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Research Article

The Synergistic Effect of Anabolic Steroid and Loading on Intercellular Calcium Signaling Via Gap Junctions in Human Supraspinatus Tendon Cells

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ABSTRACT

Objective: We hypothesized that anabolic steroid administration would act synergistically with substrate strain in two-dimensional cultures of human supraspinatus tendon cells, to upregulate the expression of connexin-43 and to increase the Ca²⁺ wave propagation through gap junctions.

Methods: Supraspinatus tendon cells were isolated intra-operatively from human specimens during shoulder arthroscopy. Cells were plated in two-dimensional spot cultures and arranged into four experimental groups: 1) non-load, non-steroid (NLNS, n=12 wells); 2) non-load, steroid (NLS, n=12 wells); 3) load, non-steroid (LNS, n=12 wells); and 4) load, steroid (LS, n=12 wells) in order to produce bioartificial tendons (BATs). The *load groups* were stretched in culture plates and the steroid groups were given nandrolone decanoate. When BATs were macro- and microscopically mature, at five days, they were evaluated with immunocytochemistry for connexin-43 staining, fluorescence microscopy for calcium imaging and mechanical stimulation with a micropipette tip manipulation for calcium propagation. Dose response test was performed in order to establish any relation between nandrolone decanoate dose and calcium signaling response. ATP was applied to the spot culture cells from all groups and all patients to determine if the cells were sensitive to extracellular ATP.

Results: Load-steroid group demonstrated the greatest density of cxn43 in comparison to all other groups. There were no significant differences between the groups considering the percentage of cells responding after mechanical stimulation (cell recruitment). The cells of load-steroid group showed a significantly greater mean peak [Ca²⁺]_{ic} compared to the values of the other groups (p<0.05). The propagation time was significantly decreased in the LS group compared with the other groups (p<0.05). There were no significant differences between the groups considering the number of cells that were responding spontaneously prior to stimulation or the number of responding cells that were oscillating after the stimulation.

Conclusion: Nandrolone decanoate and loading seem to have a synergistic effect on the upregulation of the gap junction protein cxn43 enhancing calcium signaling via gap junctions. Consecutively, anabolic steroid administration and load may enhance the formation of a better-organized cytoskeleton and particularly the actin stress monofilaments.

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Introduction

In all multicellular organisms, survival depends on an elaborate intercellular communication network that coordinates the growth, differentiation and metabolism of the multitude of cells in diverse tissues and organs. A common cell-to-cell communication pathway is the propagation of an intercellular Ca^{2+} signal [1]. Mechanical stimulation by micropipette indentation propagates a concentrically expanding Ca^{2+} signal [2]. This response has been observed in many cells' types including tendon cells [3-9]. Intercellular Ca^{2+} signaling is mediated by several different mechanisms including the release or secretion of second messengers such as ATP, the intercellular diffusion of second messengers such as inositol triphosphate (IP_3) and Ca^{2+} or a combination of these mechanisms [5, 10, 11]. Gap junctions are key components for the propagation of intercellular Ca^{2+} signals [11, 12].

Intercellular communication mediated by gap junctions plays an important role in a variety of cellular processes including cell differentiation, growth and proliferation, electrical activation of the heart and of smooth muscles, in neuronal signaling, but also in hormone secretion, auditory function, wound healing, lens transparency and immune functions [13]. Gap junctions are intercellular channels that connect the cytoplasm of two adjacent cells and thereby provide a direct diffusion pathway between the cells [1]. A gap junction is formed when a cylindrical hemichannel or connexon in one cell couples to another in an adjacent cell to create an aqueous pore between the two cells [13]. The connexon itself is composed of a hexameric assembly of proteins called connexins (Cx). There are 21 different connexin genes identified in the human genome. Cxs can either form connexons by themselves or, in some cells, they can interact with different isoforms to form heteromeric connexons [14]. The permeability and gating characteristics of gap-junction channels depend on the connexin isoforms and on their post-translational modifications [13]. Cxs have been classified according to their predicted molecular weight and named accordingly. Connexin 43 (Cxn43), a 43kDa monomer, represents the most ubiquitous connexin and is important for the propagation of intercellular Ca^{2+} signals [1, 12, 15].

Tendons are connective tissue structures that connect muscles to bone and thus experience high tensile loading. Tendon cells have the potential to use direct cell-to-cell communication to detect and coordinate responses to load [16]. In vitro, tendon cells can propagate a Ca^{2+} wave via gap junctions in response to mechanical stimulation by micropipette indentation [9]. They express the gap junction protein cxn43 and modify its expression in response to load [17]. In vitro, tendon cells upregulate collagen and DNA synthesis 16 as well as cxs expression during cyclic tensile load, and inhibition of gap junction function leads to loss of this load response [16, 18]. Consequently, gap junctions coordinate the behavior of tendon cells in their key roles of load sensing and matrix modification.

Previous studies have shown that the expression of cxn43 has been changed during inflammatory states induced by various types of injury and that downregulation of cxn43 expression can be associated with cellular dysfunction and apoptosis [19-23]. There are many reports in the literature about the deleterious effects of anabolic steroids on tendons, however, as most studies involve their systemic administration, it is

extremely difficult to differentiate between presumable direct effects and the indirect effects exerted by the increased strain caused by the hypertrophied muscles [24-26].

Dihydrotestosterone, a potent testosterone metabolite, has been shown to exert direct effects in human cultured tenocytes, and it has been previously demonstrated that nandrolone decanoate acts synergistically with loading to increase matrix remodeling and the biomechanical properties of bioartificial tendons [27, 28].

We hypothesized that anabolic steroid administration would act synergistically with substrate strain in two-dimensional cultures of human supraspinatus tendon cells, to upregulate the expression of cxn43 and to increase the Ca^{2+} wave propagation through gap junctions.

Methods

I Tissue Harvesting

Partial samples of human supraspinatus tendons were harvested from the debrided tissue of four patients during surgical rotator cuff repair. Patients were all males with a mean age of 52 years (range= 34-66 years).

II Cell Isolation

Supraspinatus tendon cells were isolated from each specimen using a previously described technique [29]. The specimens were minced into small pieces with a sterile scalpel blade and rinsed with nutrient Medium 199 (GIBCO-Invitrogen Corp, New York, NY, USA) 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, USA) with antibiotics and HEPES, buffer pH 7.2 (GIBCO-Invitrogen Corp, New York, NY, USA) for 10 minutes at 37°C with gentle agitation, to disaggregate the sample and release the cells. Cells were plated at 25k cells/cm² and grown to quiescence for 3-6 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, St. Louis, MO, USA) for 5 minutes was used to passage the cultures. Each initial cell culture was passaged 2-5 times to obtain an adequate sample for spot culture construction.

III Cell Culture

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

IV Spot-Cultures Preparation

Cells were plated in two-dimensional spot cultures at 2-3k cells per 10 µl spot in Bioflex™ culture plates (Flexcell Int., Hillsborough, NC,

USA). Each well contained two spots, and three wells were used for each treatment group per patient. The spot cultures were arranged into four experimental groups: 1) non-load, non-steroid (NLNS, n=12 wells); 2) non-load, steroid (NLS, n=12 wells); 3) load, non-steroid (LNS, n=12 wells); and 4) load, steroid (LS, n=12 wells). The cells remained in quiescence for 48 h post plating and then treated for five days.

V Anabolic Steroid Administration

After a 48-h quiescence period, where no anabolic agent was applied to the spot cultures, the *steroid groups* were given a final concentration of 100 nM nandrolone decanoate (Deca-Durabolin 200 mg/ml, Organon Inc.). Results of dilution studies were used to determine this dosage that was equivalent to the clinical prescription dose. The drug contained 200 mg nandrolone decanoate USP per ml of sterile sesame oil with 5% benzyl alcohol as preservative. The stock drug-solution was diluted 1:1 with sterile dimethylsulfoxide (DMSO). The steroid solution was made fresh daily, diluted in culture medium to 100 nM and dispensed into the culture medium. Control cultures received vehicle alone of the appropriate dilution.

VI Loading

The FX3000 FlexerCell Strain Unit™ (Flexcell Int., Hillsborough, NC, USA) was used for stretching the cells. The Bioflex™ culture plate consisted of a six-well culture plate where each well had a collagen-coated rubber growth surface. The plates were placed atop a vacuum manifold baseplate with surrounding rubber gaskets, and vacuum applied to the undersurface of each well, deforming the rubber substrate downwards to stretch it. Cultures were thus exposed to biaxial strain. The FX3000 computer controlled the intensity and duration of vacuum 1% equibiaxial strain at 1 Hz in a regime of 1 hour on, 23 hours resting, for 5 days. Media were changed daily 1 hour prior to stretching.

VII Immunocytochemistry

For cxn43 staining, cells were rinsed twice in PBS and fixed with 3.7% formaldehyde at room temperature for 15 min. Then, the fixed cells were permeabilized with 0.2% Triton X-100 in PBS containing 0.5% bovine serum albumin (BSA) (Sigma - Aldrich, St. Louis, MO, USA) and kept in dark, at room temperature, for 40 min. Cells were incubated with rabbit anti-connexin 43 antibody (Zymed Laboratories Inc, San Francisco, CA, USA) (dilution 1:100) at room temperature, for 2 hours. Then, cells were incubated with rhodamine-red™ - X goat anti - rabbit IgG (H + L) antibody (Molecular Probes Inc, Eugene, OR, USA) (dilution 1:500) in the blocking buffer, at room temperature, for 1 hour. All cells were mounted on a glass slide in a mounting medium (Biomedica Corp., Foster City, CA, USA). The preparations were examined using the Olympus BX60F5 microscope equipped with an Olympus FV12 digital camera and an Olympus MicroSuite™ B3SV image capture software (Olympus Optical Co, Japan).

VIII Calcium Imaging

On day five of treatment, cultures were rinsed twice with Earles' Balanced Salt Solution (EBSS), loaded with 5 μ M fura-2AM, a calcium fluorescent indicator (Molecular Probes Inc, Eugene, OR, USA) and incubated in the dark for 45 minutes at room temperature. Cultures were

then rinsed twice with EBSS to remove residual fura-2AM. The silicone membranes in the wells of the Bioflex™ plates excised with a scalpel and mounted in an 83 mm diameter plastic culture plate on the stage of the microscope. An Olympus BX- 51TRF upright fluorescence microscope (Olympus Optical Co, Japan) was used, equipped with a Lambda DG- 4 wavelength switcher and light guide (Sutter Instruments Corp, Novato, CA, USA), a CoolSNAP-fx CCD camera (Photometrics, Tucson, AZ, USA) and an Isee analytical imaging software (Inovis, Inc.) The cells were subjected to mechanical stimulation by micropipette indentation and intracellular calcium concentration was quantitated. Intracellular calcium levels were measured using a ratio imaging method, which entails exciting cells alternately at 340 and 380 nm observing emitted fluorescence at 510 nm and above and comparing ratios to known Ca^{2+} standards. Images were subjected to background subtraction to correct for non-uniformity across the fluorescence field. Basal Ca^{2+} values were obtained for 60 sec prior to mechanical stimulation.

IX Mechanical Stimulation

Single cells were mechanically stimulated by transient deformation of the membrane using a glass micropipette (tip diameter about 1 μ m) mounted on an MX7600L motorized manipulator (Siskiyou Design Instruments, Grants Pass, OR, USA) and guided by computer software (Siskiyou Design Instruments, Grants Pass, OR, USA).

X Dose Response Test

Dose response test was performed in order to establish any relation between nandrolone decanoate dose and calcium signaling response. Spot cultures from all groups and patients were prepared as previously mentioned and cells were stimulated by the addition of 1, 10, 100, and 1000 nM nandrolone decanoate final concentrations diluted in EBSS. Cells were stimulated with nandrolone decanoate once and the above described ratio-imaging technique was used to quantitate the $[\text{Ca}^{2+}]_{\text{ic}}$.

XI ATP Test

ATP was applied to the spot culture cells from all groups and all patients to determine if the cells were sensitive to extracellular ATP. Cells were stimulated by the addition of 1 μ M ATP final concentration diluted in EBSS. The appropriate concentration of ATP for stimulation was determined from an ATP dose response experiment with concentrations starting at 0.1 μ M and increasing to 100 μ M. Cells were stimulated with ATP once and the above described ratio-imaging technique was used to quantitate the $[\text{Ca}^{2+}]_{\text{ic}}$.

XII Calcium Signaling Parameters

Analyzing the calcium signaling data, many parameters were calculated: a) cell recruitment, b) mean peak $[\text{Ca}^{2+}]_{\text{ic}}$, c) mean average response, d) propagation time, e) response rate, e) spontaneously responding cells, and f) oscillating cells. All data were normalized to the mean basal level of $[\text{Ca}^{2+}]_{\text{ic}}$ to account for the variations from daily set up errors in light intensity and in the amount of Fura-2AM uptake. The basal level was determined by averaging the $[\text{Ca}^{2+}]_{\text{ic}}$ of the prior to mechanical stimulation condition over 60 sec. A responding cell was mathematically

defined as a cell that increased its $[Ca^{2+}]_{ic}$ four standard deviations over its basal level.

Cell recruitment was expressed by the percentage of cells responded to mechanical stimulation. This percentage was calculated by dividing the number of responding cells by the total number of cells of each trial. The mean peak $[Ca^{2+}]_{ic}$ referred to the average of the maximum $[Ca^{2+}]_{ic}$ post-stimulation for all responding cells. The mean average response for each cell referred to the $[Ca^{2+}]_{ic}$ measured every 1 sec and averaged from the time of initial rise until the return to basal level post-stimulation. The propagation time was determined, as the time required propagating a Ca^{2+} wave from a stimulated cell to an adjacent unstimulated cell. The stimulated cell is defined as the first responding cell at the time of mechanical stimulation. The time of arrival was defined as the time when the slope of the $[Ca^{2+}]_{ic}$ line of the cell changed from baseline to a new increase. The response rate of each cell was measured from the slope of the linear part of the $[Ca^{2+}]_{ic}$ graph. The mean response rate of each trial expresses the rate of intracellular calcium increase of each group. A spontaneous response was determined as the transient increase in $[Ca^{2+}]_{ic}$ prior to mechanical stimulation and considered to be a non-mechanically response. Ca^{2+} oscillations were defined as post-stimulation repetitive intracellular Ca^{2+} waves demonstrating a synchronous or asynchronous increase and decrease of the $[Ca^{2+}]_{ic}$ [30-35].

XII Statistical Analysis

Statistical analysis of data was performed using SigmaStat statistical software program (SPSS Science, Chicago, IL, USA). Level of statistical

significance was set at $p < 0.05$ and determined with a One-Way ANOVA and a Student's *t*-test. Data sets are representative of each patient cell isolate.

Results

I Immunocytochemistry

All cell processes of all groups had bright green colored foci of immunolabel for *cxn43* localized at the contact areas with other cells indicating the presence of gap junctions. The foci were clearly visible between cell bodies and between cell processes. *Cnx43* appeared to be more prominent towards the cell processes. LS group demonstrated the greatest density of *cxn43* in comparison to the other groups.

II Calcium Signaling

All concentrations of nandrolone decanoate induced an increase in $[Ca^{2+}]_{ic}$ when compared to basal values. The greatest and fastest increase was observed between 100 and 1000 nM nandrolone decanoate; therefore 100 nM nandrolone decanoate was chosen for testing the response of cells. Also, the ATP test was positive for all groups and all patients rendering the human supraspinatus tendon cells capable to increasing their $[Ca^{2+}]_{ic}$ after stimuli. This observation allowed us to consider these cell cultures appropriate for our study.

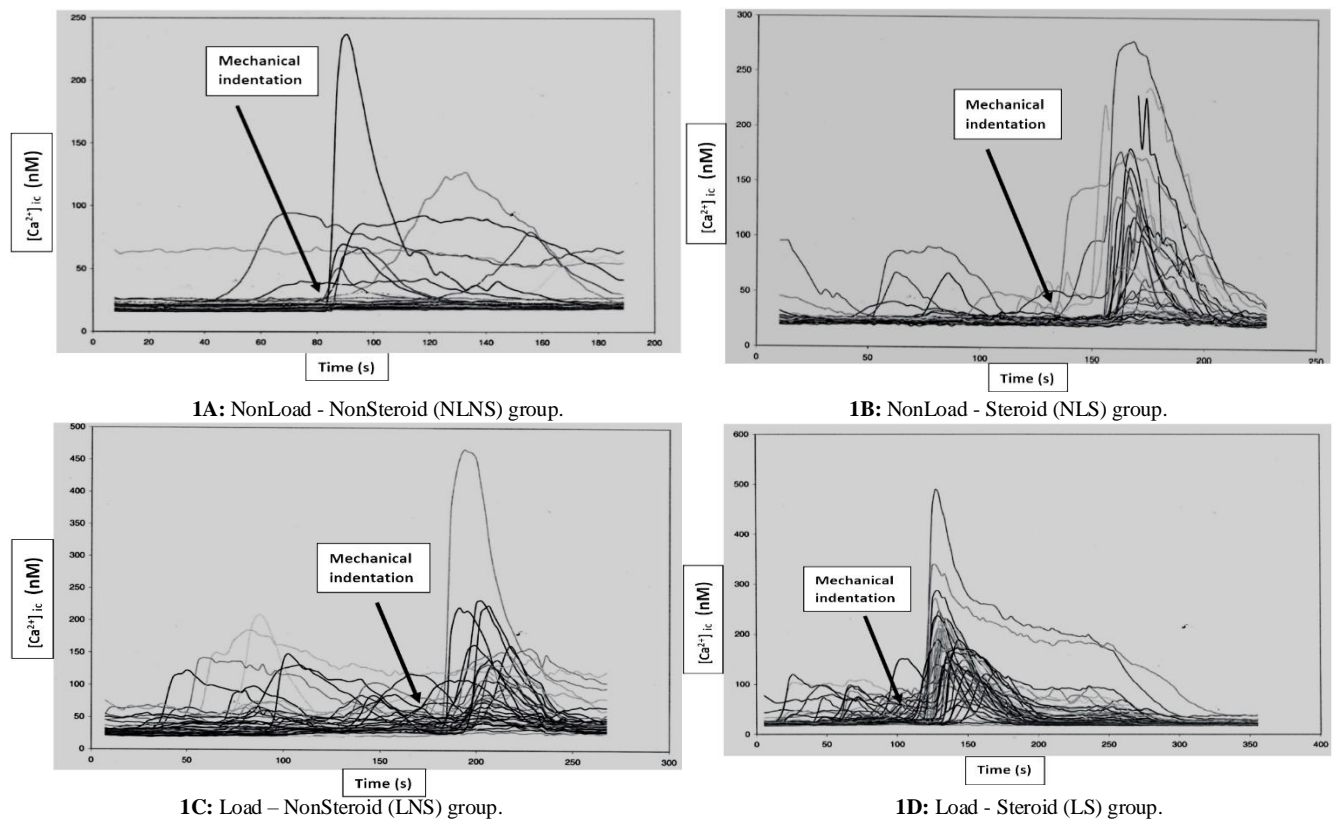


Figure 1: A-D) Mechanical stimulation by micropipette indentation, after a period of cell quiescence, induces a calcium wave that propagates to adjacent tendon cells. Once the cells are chemically stimulated there is a rapid increase in $[Ca^{2+}]_{ic}$. Each line represents an individual cell's response to stimulation.

There were no significant differences between the groups considering the percentage of cells responding after mechanical stimulation (cell recruitment). The cells of LS group showed a significantly greater mean peak $[Ca^{2+}]_{ic}$ compared to the values of the other groups ($p < 0.05$) (Figure 1). Also, LNS group showed significantly greater mean peak $[Ca^{2+}]_{ic}$ compared to the values of NLNS group ($p = 0.026$). The propagation time was significantly decreased in the LS group compared with the other groups ($p < 0.05$). The NLNS group showed increased time of Ca^{2+} wave propagation while loading alone (LNS group) and anabolic steroid administration alone (NLS group) decreased the time when acting independently. However, the differences between NLNS, NLS and LNS groups considering the propagation time were not statistically significant. There were no significant differences between the groups considering the number of cells that were responding spontaneously prior to stimulation or the number of responding cells that were oscillating after the stimulation.

Discussion

Physiotherapy protocols after tendon injury or repair are designed to improve healing, reduce adhesion formation and increase range of motion of the involved joint. Manipulation of the mechanical environment of the healing tendon may exert a biologic effect through the mechanotransduction mechanism and multiple studies have demonstrated that eccentric exercise programs are effective for the treatment of tendinopathy [36].

Parallel to motion therapy, on molecular and biochemical level, processes such as cell migration and division and matrix synthesis must provide a biomechanically sound tendon [37]. To coordinate these processes, mechanical and chemical signals must be communicated between cells through a variety of pathways utilizing mechanosensory complexes and second messengers [38, 39]. Calcium (Ca^{2+}) is one of the primary second messengers utilized by cells to transduce mechanical to biochemical signals via gap junctions regulating multiple cell functions including gene transcription, cell growth and proliferation and apoptosis [13].

The primary purpose of our study was to determine the effect of anabolic steroid administration and stretching on the intercellular calcium signaling via gap junctions. Given that gap junctions couple tendon cells electrically and chemically and coordinate responses to mechanical load, the administration of anabolic steroid may alter their role on detecting damages or allowing metabolic cooperation between cells [16, 40]. An increase of gap junction protein expression and intercellular communication could result in positive attributes after tendon injury.

There are no data about the role of connexins and gap-junctional intercellular communication in tendon healing, even though Cnx43 has been shown to play an important role in healing, yet with somewhat contradictory findings [41, 42]. In our study, cxn43 appeared to be more prominent towards the cell processes. This finding is in accordance with the findings of McNeilly and co-authors who studied the distribution of cxn43 and cxn32 in rat's deep flexor tendons [43]. They found that cxn43 was associated with cell processes while cxn32 was associated with junctions between cell bodies rather than cell processes. The authors suggested that given the different characteristics and distributions of cxn43 and cxn32 between tendons, it could be that the

two different types of junction might be responsible for sensing two different forms of loading.

We noticed that the cultures of LS group demonstrated the greatest density of cxn43 in comparison to the other groups. It seems that anabolic steroid administration and load enhance cxn43 expression in a complementary manner. Studies about the effects of mechanical strain on the expression of cxn43 have reported conflicting results, which may be either attributed to intrinsic differences between various tissues or to variations in the amount and type of strain used [17, 44-46]. In agreement with the latter speculation is the study by Maeda *et al.* The application of two different levels of static tensile strain in tenocytes isolated from Achilles tendons of Japanese white rabbits resulted in different effects in gap junction intercellular communication [47]. 4% strain resulted in enhanced gap junction intercellular communication primarily attributed to the opening of gap junction pores and accompanied by a relocation of the expression of cxn43 within the cytoplasm and near the cell nuclei, while the application of higher strain (8%) significantly reduced gap junction intercellular communication and was accompanied by reduced immunofluorescence staining for cxn43. Similarly, the application of static tensile load might differentially affect the expression of cxn43 depending on the duration of the load [17].

On the other hand, androgens have been found to alter the expression of cxn43 in tissues such as the prostate and ovaries and thus affect cell to cell communication [48, 49]. In our study, the combination of loading and steroid administration (LS) resulted in enhanced measures of intercellular communication as expressed by greater mean peak $[Ca^{2+}]_{ic}$ and decreased propagation time. This could be associated either with increased connexin expression or with changes in gap junction permeability. Our results indicate that the enhanced intercellular communication was associated with greatest density of cxn43 at the cell processes. This was not observed in the study by Maeda *et al.* where the enhanced gap junction intercellular communication was related to the opening of gap junction pores [47].

Several studies have demonstrated an interactive relationship between connexins and the actin cytoskeleton [50-53]. Functional blocking of gap junctions resulted in discordance of actin stress fibers between neighboring cells. Mechanical stimulation-induced calcium wave propagation was significantly reduced in these cells [54]. A similar association between actin and cxn43 has been described in avian and human tenocytes as well [55]. Moreover, this association seems to increase after cyclic stain and might correlate with the degree of strain. In our study anabolic steroid administration and load led to upregulation of cxn43 and to a greater number of gap junctions in comparison with the other groups. Consecutively, anabolic steroid administration and load may enhance the formation of a better-organized cytoskeleton and particularly the actin stress monofilaments. Notably, it is previously described that nandrolone administration has led to a better organized actin cytoskeleton [28].

There are many reports in the literature about the detrimental effects of anabolic steroids on tendon cells [24-26]. However, these studies are confounded by the inevitable effects of anabolic steroids on muscles and the increase in muscle force associated with chronic administration. A few studies have assessed the direct effects of anabolic steroids on tendon cells. In vitro, dihydrotestosterone has been shown to increase the

proliferation of human tenocytes from intact supraspinatus tendons after 48h and 72h treatment and to alter their morphology [27]. The proliferation rates however were similar between treated tenocytes and controls at 96h advocating an acute and provisional effect. We have previously demonstrated that the administration of nandrolone decanoate in bioartificial tendons has resulted in increased remodeling, a more organized cytoskeleton and improved biomechanical properties [28].

There are limited data about the effects of anabolic steroids on the musculotendinous unit after injury. The administration of anabolic steroids seems to protect against muscle damage caused by rotator cuff tendon release, by inhibiting the fatty infiltration that usually follows after such injuries [56, 57]. Notably, this effect might be influenced by the timing of administration, since once the fatty infiltration has been established, anabolic steroids are unable to reverse the damage [58]. However, administration of nandrolone decanoate locally on the rotator cuff after incision and reconstruction surgery exerted negative effects inhibiting the healing process [59]. It is possible that, just as with mechanical load, the dose of anabolic steroids, as well as the timing of administration might influence their effect on tendon healing.

In conclusion, we demonstrated that nandrolone decanoate and loading seem to have a synergistic effect on the upregulation of the gap junction protein cxn43 enhancing calcium signaling via gap junctions. The importance of mechanical stimulation and the anabolic effect of biological or pharmaceutical factors in normal homeostasis of tissues during post-injury repairing process may be integral in advancing therapeutic approaches and motion therapy regimens. Further investigations are needed to establish the role of anabolic steroids in conjunction with motion therapy in the management of complex and challenging tendon injury cases.

Limitations

There were some limitations considering our culture mechanostimulus instrumentation. The state of strain achieved varied as a function of position on the substrate culture surface (strain heterogeneity). Also, at any given site on the substrate culture surface, the state of strain varies as a function of direction (strain anisotropy). Moreover, motion of the substrate induces motion of the overlying liquid nutrient medium. This motion exerts unintended reactive stresses upon the culture layer [60]. Finally, the loading regime was only 1% stretch at 1 Hz for only 1h per day for 5 days. That regime may seem to be less aggressive than other applied and consecutively less effective. However, it is demonstrated that acute or excessive load breaks gap junction. Excessive strain greater than 5% cell elongation at 1 Hz for 3-7 days leads to smaller and less numerous cxn43 than non-stretched cells. Also, stretching cells discontinuously, allowing at least 18h of non-load stimulates connexin protein expression [61].

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