via placement of Kirschner wires through the femoral condyle and the distal tibia, and the medial gastrocnemius tendon was coupled to a force transducer using a 4-0 silk suture. A bipolar electrode delivered three supramaximal stimulations (5 V, 1 Hz) to the sciatic nerve (2-min rest intervals between stimulations). Contraction strength was converted to digital data using ADI Instruments Power Lab system (ADI Instruments, Inc., Colorado Springs, CO, USA) and recorded using an Apple laptop computer (Apple Computer, Inc., Cupertino, CA, USA). Data were analyzed for statistical significance using Student's *t* test with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

The medial gastrocnemius muscles were harvested and weighed and measured (largest diameter) prior to fixation in 4% formaldehyde. Histological preparation with hematoxylin and eosin staining allowed for individual muscle fiber cross-sectional area analysis. Average cross-sectional areas were calculated from direct measurements (using ImageJ 1.42 software found on the NIH website) of 20 muscle fibers randomly selected within ten high-power ($\times 20$) fields per muscle sample (200 measurements per sample).

At the conclusion of testing, all animals were euthanized with an intraperitoneal injection of 150 mg Euthasol and were disposed of per our institution's policy.

Results

The results of our study show an improvement in muscle contraction force with the administration of nandrolone. Muscle forces were analyzed as a percentage of normal contraction force (using the sham group as normal). The experimental group achieved 72% of the muscle contraction force of the sham group. The control group achieved 57% of the contraction force compared to the sham group (Fig. 1). This difference between the experimental and control groups was statistically significant ($p<0.05$). The average muscle diameter for the experimental group was 6.9 mm compared to 6.3 mm for the control group. This was not statistically significant, but shows a trend for larger muscle diameters for the experimental versus the control group (Fig. 2). Likewise, the average muscle belly weight for the experimental group was 452.5 mg compared to the control group, which averaged 368.1 mg. This again was

Fig. 1 Mean muscle contraction force generated with supramaximal stimulation of sciatic nerve. The experimental group had a statistically greater contraction than the control group ($p < 0.05$)

not statistically significant, but just as with the muscle diameters, it shows a trend towards larger muscle belly weights for the experimental compared to the control group (Fig. 3). The muscle belly diameters and weights in the sham group averaging 8.2 mm and 633.7 mg, respectively, were statistically higher than both the experimental and control groups ($p < 0.05$).

Histological analysis revealed a trend towards larger cross-sectional areas of individual muscle fibers for the experimental group ($1,917 \mu\text{m}^2$) versus the control group ($1,655 \mu\text{m}^2$; Fig. 4). Both the experimental and control groups had smaller mean areas compared to the sham group which averaged $3,003 \mu\text{m}^2$ ($p < 0.05$; Fig. 5).

Discussion

Despite advances in our understanding of nerve regeneration, treatment outcomes have not seen an equivalent improvement [2, 26, 29, 36, 42]. Indeed, the literature demonstrates too many “almost good” results following major nerve repairs. Sakallariades reported 50% of his patients obtained M2 or M3, and only 12% obtained M4 strength following primary median nerve repair [42];

Fig. 2 Average muscle diameter was statistically higher in the sham group only ($p < 0.05$)

Fig. 3 Muscle belly weight was statistically higher in the sham group compared to the experimental and control groups. The experimental group averaged a higher muscle belly weight than the control group, but this did not reach statistical significance

Jongen and van Twisk reported 60% M3 recovery following primary repair of ulna nerves [26]; and Mellesi reported 31% M3 level recovery following median, ulna, and radial nerve grafting [36]. Although nerve repair, regeneration, and recovery are complex and multifactorial processes, long delays in muscle reinnervation is a major factor in these suboptimal outcomes [2, 29]. Often seen in clinical scenarios due to late surgical intervention or to prolonged axonal regeneration lengths, prolonged recovery time leaves the reinnervated muscles in a difficult or impossible to reverse, weakened state. Therapeutic modalities aimed at strengthening these atrophic muscles might improve outcomes and push the “almost good” results to functionally useful recovery. Anabolic steroids have been shown to improve muscle mass and strength in other medical conditions and may have potential in this application. Based on this, we hypothesized that systemic anabolic steroid administration following reinnervation of atrophic muscle will amplify strength recovery.

One obstacle in solving the problem of denervation atrophy is that the pathophysiology is so poorly understood. Prevailing theories point to alterations in contractile protein concentrations and composition [10, 20, 25] as well as the degradation of the satellite cell population. Satellite cells, which are reparative mononuclear progenitor cells found in muscle tissue, normally divide, proliferate [1, 40, 46], and become incorporated into damaged or denervated muscle fibers [1, 24, 38] to preserve or rebuild mass. With prolonged denervation, however, this pool of cells ultimately becomes exhausted. Loss of this important regenerative component may explain why denervation atrophy eventually becomes irreversible [1, 17, 29].

Testosterone and anabolic steroid usage in improving muscle mass and strength for both medical and nonmedical purposes seem to be increasing [13]. Their anabolic effects have been anecdotally accepted by body builders and

Fig. 4 Representative slides of muscle cross-sections

athletes well before the scientific community could do so [6, 13, 16]. However, more recent studies have been able to demonstrate the positive effects of supraphysiological doses of testosterone on muscle mass and strength in healthy men [5, 48]. Furthermore, reduction in muscle atrophy associated with wasting conditions such as HIV infection or age-related sarcopenia has been demonstrated in several recent studies [4, 8, 41, 44, 45, 50]. Improved muscle size and strength have been reported in female rabbits following 12 weeks of anabolic steroid administration [43] and in a sedentary rabbit model treated with nandrolone decanoate [54].

Our study design incorporated a common, reliable small animal model in which denervation atrophy could be artificially created, halted, and pharmacologically treated. Sample size (to achieve 90% Power) was determined using GraphPad StatMate software (GraphPad Software, Inc., La Jolla, CA, USA) referencing standard deviations from previous rodent studies on anabolic steroids and denervation atrophy progression [59] and a clinically relevant expected improvement of 15% for our study. Young female rats were specifically chosen to minimize both senescence-

related problems associated with long studies and steroid-induced hypogonadism. Denervation atrophy, additionally, has been demonstrated in rodents to occur in as little as 1 month [29]. The 3-month delay between nerve resection and repair in our study was meant to augment this effect and ensure a well-established atrophy. Indeed, the contraction force deficits found in the untreated (control) group in our study were similar to those reported by Aydin et al. in a similar rodent denervation atrophy model [2]. After this 3-month delay, however, end-neuroma formation and normal nerve retraction necessitated the use of an interposition nerve graft though the specific nerve gaps were not measured. The choice of a 1-cm autograft from the contralateral tibial nerve helped maintain consistency between animals and would not be expected to compromise nerve regeneration in this type of model [5]. From the site of repair, regenerating axons still needed to travel approximately 3 cm to the muscle before denervation atrophy was halted. At an expected rodent axonal regeneration rate of 1.5 mm/day [28], a 1-month delay in steroid treatment was meant to minimize any potential confounding positive effects of the steroids on axonal regeneration, as demonstrated in previous rodent nerve crush models [9, 30, 31]. Lack of effect on axon regeneration was not validated, however, and future study will need to differentiate between neurotrophic and anabolic effects of steroid treatment.

Nandrolone was chosen as our treatment drug based on commercial availability, a favorable anabolic/androgenic ratio, and previous incorporation of this steroid in similar animal studies. Nandrolone and other anabolic steroids are commonly dissolved in an oil base carrier such as sesame seed, peanut, or cottonseed oil [3, 7, 12, 27, 32–34, 37, 53, 57], and while no pharmacologic effects were expected from the cottonseed oil carrier used, it was administered to both the steroid and nonsteroid groups to control for any unforeseen effect. Dosage and length of administration were calculated based on the Food and Drug Administration recommendations for human usage [59]. Though

Fig. 5 Individual muscle fiber cross-sectional area (CSA) average was higher for the sham group compared to the experimental and control groups ($p < 0.05$). There was a trend for larger cross-sectional areas for the experimental group compared to the control group, but this was not statistically significant

steroid levels were not measured, the osmotic pump delivery system used in this study is considered well-validated and reliable [55].

Our results were interesting in that the anabolic steroid-treated group demonstrated improved force contractions but were not significantly less atrophic based on morphologic or histological muscle measurements. This is in contrast to the findings of Yeagle et al. who found no difference in the return of contractile properties in rat extensor digitorum longus muscles following crush injury to the peroneal nerve in castrated versus testosterone-supplemented groups [58] or Gordan et al., who found no effect on anterior tibial muscle atrophy in a similar study following sciatic nerve transection [19]. However, in these studies, denervation atrophy had not developed, and differences in treated and untreated groups may have been more subtle. Zhao et al., however, found that nandrolone did significantly reduce the progression of chronic denervation atrophy in rats (based on muscle weight and atrophic gene expression) when administered 28 days post-nerve transection. Interestingly, similar to Gordan's and Yeagle's studies, there was no effect on muscle atrophy with more acute denervation [59]. Zhao's study, however, unlike ours, did not involve reinnervation of the atrophic muscle and, while their findings in general support our own, this represents an important difference in the two models. Our study took muscle in a severe catabolic state (denervation atrophy), reversed it by reinnervation, and demonstrated augmentation of contraction recovery with anabolic steroid administration. Additionally, the dosage of anabolic steroid used in our study (though the same used in Zhao's) was actually quite low in comparison to the spectrum of dosages described throughout the animal literature (3.75, 6, 15, and 20 mg/kg) [7, 27, 32, 34, 53, 57]. We cannot assume that a higher dose would result in a larger effect, but certainly, a dose–response curve would be an important variable to look at in future studies.

A physiological explanation for our observations at this point would only be conjecture. Testosterone administration has been shown to increase protein and ribosomal synthesis while decreasing protein breakdown in muscle cells [14, 15, 56]. This mechanism, along with links to increased replication [23, 39, 47] and integration of satellite cells [48, 49], should result in an increased number of nuclei and enlargement of muscle cells [48, 49]. Why no statistically significant increase in muscle cell size or muscle body size was found in our steroid-treated group despite the positive effects on contraction strength is not clear. Since there was a trend toward larger muscle body and cell size, the failure may be as simple as inadequate sample sizes. Future studies, however, will need to focus on the physiologic effects of anabolic steroid administration in addition to the functional ones.

This study is the first that we are aware of in which a pharmacologic agent, such as anabolic steroids, has been demonstrated to augment strength recovery following reinnervation of an atrophic, denervated muscle. Confounding factors, which cloud our ability to definitively interpret the results, include potential unintended neurotrophic effects, omission of a dose–response curve, and a failure to demonstrate a statistically significant effect on muscle atrophy. The concept, however, seems to have potential for improving outcomes following peripheral nerve repair, and further study is warranted.

Conflict of interest The authors declare that they have no conflict of interest.

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