Original Article

Effects of testosterone replacement therapy on skeletal muscle after spinal cord injury

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Study design: Randomized control.

Objective: To examine the effects of testosterone replacement therapy (TRT) on skeletal muscle 11 weeks after complete SCI.

Setting: Athens, Georgia USA.

Methods: Soleus (SOL), gastrocnemius (GA), tibialis anterior (TA), vastus lateralis (VL) and triceps brachii (TRI) muscles were taken from twelve young male Charles River rats 11 weeks after complete SCI (T-9 transection, $n=8$) or sham surgery ($n=4$). Rats received either TRT (two 5 cm capsules, $n=4$) or empty capsules ($n=8$) implanted at surgery. Muscle samples were sectioned and fibers analyzed qualitatively for myosin ATPase and quantitatively for succinate dehydrogenase (SDH), α -glycerol-phosphate dehydrogenase (GPDH) and actomyosin ATPase (qATPase) activities using standard techniques.

Results: SCI decreased average fiber size ($49 \pm 4\%$) in affected muscles and the percentage of slow fibers in SOL ($93 \pm 3\%$ to $17 \pm 2\%$). In addition, there was a decrease in SDH and an increase in GPDH and qATPase activities across the four hind-limb muscles of the SCI animals. Fiber size in the TRI was increased ($31 \pm 2\%$) by SCI while enzyme activities were not altered. Average fiber size across the four hind limb muscles was decreased by only 30% in TRT SCI animals and their SOL contained $39 \pm 2\%$ slow fibers. TRT also attenuated changes in enzyme activities. There was no effect of TRT on the TRI relative to SCI.

Conclusions: TRT was effective in attenuating alterations in myofibrillar proteins during 11 weeks of SCI in affected skeletal muscles.

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Introduction

It has been estimated that there are approximately 200 000 patients with spinal cord injury (SCI) in the United States alone, with roughly 10 000 new injuries occurring each year. Additionally, the relative number of new injuries is rising and expected to increase by 20% by 2010, compared to 1994 prevalence.¹ SCI is associated with a decrease in perceived quality of life and an increase in several health-related risk factors. Interventions aimed at reducing some of the health risks associated with SCI have been examined over the

past two or so decades. As a result of this search and the changes in care and treatment of patients with SCI, the expected life span of this population has increased to approximately the same as for able-bodied persons. However, many health problems remain for this patient group that may be related to the extensive loss of the mass of affected skeletal muscle after injury.

One possible treatment that has received little attention with respect to SCI and sarcopenia is testosterone replacement therapy (TRT). TRT is used to combat a variety of health problems in the aging male. The decreases in strength, muscle mass, reproductive function and bone density with aging have been linked to lower circulating androgens,

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specifically testosterone.² Although the benefit to risk analysis of TRT for able-bodied individuals is sometimes questioned because of the potential for increased hematopoiesis, liver toxicity and prostatic cancer,³ the potential of this treatment to aid SCI patients has not been examined.

Previous research has shown that TRT can ameliorate the atrophic response resulting from hind-limb unloading in rats,⁴ a model that evokes decreases in plasma testosterone similar to those seen in animals with SCI. EMG activity of involved muscles in hind-limb suspended rats is 'normal' after 48 h of unloading while affected skeletal muscles after SCI can be electrically silent. Thus, the lack of 'activation and associated events', for example active muscle shortening and subsequently lengthening of associated antagonists, could result in different effects of TRT in these two models of muscle atrophy.^{5,6}

In light of the aforementioned, the purpose of this study was to evaluate the effects of TRT on rat skeletal muscles after SCI. It was hypothesized that TRT would at the least attenuate atrophy after SCI based on the effects of androgen replacement in other models of sarcopenia. This study will not address the concerns surrounding testosterone therapy in humans.³ However, it will hopefully serve as an initial step in assessing the efficacy of TRT for reducing several increased health risks after SCI. An increase in affected muscle mass in chronic SCI could increase exercise capacity, and thereby reduce the risk for cardiovascular disease.⁷ A therapeutically induced increase in muscle mass could also result in increased insulin sensitivity, venous return and metabolic rate with corresponding potential for more favorable body composition and increased bone density, all would have a positive influence on health.

Methods

The soleus (SOL), gastrocnemius (GA), tibialis anterior (TA), vastus lateralis (VL) and triceps brachii (TRI) muscles were taken from 12 young male Charles River rats (131 days old), 11 weeks after complete SCI (T-9 transection, $n=8$) or sham surgery ($n=4$). A complete surgical transection of the spinal cord at T-9 with a foam barrier placed between the severed ends of the cord was performed. Rats received either TRT (two 5 cm testosterone capsules, $n=4$), or empty capsule implantation ($n=8$) at the time of surgery, as described previously.⁸ Rats were housed singly in hanging wire cages with Purina Rat Chow and water ad libitum. Animals were cycled three times per day until bladder function returned after recovery from spinal shock (~2 weeks). There was no recovery of voluntary motor function in the hind-limbs of the rats, although some spinal reflexes did return after recovery from spinal shock.

Excised muscle tissue was mounted in an embedding medium and subsequently frozen in 2-methyl butane cooled in dry ice. Samples were stored at -70°C until analyzed.

Histochemical analyses

Samples were removed from the freezer and placed in a cryostat microtome at -20°C to warm. Serial sections (6, 10 or 14 μm) were cut and placed onto coverslips for immediate assay by both qualitative and quantitative histochemical procedures. Images of the sections were acquired using a Sony xc 77 CCD camera attached to an Olympus bh-2 microscope linked to a Macintosh Quadra 800 computer. Images were saved and analyzed using NIH image software (written by Wayne Rasband at the US National Institutes of Health, zippy.nimh.nih.gov), as done previously.^{9,10}

Sections (10 μm) were assayed qualitatively for myofibrillar adenosine triphosphatase activity using the techniques of Brooke and Kaiser¹¹ and of Guth and Samaha.¹² Single fibers were analyzed for type by microdensitometric determination of optical density. The myosin ATPase composition, represented by the optical density, which is based on the pH lability of the fiber, is directly correlated to myosin heavy chain composition of a given fiber.¹³

Quantitative histochemical determination of succinic dehydrogenase activity (SDH) and α -glycerol phosphate dehydrogenase activity (GPDH) were used as estimates of oxidative and glycolytic energy supply, respectively, essentially as done previously.^{9,10} SDH and GPDH activities were determined by the microdensitometric technique described by Blanco *et al.*,¹⁴ and Martin *et al.*,¹⁵ respectively. Images were captured using the same tools as for fiber typing with the exception that a narrow pass interference filter with peak emission of 570 nm was used so as to assess maximal absorption of Nitro-blue tetrazolium-diformazan, the *in-vitro* reaction end product. Enzyme activities were obtained from the difference in optical density between samples incubated in the presence and absence of substrate and are expressed as μmol fumerate/l tissue/min and μmol glycerol-3-phosphate/l tissue/min for SDH and GPDH activity, respectively.

The method of Blanco and Sieck (1992) was modified and used for quantitative determination of actomyosin adenosine triphosphatase (qATPase) activity in single fibers, essentially as done previously for human skeletal muscle.⁹ Briefly, 6 μm serial sections were incubated in one of six solutions of different ATP concentrations (0–3 mM). The use of incubations of different concentrations is necessary because, in contrast to the assays for SDH and GPDH, the qATPase reaction is non-substrate limited. The Michaelis-Menten model of enzyme kinetics was used to analyze the data and the reciprocal of the y-intercept of the Lineweaver-Burk plot was taken as the maximum velocity of the qATPase in OD/min. Enzyme activity, expressed as mmol Pi per liter of tissue per min, was determined using the Lambert-Beer equation with a molar extinction coefficient for lead-sulfide of $1450 \text{ M}^{-1} \text{ cm}^{-1}$.

Images saved for SDH, GPDH and qATPase assays were matched to those saved for fiber typing, thus allowing values obtained for individual fibers in serial

sections to be matched and expressed relative to fiber type and fiber size. This provides both a qualitative indicator of fiber properties via their type, and quantitative estimates of both anaerobic and aerobic energy supply via GPDH and SDH activities, respectively, and energy demand via qATPase activity.

A minimum of 100 fibers of a given type were analyzed and averaged for calculation of fiber type specific values for each animal. These averaged values were used as the dependent measures for each variable. Determination of the effect of TRT on average muscle fiber cross-sectional area (CSA) was assessed using a two-way (muscle $\times$ group) analysis of variance (ANOVA). Group differences in fiber type specific CSA and in SDH, GPDH and qATPase activities were examined using a three-way (muscle $\times$ group $\times$ fiber type) repeated measures ANOVA. Differences were considered significant at $P < 0.05$.

Results

Muscle characteristics in control rats

The CSA of individual fibers followed an overall hierarchy with type IIx > IIa > I ($P < 0.01$). Additionally, type IIx fibers accounted for a greater per cent of the fibers in the muscles studied than type IIa or type I fibers ($P < 0.01$). Accordingly, calculation results for the relative area of these muscles occupied by a given fiber type followed the same hierarchy as FT%. With respect to individual muscles, the hierarchy for the SOL in both FT% and relative area differed from the group data, with type I > IIa > IIx. The other muscles all resembled the group data with type IIx fibers occupying more of the muscle than either type I or type IIa ($P < 0.01$).

The SDH, GPDH and qATPase activities of individual fibers also followed hierarchies. The GPDH and qATPase activities followed the same hierarchy as did CSA with type IIx > IIa > I ($P < 0.01$), while the SDH activities of type I and IIa fibers were significantly higher than type IIx fibers ($P < 0.01$). Muscle effects for SDH followed a hierarchy with SOL > GA > VL = TRI > TA ($P \leq 0.01$), expectedly almost opposite of that for GPDH and qATPase with TA, TRI and VL > GA > SOL ($P < 0.02$).

Effects of SCI on rat muscle

Affected muscle fibers in SCI animals were approximately 49% smaller ($P < 0.01$, Figure 1) than those in control animals. A muscle $\times$ fiber type interaction indicated that fibers in the SOL showing the greatest and those in the TA the least atrophy ($P < 0.01$). Fibers in the TRI, in contrast, were $31 \pm 2\%$ bigger than in CON animals.

The percentage of type I fibers was lower in the SCI compared to the CON animals ($P < 0.01$) and these fibers occupied a smaller relative area of the affected muscle ($P < 0.01$). Muscle $\times$ fiber type $\times$ group interac-

Figure 1 Average fiber size. Values are expressed in μm^2 . Mean data $\pm$ s.e. are given for control (CON), spinal cord injured (SCI) and testosterone treated spinal cord injured (TRT SCI) groups in the soleus (SOL) gastrocnemius (GA), tibialis anterior (TA), and vastus lateralis (VL) muscles. *Indicates values significantly greater than values for the same muscles in other groups. #Indicates values significantly greater than values for the same muscles in SCI group

tion showed that transition to a faster muscle was significant only for the SOL. This reflected the inherently low percentage of slow fibers in the other muscles.

Average fiber SDH activity was lower in hind-limb muscles while average fiber GPDH and qATPase activities were higher in the SOL of SCI affected animals ($P < 0.01$). There were no differences in the activities of these enzymes in the TRI of CON and SCI animals. Fiber type specific results indicate the greatest reduction in SDH activity resulting from SCI in the fiber type with the highest initial activity, the type IIa fibers ($P < 0.01$). There was not significant group $\times$ fiber type interaction with respect to GPDH or qATPase activity ($P \geq 0.17$).

Effects of testosterone replacement therapy

Plasma testosterone concentrations were 3.83 ± 1.11 ng/ml in the sham surgery rats, 1.34 ± 0.31 in the SCI rats without TRT and 5.67 ± 0.83 in SCI rats with testosterone capsules. Plasma testosterone levels were higher in the SCI rats receiving testosterone than in those that did not ($P < 0.01$), but they were not significantly different from those in sham rats. With respect to the health of the animals, there was an initial loss of body weight after SCI, but the rats continued to gain weight over the 11 weeks without becoming obese. There were no other side effects of the treatment in this group of rats, although we have noticed prostate enlargement in other groups of testosterone treated rats.

The effect of TRT on average fiber CSA in the hind-limb was an attenuation of the atrophic response resulting from SCI. Average fiber CSA in the TRT SCI animals was higher and lower compared to SCI and CON animals, respectively ($P < 0.01$, Figure 1). This attenuation in fiber atrophy was demonstrated across all fiber types ($P = 0.002$), and in all muscles except the TA ($P < 0.01$). The most dramatic effect was observed in the SOL, with fibers in the TRT SCI animals being 58% larger than in SCI animals (Figure 1). Additionally, TRT SCI animals retained 152% more slow fibers in the SOL than SCI animals ($P < 0.01$, Figure 2). There was no additional increase in size resulting from TRT in the TRI, compared to SCI alone (Figure 1).

TRT attenuated the overall decrease in SDH activity relative to SCI animals. This resulted in a hierarchy with $\text{CON} > \text{TRT SCI} > \text{SCI}$ ($P < 0.01$, Figure 3). The SCI animals also had higher values for overall GPDH and qATPase activities than CON and TRT SCI animals, thus $\text{SCI} > \text{TRT SCI} = \text{CON}$ ($P = 0.02$).

Discussion

The results of this study reveal new and important findings with respect to TRT after SCI. Additionally, many of the findings in the present work examining normal as well as SCI affected muscles are consistent with previous literature and lend validity of this work. First, the hierarchies in fiber size and fiber type percentage agree with existing literature.¹⁶ Likewise, the reduction in fiber size, SDH activity and percentage of type I fibers as well as the increase in GPDH activity resulting from SCI are within the range of reported values for animals of similar age and duration of injury.^{16,17,18}

The most important finding of this study was that TRT ameliorated the decrease in fiber CSA resulting from SCI. TRT also attenuated the slow to fast fiber type shift as well as the decrease in oxidative enzyme activity. To our knowledge, this is the first study to investigate the potential of TRT to prevent atrophy in SCI. TRT in aging sarcopenia and in other diseases with muscle wasting (for example, AIDS) results in favorable effects on bone, muscle size and strength in both low-average and hypogonadal men.¹⁹ Increases in muscle CSA were equal, if not greater in TRT only groups than in exercise groups without TRT.²⁰ These data and ours for SCI both demonstrate a positive effect of TRT on muscle size without traditional overload.

The mechanisms responsible for the increased muscle size resulting from TRT remain unclear. In addition, the positive responses to TRT after SCI demonstrated in this study most likely resulted from preservation of existing muscle proteins and not the hypertrophic responses thought to occur in other androgen treated populations. Nonetheless, whether the positive responses to TRT stem from a testosterone deficiency, a tissue-specific or relative resistance to

Figure 2 Images taken from the soleus muscle stained histochemically for myofibrillar adenine triphosphatase (hATPase) CON (panel A), TRT SCI (panel B) and SCI (panel C)

Figure 3 Changes in enzyme activities. Values are expressed as per cent difference from control (CON) values. Mean data $\pm$ s.e. are given for SCI and TRT SCI groups in the SOL, GA, TA, and VL muscles. #, Different than SCI. *, Different than control

androgen replacement in these populations, or some other systemic response, TRT has been shown to have beneficial effects in populations with below average testosterone levels. This is promising for patients with SCI who show an inverse correlation between duration of injury and testosterone levels.²¹

Although the data in the present work are promising, TRT did not completely ameliorate the atrophic response associated with this type of injury. Thus, additional treatment interventions aimed at further attenuating loss and/or producing skeletal muscle hypertrophy in this population may be needed. Previous studies using electrically assisted cycling or walking in long term SCI populations report minimal muscle growth. However, data generated using electrical stimulation protocols designed to mimic resistance training in short term patients have been extremely successful.²² These results, when combined, bear similarity to training protocols in healthy

populations comparing traditional resistance and endurance training: hypertrophy an obvious response to the former and not the latter. Thus, the plasticity in muscle size is seemingly affected by much the same stimuli in able-bodied and SCI affected muscle, at least in the first year after injury.

Another interesting finding of this work was hypertrophy of the TRI after SCI. It seems reasonable that this muscle was 'overloaded' after injury as the animals 'ambulated' about their cage. This growth occurred in spite of the compromised androgen status of these animals. Thus local factors appeared sufficient to evoke hypertrophy in unaffected skeletal muscle, but this was not augmented by TRT. Whether such growth could be evoked by resistive overload of affected skeletal muscle after SCI is not clear. The hypertrophy we found with resistive loading was in patients about one year after injury, and thus likely not testosterone deficient.²² It is also not clear if a combination of TRT and resistive overload would be additive in long term patients who would likely be androgen deficient. The inability of TRT to augment the 30% increase in size of unaffected, apparently overloaded skeletal muscle after SCI may not be the same as increasing the size of extremely atrophied, affected skeletal muscle after injury.

The results of this paper indicate several potential benefits of TRT in patients with SCI, namely attenuation of transformation of affected skeletal muscle to smaller, faster and probably more fatigueable contractile machinery. These benefits include the potential for increased venous return, increased insulin sensitivity, and increased metabolic rate, all resulting from the maintenance of skeletal muscle characteristics after SCI. Although a myriad of health related problems occur as a result of SCI, the potential to reduce the severity of these with TRT is an exciting possibility; one that demands future research in these patients and others suffering from chronic disease and/or disability. Because TRT may impose additional health risks, responses to intermittent therapy could be specifically addressed.

References

- 1 Phillips WT, Kiratli BJ, Sarkarati M, Weraarchakul G. Effects of Spinal Cord Injury on the Heart and Cardiovascular Fitness. *Curr Prob Cardiol* 1998; **23**: 644–717.
- 2 Wimalawansa SM, Wimalawansa SJ. Simulated weightlessness-induced attenuation of testosterone production may be responsible for bone loss. *Endocrine* 1999; **10**: 253–260.
- 3 Basaria S, Dobs AS. Risk versus benefit of testosterone therapy in elderly men. *Drugs and Aging* 1999; **15**: 131–142.
- 4 Wimalawansa SM, Chapa MT, Wei JN, Westlund KN, Quast MJ, Wimalawansa SJ. Reversal of weightlessness-induced musculoskeletal losses with androgens: quantification by MRI. *J Applied Physiol* 1999; **86**: 1841–1846.

- 5 Alford EK, Roy RR, Hodgson JA, Edgerton VR. Electromyography of rat soleus, medial gastrocnemius, and tibialis anterior during hind limb suspension. *Exp Neurol* 1987; **96**: 635–649.
- 6 Alaimo MA, Smith JL, Roy RR, Edgerton VR. EMG activity of slow and fast ankle extensors following spinal cord transection. *J Applied Physiol* 1984; **56**: 1608–1613.
- 7 Hopman MTE, Dueck C, Monroe M, Phillips WT, Skinner JS. Limits to maximal performance in individuals with spinal cord injury. *Int J Sports Medicine* 1998; **19**: 98–103.
- 8 Ottenweller JE, Li M, Giglio W, Anesetti R, Pogach LM, Huang HFS. Alteration of Follicle-Stimulating Hormone and Testosterone regulation of messenger Ribonucleic Acid for Sertoli Cell proteins in the rat during the acute phase of spinal cord injury. *Biol Reprod* 2000; **63**: 730–735.
- 9 Gregory CM, Vandenborne K, Dudley GA. Metabolic enzymes and phenotypic expression among human locomotor muscles. *Muscle Nerve* 2001; **24**: 387–393.
- 10 Castro MJ, Apple DF, Staron RS, Campos GER, Dudley GA. Influence of complete spinal cord injury on skeletal muscle within 6 mo of injury. *J Applied Physiol* 1999; **86**: 350–358.
- 11 Brooke MH, Kaiser KK. Three ‘Myosin Adenosine Triphosphate’ systems: The nature of their pH ability and sulfhydryl dependence. *J Histochem Cytochem* 1970; **18**: 670–672.
- 12 Guth L, Samaha J. Qualitative differences between Actomyosin ATPase of slow and fast mammalian muscle. *Exp Neurol* 1969; **25**: 138–152.
- 13 Staron RS, Pette D. Correlation between myofibrillar ATPase activity and myosin heavy chain composition in rabbit muscle fibers. *Histochemistry* 1986; **86**: 19–23.
- 14 Blanco CE, Sieck GC, Edgerton VR. Quantitative histochemical determination of succinic dehydrogenase activity in skeletal muscle fibers. *Histochem J* 1988; **20**: 230–243.
- 15 Martin TP, Vailas AC, Durivage JB, Edgerton VR, Castleman KR. Quantitative histochemical determination of muscle enzymes: biochemical verification. *J Histochem Cytochem* 1985; **33**: 1053–1059.
- 16 Grossman EJ, Roy RR, Talmadge RJ, Zhong H, Edgerton VR. Effects of inactivity on myosin heavy chain composition and size of rat soleus fibers. *Muscle Nerve* 1998; **21**: 375–389.
- 17 Roy RR, Baldwin KM, Edgerton VR. The plasticity of skeletal muscle: Effects of neuromuscular activity. *Exerc Sport Sci Rev* 1991; **19**: 269–312.
- 18 Gordon T, Pattullo MC. Plasticity of muscle fiber types and motor unit types. *Exerc Sport Sci Rev* 1990; **21**: 331–362.
- 19 Tenover JS. Effects of testosterone supplementation in the aging male. *J Clin Endocrinol Metabol* 1992; **75**: 1092–1098.
- 20 Bhasin S, Storer TW, Berman N, Callegari C, Clevenger BA, Phillips J, Bunnell T, Tricker R, Shirazi A, Casabari R. The effects of supraphysiological doses of testosterone on muscle size and strength in men. *New England J Med* 1996; **335**: 1–7.
- 21 Bauman WA, Spungen AM, Adkins RH, Kemp BJ. Metabolic and Endocrine changes in persons with spinal cord injury. *Asst Technologies* 1999; **11**: 88–96.
- 22 Dudley GA, Castro MJ, Rogers S, Apple Jr DF. A simple means of increasing muscle size after spinal cord injury. *Eur J Appl Physiol* 1999; **80**: 394–396.