Nandrolone Decanoate and Load Increase Remodeling and Strength in Human Supraspinatus Bioartificial Tendons

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Background: To date, no studies document the effect of anabolic steroids on rotator cuff tendons.

Study Design: Controlled laboratory study.

Hypothesis: Anabolic steroids enhance remodeling and improve the biomechanical properties of bioartificially engineered human supraspinatus tendons.

Methods: Bioartificial tendons were treated with either nandrolone decanoate (nonload, steroid, n = 18), loading (load, nonsteroid, n = 18), or both (load, steroid, n = 18). A control group received no treatment (nonload, nonsteroid [NLNS], n = 18). Bioartificial tendons’ remodeling was assessed by daily scanning, cytoskeletal organization by staining, matrix metalloproteinase–3 levels by ELISA assay, and biomechanical properties by load-to-failure testing.

Results: The load, steroid group showed the greatest remodeling and the best organized actin cytoskeleton. Matrix metalloproteinase–3 levels in the load, steroid group were greater than those of the nonload, nonsteroid group (P < .05). Ultimate stress and ultimate strain in the load, steroid group were greater than those of the nonload, nonsteroid and nonload, steroid groups (P < .05). The strain energy density in the load, steroid group was greater when compared to other groups (P < .05).

Conclusions: Nandrolone decanoate and load acted synergistically to increase matrix remodeling and biomechanical properties of bioartificial tendons.

Clinical Relevance: Data suggest anabolic steroids may enhance production of bioartificial tendons and rotator cuff tendon healing in vitro. More research is necessary before such clinical use is recommended.

Keywords: anabolic steroids; supraspinatus tendon; mechanical loading; material properties; bioartificial tendons

Inappropriate anabolic steroid use is widespread among competitive athletes. The consumption of these synthetic derivatives of testosterone is perceived as a method to improve strength and athletic performance. Modern anabolic steroids maintain the anabolic effects of testosterone such as growth promotion and tissue repair while minimizing its androgenic effects—the production of male secondary sex characteristics.

The mechanism of action of anabolic steroids is related either to nitrogen balance and protein synthesis or to activation of androgen receptors in skeletal muscles. These androgen receptors, once activated, can lead to increased muscle mass and improved strength via increased protein synthesis and metabolism. An increased incidence of tendon rupture has been reported in athletes and bodybuilders who use anabolic steroids. Several clinical and animal studies suggest that anabolic steroids may be associated with detrimental
effects on the mechanical properties of connective tissue. In those studies, exercise (load) and anabolic agents were hypothesized to synergistically undermine the structural properties of tendon tissue and predispose the tendon to failure.

Although it has been demonstrated that anabolic steroids predispose tendons to injury by altering the crimp pattern and biomechanical properties of tendons, others have suggested that tendons are indirectly at risk for injury. The indirect effect is believed to be, in part, due to the increased force exerted by hypertrophied muscle tissue on weakened tendon attachments.

The detrimental effects of anabolic steroids have been well documented in both the scientific and lay literature. However, the clinical use of anabolic steroids is an important adjunctive intervention in patients with burns, extensive surgery, failure to thrive, and certain dwarfism syndromes.

The beneficial effects of anabolic steroids may have important implications in the treatment of difficult clinical entities in orthopaedic surgery. One such entity is the massive rotator cuff tear. Numerous authors have reported on the relatively poor results after repair of massive rotator cuff tears. Revision rotator cuff surgery has also been shown to have less successful outcomes than primary repairs.

Given the difficulty attaining uniformly good results in massive or recurrent postsurgical rotator cuff tears, it is reasonable to consider adjunctive methods to enhance the repair and healing process in these difficult clinical problems. Many authors have attempted to increase the strength of repair through maximizing the biomechanical integrity of the repair site. Others have attempted to augment the deficient tissue in massive or postsurgical tears with carbon filaments, allograft, or xenograft tissue.

To date, there have been no studies documenting the acute effect of anabolic steroids on human rotator cuff tendon cells. Given the known beneficial effects of anabolic steroid administration on protein synthesis, nitrogen balance, and catabolic states, we hypothesized that anabolic steroid administration in vitro would enhance matrix remodeling and improve the biomechanical properties of biotissue engineered human supraspinatus tendons (bioartificial tendons [BATs]). Our study is the first to analyze the effects of anabolic steroids in a human tendon cell model. The 3-dimensional tenocyte-populated BAT matrix is an ideal model to study cell interactions and responses to pharmacological agents and mechanical load. BATs can maintain viable cells and a tendon-like architecture for up to 1 month, thus allowing investigators significant time to observe the effects of a multitude of biochemical and biomechanical interventions.

METHODS

Partial samples of human supraspinatus tendons were harvested from the debrided tissue of 6 patients during open or arthroscopic surgical repair of medium and large chronic rotator cuff tears. There were 5 men and 1 woman with a mean age of 52 years (range, 34-66 years).

Preparation of Cell Cultures

Supraspinatus tendon cells were isolated from each specimen using a previously described technique. The specimens were minced into small pieces with a sterile scalpel blade and rinsed with nutrient Medium 199 (GIBCO-Invitrogen Corp, New York, NY) to remove red blood cells. The minced tendons were then digested with 0.1% collagenase in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma-Aldrich, St Louis, Mo) with antibiotics and HEPES buffer, pH 7.2 (GIBCO-Invitrogen Corp, New York, NY) for 10 minutes at 37°C with gentle agitation, to disaggregate the sample and release the cells. Cells were plated at 25 k cells/cm² and grown to quiescence for 3 to 6 days until confluent. The cultures were kept at 37°C in a 5% CO₂ humidified incubator, and the media were changed every third day. Treatment with 0.05% trypsin-EDTA solution (Sigma-Aldrich) for 5 minutes was used to passage the cultures. Each initial cell culture was passaged 2 to 5 times to obtain an adequate sample for BAT construction.

The cells were cultured in the Medium 199 with 10% fetal bovine serum (Sigma-Aldrich); 0.1 mM ascorbate-2-phosphate (Sigma-Aldrich); 15 mM sodium pyruvate (GIBCO-Invitrogen Corp); 20 mM HEPES buffer; pH 7.2 (GIBCO-Invitrogen Corp); Insulin-Transferrin-Sodium Selenite supplement (Roche Diagnostics GmbH, Mannheim, Germany); and penicillin (100 units/mL)–streptomycin (100 µg/mL)–amphotericin-B (0.25 µg/mL) antibiotics (GIBCO-Invitrogen Corp).

BAT Construction

BAT units were constructed by using a previously described technique. The isolated supraspinatus cells, at a concentration of 200 k cells per base-neutralized Vitrogen, contain 10% fetal bovine serum in DMEM. Circular TissueTrain Loaders were secured beneath each of the 6 wells of a flexible bottom of a linear TissueTrain culture plate (Flexcell Int, Hillsborough, NC) (Figure 1). For each construct, 155 µL of the cell-matrix mixture were dispensed into a 20×1 × 3-mm trough in the membrane after vacuum was applied to the flexible well bottom to draw the membrane downward. Flexible and inelastic nylon mesh anchors were bonded to the membrane at east and west poles. Tubs from each anchor (anchor stems) connected to each end of the trough acted as a bonding region for collagen gel and cells. The vacuum was released after gelation. Four experimental groups were used in the study: nonload, nonsteroid (NLNS, n = 18); nonload, steroid (NLS, n = 18); load, nonsteroid (LNS, n = 18); and load, steroid (LS, n = 18). Each group contained 3 BATs per patient.

Anabolic Steroid Administration

After a 48-hour quiescence period in which no anabolic agent was applied to the BAT cultures, the steroid groups were given a final concentration of 100 nM nandrolone decanoate (Deca-Durabolin 200 mg/mL, Organon Inc, West Orange, NJ). Dilutional analysis was performed to determine a concentration that was equivalent to the dose rec-
The drug contained 200 mg nandrolone decanoate USP per mL of sterile sesame oil with 5% benzyl alcohol as a preservative. The stock drug solution was diluted 1:1 with sterile dimethylsulfoxide. The steroid solution was made fresh daily, diluted in the culture medium to 100 nM, and dispensed into the culture medium. Control cultures received the vehicle alone of the appropriate dilution.

**Mechanical Loading of BATs**

No load was applied during the first 48 hours after BAT formation. After this quiescence period, an FX3000 FlexerCell Strain Unit (Flexcell Int) was used to mechanically load the cells. The 6-well linear TissueTrain plates were placed atop a vacuum manifold base plate with surrounding rubber gaskets, and the vacuum was applied to the undersurface of each well, deforming the rubber substrate downward to stretch it (Figure 2). A 6-place loading station with arcangle-shaped (a rectangular geometric shape with short, curved ends) loading posts was placed beneath each TissueTrain culture plate. The long axis of the arcangle loading post was perpendicular to the long axis of the BAT, leaving stems of the flexible anchors connecting with the trough receiving cells and collagen gel. Downward deformation of the membrane-attached anchors transformed uniaxial load to the BATs. The FX3000 computer controlled the load regime (1% uniaxial strain at 1 Hz in a regime of 1 hour on, 23 hours rest, for 7 days).

**Figure 1.** Schematic of the function of the linear TissueTrain culture plate. Supraspinatus cells within a collagen matrix were placed on the rubber membrane overlying the trough bay. A vacuum applied to the undersurface of the rubber membrane provided the mechanical load to the system.

**Figure 2.** Enlarged schematic of the function of the Bioflex well. Vacuum traction applied to the rubber membrane on the undersurface of the well provided mechanical load to the cell-matrix culture.
Dimensional Analysis

The total surface area as well as the width of the BATs were measured daily to estimate the remodeling index of the BAT constructs. The kinetics of BAT contraction were assessed by digital scanning and analyzing the captured images using IMAQ Vision Builder dimensional analysis software (National Instruments Corp, Austin, Tex). We measured only 2 dimensions (surface area) of the 3-dimensional BAT constructs. Given that BATs become cylindrical during remodeling, the aspect ratio of their midsection was equal to 1, substantiating the 2-dimensional image in our experiments. Furthermore, we compared our results with those of the cross-sectional area analysis to reveal any differences in the measurement techniques.

4',6-Diamidino-2-Phenylindin Dihydrochloride and Rhodamine Phalloidin Staining

On day 7 of treatment, the time period by which BATs had achieved a mature, organized, and consolidated collagen matrix, BATs were collected and stained with 4',6-diamidino-2-phenylindin dihydrochloride (DAPI) and rhodamine phalloidin. DAPI binds to DNA and stains nuclei, whereas rhodamine phalloidin was used to stain the actin cytoskeleton.30,53 Initially, cells were rinsed twice in phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde at room temperature for 30 minutes. Then, the fixed cells were permeabilized with 0.2% Triton X-100 in PBS containing 0.5% bovine serum albumin (Sigma-Aldrich) and kept in the dark, at room temperature, for 30 minutes.

Matrix Metalloproteinase–3 Measurements

Daily samples of the media were examined for matrix metalloproteinase–3 (MMP-3) using the Biotrak MMP-3 Human ELISA System (Amersham Pharmacia Biotech, Piscataway, NJ). The assay was based on a 2-site ELISA “sandwich” format, which provided a quantitative assessment of the MMP-3 enzyme. The concentration of MMP-3 in the samples was determined by interpolation from a standard curve generated by plotting the mean optical density against ng/mL standard.
within the Flexcell Vision Acquisition System program (Flexcell Int, Hillsborough, NC). This software program used the diameters measured from each camera to calculate the specimen’s cross-sectional area. All data were analyzed in the LabVIEW software (National Instruments Corp).

Five parameters were determined from the stress-strain and force-elongation curves: (1) load to failure, (2) ultimate stress, (3) ultimate strain, (4) modulus of elasticity, and (5) strain energy density. The modulus of elasticity (Young’s modulus) was measured from the slope of the linear part of the stress-strain curve. The strain energy density was the strain energy to ultimate load divided to grip-to-grip distance and to the cross-sectional area of each BAT.

Statistical Analysis

Statistical analysis was performed using SigmaStat software (SPSS Inc, Chicago, Ill). Data were analyzed with a 1-way analysis of variance and the Student $t$ test. Non-parametric 1-way analysis (Kruskal-Wallis) was also used for arbitrary distributions. $P < .05$ was selected to indicate statistical significance.

RESULTS

Dimensional Analysis

There was a rapid decrease in width and the total area of all BAT units during the first 2 days of culture. The rate of contraction was the same in all groups during this period (Figure 4). By the fourth day of treatment, BATs in the LS group showed a statistically significant decrease in width compared to the other groups ($P < .05$). NLNS, nonload, nonsteroid; NLS, nonload, steroid; LNS, load, nonsteroid.

Figure 4. Bioartificial tendons were scanned daily to estimate their remodeling rate. The load, steroid (LS) group (right column) demonstrated the greatest rate of remodeling. NLNS, nonload, nonsteroid; NLS, nonload, steroid; LNS, load, nonsteroid.

Figure 5. Dimensional analysis of bioartificial tendons (BATs) representing change in width over time. From day 4 of treatment, BATs in the load, steroid (LS) group showed a significant decrease in width compared to the other groups ($P < .05$). NLNS, nonload, nonsteroid; NLS, nonload, steroid; LNS, load, nonsteroid.

The load, steroid (LS) group showed a statistically significant decrease in width compared to the NLNS (34%), NLS (30.7%), and LNS (36.7%) groups ($P = .022$) (Figure 5). Also, BATs in the LS group demonstrated a significant decrease in surface area measurement when compared to the NLNS (17.78%), NLS (17.78%), and LNS (14.1%) groups ($P < .05$) (Figure 6). By the seventh day of treatment, BATs in the LS group showed the greatest reduction in width compared to the NLNS (47.9%), NLS (46.5%), and LNS (59.2%) groups ($P < .05$). Also, BATs in the LS group showed the lowest values...
for surface area when compared to the NLNS (27.94%), NLS (30.5%), and LNS (30.5%) groups ($P < .05$). The cross-sectional area of the LS group was half the size of that of all other groups ($P < .05$) (Figure 7).

**DAPI and Rhodamine Phalloidin Staining**

The BATs incubated in the presence of anabolic steroid (NLS, LS) had a more organized cytoskeleton than control BATs, with actin filaments arranged in an elongated manner corresponding to the direction of collagen fiber alignment. BATs in group LS, subjected to both steroid and load, showed the highest level of actin fiber elongation (Figure 8).

**MMP-3 Expression**

All experimental groups showed peak MMP-3 levels on the second day of treatment (Figure 9). On the second and third days of treatment, MMP-3 levels of the LS group were significantly greater than those for the NLNS group (24.42%) ($P = .029$). By the sixth day of treatment, all MMP-3 levels returned to baseline with no significant differences observed among the groups.

**Biomechanical Testing**

Biomechanical data analyses are shown in Table 2. BATs in the LS group demonstrated a significantly greater ultimate stress when compared to the NLNS (61.4%, $P = .032$) and NLS (58.0%, $P = .039$) groups (Figure 10). Also, BATs...
in the LS group showed significantly greater ultimate strain when compared to results for the NLNS (37.3%, $P = .014$) and the NLS (33.5%, $P = .02$) groups (Figure 11). The strain energy density in the LS group was significantly greater than the energy volume in the NLNS (115%), NLS (96.9%), and LNS (71%) groups ($P < .05$) (Figure 12).

DISCUSSION

Even though numerous authors have questioned the role of anabolic steroids and load on skeletal muscle, few have reported on the effect of these factors on collagen structures. Alway and Starkweather\textsuperscript{1} studied the effects of anabolic steroids and stretch on the anterior latissimus dorsi muscles of Japanese quail. They found that anabolic steroids did not act synergistically with load to increase muscle mass or induce collagen fiber formation. In contrast, many studies have suggested that anabolic steroids provide a positive effect on muscle weight and muscle development in mammals.\textsuperscript{19,22,27}

Wood et al\textsuperscript{60} treated rat tendons with anabolic steroids and exercise and found alterations in collagen structure. The authors demonstrated that the length of the collagen crimp pattern was shortened, whereas the crimp angle was greater. They concluded that these dysplastic collagen fibrils might render tendons weaker and more prone to injury. Michna\textsuperscript{38} came to the same conclusion, suggesting that altered cross links might weaken the collagen of mouse tendons treated with anabolic agents and exercise. These findings are in contrast with those of Miles et al.,\textsuperscript{39} who did not observe any anomalies in collagen structure when mouse tendons were treated with anabolic steroids and stretch.

Even less data are available regarding the effects of exercise and anabolic steroid administration on human collagen structures. Most publications relating chronic steroid abuse to tendon injury consist of anecdotal reports and case studies.\textsuperscript{11,29,31,33,54} No convincing evidence of the
beneficial or detrimental effects of anabolic steroids on human nonmuscular connective tissues has been reported in controlled laboratory investigations.

In our study, the LS group showed the greatest and earliest rate of remodeling of the collagen-I matrix with a statistically significant difference observed on day 4 of treatment ($P < .05$). The biochemical analysis of collagen content and cross-links may be the next step in the investigation of the mechanism involved in the changes attributed to exercise and anabolic steroid administration.

We also demonstrated that the combination of loading and steroid administration led to the development of a more organized actin cytoskeleton when compared to the other 3 groups. Actin maintains the integrity of the longitudinal tendon cell rows and generates contractile forces within the tendon. In our study, the LS group demonstrated the greatest number of actin filaments. Stress fibers were arranged in the most elongated manner corresponding to the direction of collagen fiber alignment. The combination of anabolic steroid and load enhanced the integrity of the bioartificial constructs and their contractile capability. Also, because the actin network is involved in the generation of load-related signals, the synergistic effect of anabolic steroid and load may improve the communication to neighboring cells via the gap junction network.

Woo et al reported a positive effect of long-term exercise on the mechanical properties of swine tendon. The stiffness and the ultimate load of the tendons increased as a result of exercise training. However, the animals were not given anabolic steroids. Miles et al suggested that the use of anabolic steroids produced a stiffer tendon that failed with less elongation. Michna combined an exercise regimen with anabolic steroid administration and found that the biomechanical properties of the tendons deteriorated. They attributed their findings to degenerative changes at the musculotendinous junction.

Our study revealed that anabolic steroid administration alone did not affect BAT flexibility and strength. However, the combination of stretch and anabolic steroid administration enhanced the ultimate stress and strain of the bioartificial supraspinatus tendon when compared to the NLNS and NLS groups. The combination of these 2 treatments also led to an increase in the elastic modulus when compared to the NLS group, thus demonstrating an improvement in flexibility when compared to the BATs treated only with steroid. The LS treatment group was not found to have greater load to failure when compared to the other groups, likely due to the number of specimens available for analysis. However, because the strength of a tendon is proportional to its elastic modulus, one might infer that the synergistic effect of anabolic steroid and load increased the strength of the BATs. In contrast, anabolic steroid alone or stretching alone did not dramatically alter the biomechanical properties of the tendons when compared to the control NLNS group. Finally, the combination of loading and anabolic steroid administration significantly increased the energy absorbed before mechanical failure.

MMP-3 (stromelysin-1) is an important enzyme in tissue repair and matrix remodeling, and increased expression has been associated with cartilage destruction in osteoarthritis and degenerative disc disease. In tendons, MMP-3 is likely involved in matrix remodeling by activating MMP-1 to degrade collagen. Other authors have shown that reduced activity of MMP-3 may represent a failure of the normal matrix remodeling process. An increase in proteolytic activity may lead to tendon degeneration and rupture.

In our study, the LS group demonstrated the earliest increase and highest levels of MMP-3, suggesting that the combination of loading and anabolic steroid accelerates matrix remodeling. During the first 5 days of treatment, MMP-3 protein levels in the LS group were greater than in the NLNS group. However, after the fifth day of treatment, MMP-3 protein levels in the LS group were greater than in the NLNS group. However, after the fifth day of treatment, the levels of MMP-3 in all groups returned to baseline. This finding suggests that the increase of MMP-3 protein secretion was transient. The cause of the transient increase in MMP-3 levels may be the activation of tissue inhibitors of MMPs. These natural inhibitors regulate the MMP activity to avoid excessive tissue damage.

There were several limitations to our study. Our findings may be explained by deviations from previously described laboratory techniques. Previous investigators used animal models in vivo or in vitro. Some authors used bundles of 3 or 4 preconditioned tendon fascicles for testing, whereas others used the whole tendon. We used bioengineered human tendons, which is a unique model to study specific tendon cell-matrix interactions, responses to mechanical loading, and reactions to pharmacological agents.
A second limitation to our study was that we tested the experimental groups after only 7 days of treatment. Rodeo et al studied the effects of bone morphogenetic protein-2 on tendon healing in a bone tunnel. Although the authors noted an acceleration in healing at 2 weeks, there was no difference in ultimate strength between the experimental or control groups at 4 or 8 weeks posttreatment. Increasing the time between treatment and analysis, or increasing the time of treatment with anabolic steroid and load, will be another avenue of study to determine whether the effects will remain significant over a longer period of time.

Another limitation of our study is that our model fails to account for the complex interaction between humeral bone, subacromial bursa, and tendon during rotator cuff healing. Uthhoff et al, in a rabbit model, investigated the origin of cells during healing of the supraspinatus tendon bone, subacromial bursa, and tendon during rotator cuff healing. Uhthoff et al, in a rabbit model, investigated the origin of cells during healing of the supraspinatus tendon to the repair site. Although our study did not specifically address the histologic or biomechanical effects of anabolic steroid at the repair site, an improvement in the intrinsic biomechanical properties of the rotator cuff tendon may allow it to better withstand tension and suturing in the tendon itself.

CONCLUSIONS

The combination of loading and anabolic steroid administration significantly enhanced BAT remodeling (on the basis of surface area measurements and cross-sectional analysis), improved flexibility by increasing maximum deformability and the ultimate stress sustained before mechanical failure, and increased the energy absorbed before failure when compared to untreated controls. Also, the production of greater levels of MMP-3 in this group suggests that anabolic steroid coupled with stretch may enhance collagen matrix remodeling. Finally, the synergistic effect of the combined treatment led to a better organized cytoskeleton when compared to the other groups.

We have presented a 3-dimensional, in vitro rotator cuff tendon model that may have potential applications in determining the effects of anabolic steroids and other agents on tendon healing. The results of this study must be interpreted with caution, and further in vitro and in vivo research is needed to confirm these preliminary findings.

ACKNOWLEDGMENT

The authors wish to thank Paul Weinhold (Department of Biomedical Engineering) for assistance with the biomechanical data analysis and Flexcell International Corporation for use of their fluorescence microscope and micro-mechanical-testing machine.

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