Effect of Nandrolone Decanoate on Skeletal Muscle Repair

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Key words
- muscle injury
- cryoinjury
- nandrolone decanoate
- MyoD
- myogenin

Abstract

This study analyzed the effect of nandrolone decanoate (ND) on muscle repair and the expression of myogenic regulatory factors following cryoinjury in rat skeletal muscle. Adult male Wistar rats were randomly divided into 4 groups: control group, sham group, cryoinjured group treated with ND and non-injured group treated with ND. Treatment consisted of subcutaneous injections of ND (5 mg/kg) twice a week. After sacrifice, the tibialis anterior muscle was removed for the isolation of total RNA and analysis of myogenic regulatory factors using real-time PCR as well as morphological analysis using the hematoxylin-eosin assay. There was a significant increase in MyoD mRNA after 7 days and in myogenin mRNA after 21 days in the cryoinjured ND group in comparison to other groups in the same period. The morphological analysis revealed no edema or myonecrosis after 7 days as well as no edema or inflammatory infiltrate after 14 days in the cryoinjured ND group. In conclusion the anabolic steroid nandrolone decanoate can modulate the muscle repair process in rats following cryoinjury by influencing the expression of regulatory myogenic factors and phases of muscle repair.

Introduction

Skeletal muscle has a considerable ability to adapt to physiological conditions, such as postnatal growth, exercise training and stretching as well as repair following injury due to a direct trauma (e.g., lacerations and bruises) or indirect causes, such as ischemia and neurological dysfunction [5, 10, 31]. The enhancement of muscle regeneration and prevention of fibrosis are the main objectives in improving muscle healing following injury [11, 19].

After an injury, the muscle begins a repair process similar to myogenesis; however, the cells involved are satellite cells rather than myogenic progenitor cells [10, 31]. Following activation, satellite cells undergo a series of stages involving proliferation, differentiation and myoblast fusion in the myofibers to repair muscle damage or constitute a new muscle fiber [9, 15, 18, 21, 22, 24, 27, 28]. Therefore, satellite cells are quiescent myoblasts that are critical to muscle regeneration.

During each stage of the repair process, these cells express distinct myogenic regulatory factors (MRFs), such as MyoD, myf-5, myogenin and MRF4. Myf5, MyoD and MRF4 are associated with satellite cell activation and proliferation, whereas myogenin reflects terminal muscle cell differentiation [25, 28, 33, 37, 38]. Alterations in the expression of both MRFs and other cell cycle-regulating proteins can alter the ability of skeletal muscle to regenerate following injury [28]. Moreover, the deletion or inhibition of MyoD expression results in the downregulation of M-cadherin, which is a critical protein involved in cell adhesion and myoblast fusion in skeletal muscle, and myogenin knockout results in severe skeletal muscle deficiency due to the inability of skeletal myoblasts to fuse and form mature myofibers [28].

Anabolic androgenic steroids are commonly used to affect muscle mass and markers of muscle growth. Nandrolone decanoate (ND) is a derivative of testosterone, an anabolic steroid present in small amounts in the human body, and is usually marketed as Deca-Durabolin. Nandrolone binds to the androgen receptor to a greater extent than testosterone, but has a lesser overall effect on muscle hypertrophy and there is the possibility that the increase in the expression of androgen receptors is important to the adaptation and constitution of muscle fibers, possibly through

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the protein regulation of the cell cycle and activity of satellite cells [28]. The effect of exogenous testosterone on muscle regeneration has been investigated through different types of muscle injury.

An analysis of the effects of ND on the extensor digitorum longus (EDL) and soleus muscles following myotoxic injury revealed an increase in mass of the soleus muscle as well as a decrease in the relative amount of fast myosin heavy chain protein in regenerating EDL muscle. Furthermore, following a contusion injury in the gastrocnemius muscles, ND was found to induce an increase in strength in treated animals after 14 days [26]. Another study analyzed the tibialis anterior muscle following injury induced by a myotoxin (bupivacaine) and found that ND caused an increase in the incidence of small-diameter and large-diameter fibers after 14 and 28 days, respectively [8]. However, a number of questions remain regarding the role of testosterone in muscle regeneration, which depends on the type of injury inflicted, the outcome variables used to assess regeneration and the type of muscle examined [26, 36].

The aim of the present study was to evaluate the effects of the anabolic steroid nandrolone decanoate on the skeletal muscle repair process and on the expression of the myogenic regulatory factors MyoD and myogenin following cryoinjury.

Methods

The experimental protocols used in this study were in compliance with the principles of laboratory animal care formulated by the Brazilian College of Animal Experimentation (COBEA) and with the ethical standards of the journal [17] and received approval from the Ethics Committee of the Universidade Nove de Julho (São Paulo, SP, Brazil) under protocol number: 12/2009. Adult male Wistar rats (n = 100) weighing 230 ± 25.45 g at the beginning of the procedure were maintained under controlled room temperature (22 °C) and relative humidity (40 %), with a 12-h night/day cycle. All animals had free access to a solid ration and water before and during the experimental period.

The animals were randomly divided into 4 groups: control without injury (n = 10); sham group – only surgical incision and exposure of the tibialis anterior (TA) muscle (n = 10); cryoinjured group treated with nandrolone decanoate or vehicle (peanut oil plus benzyl alcohol) (n = 40); and non-injured group treated with nandrolone decanoate or vehicle (peanut oil plus benzyl alcohol) (n = 40). The control group was sacrificed on Day 1 after beginning the experiment. The cryoinjured groups with and without treatment were analyzed on Days 1, 7, 14 and 21 following the injury procedure. Short-term muscle remodeling was evaluated on Days 1 and 7, whereas long-term muscle remodeling was evaluated on Days 14 and 21.

The surgical procedures were performed based on those described by Miyabara et al. [29] with the administration of 1 ml/kg of 1 % ketamine HCL (Dopalen, Vetbrands, Sao Paulo, Brazil) and 2 % xylazine (Anasedan, Vetbrands, Sao Paulo, Brazil). The TA muscle was surgically exposed and submitted to the cryoinjury procedure, which consisted of the application of a round metal probe (3 mm in diameter) that had been cooled in liquid nitrogen to the surface of the exposed TA and maintaining it in this position for 10 s. After the frozen muscle had thawed, the procedure was repeated on the same area for additional 10 s.

The cryoinjured area was macroscopically identified as a firm, white, disk-shaped region. Only the left TA muscle was injured and the right side served as the control. The wounds were closed with polyamide sutures and the animals were kept for several hours on a warm plate (37 °C) until they had recovered from the effects of the anesthetic in order to prevent hypothermia.

ND treatment

The animals received either Deca-Durabolin® (nandrolone decanoate; Organon do Brasil, São Paulo, Brazil) or the vehicle only (peanut oil plus benzyl alcohol (1:1.5). Doses of 5 mg/kg of the body mass (supraphysiological dose) were injected subcutaneously in the back of the rats twice a week. This dosage is similar to that frequently used by athletes [27]. Treatment began 1 h after the cryoinjury procedure.

After the experimental period of each group, the animal was sacrificed with an overdose of anesthetics (ketamine and xylazine (1:2)). The left and right TA muscles were removed, weighed, and immediately frozen in liquid nitrogen-cooled isopentane and stored in liquid nitrogen.

Total RNA isolation

Frozen TA muscle tissue was homogenized and total RNA was isolated using cold Trizol Reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Total RNA was quantified by spectrophotometry and RNA samples were treated with DNAse (Invitrogen Carlsbad, USA) to avoid contamination with genomic DNA. All solutions were prepared with 0.01 % diethyl pyrocarbonate-treated water (DEPC, Sigma, USA), while glassware and plasticware were treated against RNase using standard procedures.

cDNA synthesis and real-time PCR

One microgram of total RNA was used for cDNA synthesis and real-time polymerase chain reaction (PCR) analysis of gene expression. Contaminated DNA was removed using DNase I (Invitrogen, Brazil) at a concentration of 1 unit/µg RNA in the presence of 20 mM Tris-HCl, pH 8.4, containing 2 mM MgCl2, for 15 min at 37 °C, followed by incubation at 95 °C for 5 min for enzyme inactivation. Reverse transcription (RT) was carried out in a 200-µl reaction in the presence of 50 mM Tris-HCl, pH 8.3, 3 mM MgCl2, 10 mM dithiothreitol, 0.5 mM dNTPs and 50 ng of random primers with 200 units of Moloney murine leukemia virus-reverse transcriptase (Invitrogen, Brazil). The reaction conditions were 20 °C for 10 min, 42 °C for 45 min and 95 °C for 5 min.

Real-time PCR was carried out using the SYBRGreen kit (Applied Biosystems, USA) in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA). The thermal cycling conditions were 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The experiments were performed in triplicate for each datum point. MyoD and myogenin mRNA abundance was quantified as a relative value compared with an internal reference (GAPDH), the abundance of which was believed not to change between the varying experimental conditions. The primers used for real-time PCR were as follows: GAPDH (GenBankTM accession number NM 017008) sense 5′- TGCACCACCAACTGCTTAGC -3′ and anti-sense GCCCCACGGCCATCA -3′; MyoD [13] sense 5′ GGA GAC ATC CTC AAG CGA TGC and anti-sense AGC ACC TGG TAA ATC GGA TTG (product: 80 pb); Myogenin - sense 5′ACT ACC CAC CGT CCA TTC AC- 3′ and anti-sense 3- TCG GGG CAC TCA CTG TCT CT -5 (product: 233 pb) (Genbank accession number M24393) [7, 16]. One microliter of RT reaction was used for real-time PCR.

Quantitative values for MyoD, myogenin and GAPDH mRNA transcription were obtained from the threshold cycle number at which the increase in the signal associated with an exponential growth of PCR products begins to be detected. Melting curves were generated at the end of every run to ensure product uniformity. The relative target gene expression level was normalized on the basis of GAPDH expression as an endogenous RNA control. ΔCt values of the samples were determined by subtracting the average Ct value of MyoD and myogenin mRNA from the average Ct value of the internal control GAPDH. As it is uncommon to use ΔCt as a relative data due to this logarithmic characteristic, the 2-ΔCt parameter was used to express the relative expression data.

Morphological analysis

The muscle samples (control and nandrolone decanoate/vehicle treated) were also used for morphological analysis. For this procedure, TA muscles were immediately frozen in molten isopentane and stored in liquid nitrogen. Frozen specimens were cut into 10-μm cross sections with a cryostat (Leica CM3050, Nussloch, Germany). Tissue specimens were stained with hematoxylin-eosin for routine histological examination performed under conventional light microscopy (Zeiss Axioplan 2) by 2 calibrated examiners.

The qualitative analysis of histological sections stained with HE included a description of the stages of tissue repair, involving the presence and type of inflammatory infiltrate, edema, necrosis and immature fibers. The semi-quantitative analysis involved the rating of the inflammatory infiltrate, edema, necrosis and immature fibers as follows: absent (Grade 0), mild (Grade 1), moderate (Grade 2) and severe (Grade 3), considering mild to mean up to 25%, moderate 25–50% and severe more than 50% of the respective analyzed items in the fields examined [35].

Statistical analysis

MyoD and myogenin RNAm data are presented as mean ± standard deviation (SD) values. Comparisons between groups were made using one-way analysis of variance (ANOVA). Tukey's test was used to determine significant differences between experimental groups. A p-value <0.05 was considered statistically significant. Data analysis was performed with the aid of the GraphPad Prism 4.0 statistical software (GraphPad Software, San Diego, CA, USA).

Results

Expression of myogenic regulatory factors

One day following cryoinjury, no differences in MyoD mRNA were found between experimental groups. However, after 7 days, there was a significant increase in MyoD mRNA in cryoinjuried group treated with ND in comparison to the control, cryoinjury without ND and ND without injury groups in the same period (**Fig. 1**). At 14 and 21 days, no significant differences were found between groups and MyoD mRNA values were similar to those on the Day 1 evaluation.

One day following cryoinjury, no significant differences in myogenin mRNA were found between experimental groups. Likewise, no significant differences were found between groups on Days 7 and 14 and values were similar to those on the Day 1 evaluation. However, after 21 days, there was a significant increase in myogenin mRNA expression in the cryoinjury group treated with ND in comparison to the control, cryoinjury without ND and ND without injury groups in the same period (**Fig. 2**).

Quantitative morphological analysis

The qualitative morphological analysis revealed that the muscles in the control group exhibited a normal histological appearance, with the presence of fibers with peripheral nuclei and no signs of injury or inflammation (**Fig. 3a**). These results were similar to those observed in the vehicle control group and ND control in all experimental periods.

The sham group exhibited mild, predominantly mononuclear inflammatory infiltration, few degenerated muscle cells (myonecrosis) and edema foci located on the surface of the surgically exposed muscle.

After one day, the cryoinjured group, cryoinjured group treated with vehicle and cryoinjured group treated with ND exhibited similar results, with marked edema between muscle fibers and mild infiltration of neutrophils and macrophages scattered between the fibers, which were largely necrotic (myonecrosis) (**Fig. 3b**).

After 7 days, the cryoinjured group and cryoinjured group treated with vehicle exhibited a reduction of inflammation, scarce myonecrosis and the emergence of a large number of new, immature muscle fibers (**Fig. 3c**). In the same period, the cryoinjured group treated with ND exhibited muscle fibers with a greater degree of maturation as well as reduced edema and myonecrosis (**Fig. 3d**).

After 14 days, the cryoinjured group, cryoinjured group treated with vehicle and cryoinjured group treated with ND exhibited similar morphological repair, with a reduction in edema and inflammatory infiltrate as well as the replacement of the entire injured area by muscle cells with a separated central nucleus, denoting tissue renewal (**Fig. 3e**).

At 21 days, the cryoinjured group, cryoinjured group treated with vehicle and cryoinjured group treated with ND exhibited muscle tissue with normal morphology, revealing complete repair with no inflammatory signs and rare cells with a central nucleus (**Fig. 3f**).

Semi-quantitative morphological analysis

As expected, the semi-quantitative morphological analysis revealed that the muscles in the control group exhibited a normal histological appearance, with the absence of inflammatory aspects, edema, myonecrosis and new, immature muscle fibers associated with repair after injury (**Fig. 4**). The sham group exhibited edema, myonecrosis and Grade 1 inflammatory infiltration, with no new, immature fibers (**Fig. 4a**).

On Day 1, all cryoinjured groups exhibited edema, myonecrosis and moderate to intense inflammatory infiltration (**Fig. 4a**). After 7 days, no edema or myonecrosis was detected in the cryoinjured group treated with ND, while the other cryoinjured groups exhibited slight edema and myonecrosis. Inflammatory infiltrate was lesser in the group treated with ND (mild) in comparison to other groups (moderate). An intense degree of new, immature fibers was identified in all groups in this period (**Fig. 4b**).

On Day 14, no edema or inflammatory infiltrate was found in the group treated with ND, unlike what occurred in the other groups in the same period. No myonecrosis was observed in any group, whereas new, immature fibers were found in all groups. These fibers were present to a mild degree in the group treated with ND and a moderate degree in the cryoinjured group treated with vehicle (**Fig. 4c**).

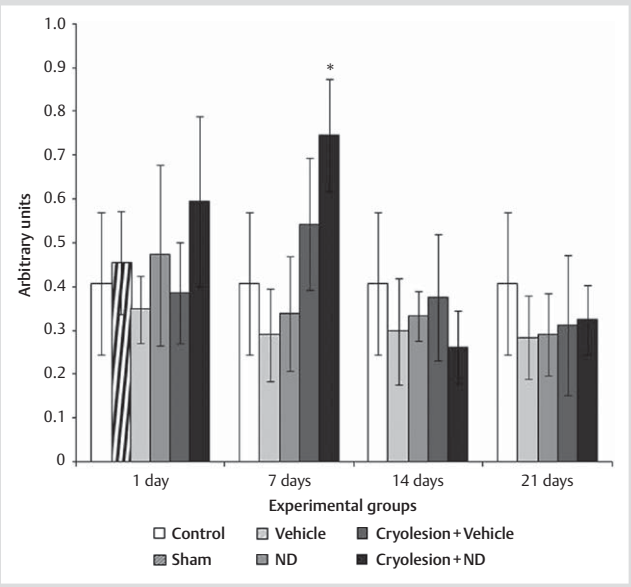

Fig. 1 MyoD mRNA analysis comparing control condition and injured (cryoinjury) and non-injured groups with and without ND/vehicle; *significant difference ($p \leq 0.05$, ANOVA/Tukey).

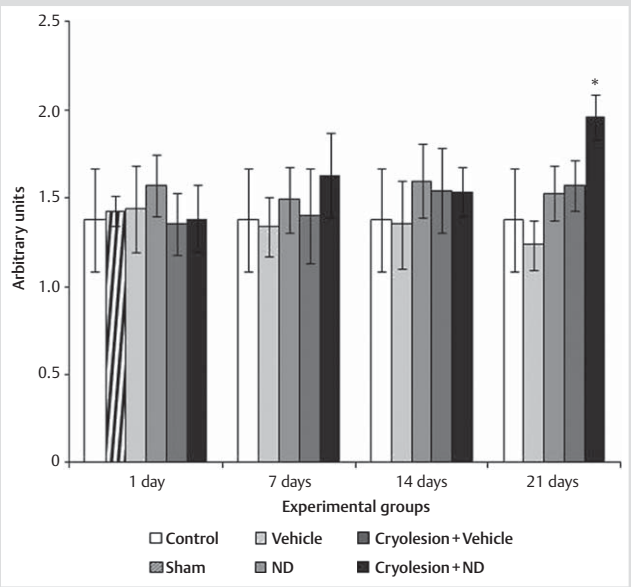

Fig. 2 Myogenin mRNA analysis comparing control condition and injured (cryoinjury) and non-injured groups with and without ND/vehicle; *significant difference ($p \leq 0.05$, ANOVA/Tukey).

On Day 21, no edema, myonecrosis or inflammatory reaction was found in any of the groups, whereas a mild degree of new, immature fibers was found in all groups (**Fig. 4d**).

Discussion

Different therapeutic modalities are employed to provide a muscle repair process of better quality and shorter duration [6,12]. The use of ND by athletes and non-athletes alike has increased due to its association with improved performance through an increase in muscle size and strength [14,28]. The results of the present study suggest that ND contributes favorably to the muscle repair process, as evidenced by the significant increase in the expression of MyoD 7 days following injury in comparison to the groups without the use of ND. MyoD is expressed in the early stages of the muscle repair process and is involved in the activation of satellite cells [23,28,34,37,38].

The findings of the present study corroborate those reported by Souza et al. [32] who found that the use of this same anabolic steroid accelerated muscle regeneration and increased mRNA expression of MyoD in mice between 7–14 days, using an injury model of myonecrosis caused by snake venom (*Bothrops jararacussu*). These results suggest that ND accelerates muscle regeneration and indicate the involvement of MyoD in the muscle repair process. However, Jin et al. [20] reported that immunohistochemistry analysis revealed that MyoD appeared after 18 h and reached its peak after 48 h following myonecrosis induced by the administration of anesthesia (bupivacaine hydrochloride) in dystrophic rats, whereas myogenin remained unchanged in the first 24 h and reached its peak expression after 72 h, confirming the expression of MyoD in the early stages and myogenin in the later stages of the muscle repair process.

Moreover, 21 days following injury, treatment with nandrolone induced an increase of myogenin mRNA, which is directly related to the myogenic differentiation of muscle cells [1,28]. This finding also could be related to the anabolic effect of ND, since studies have demonstrated that MyoD and myogenin mRNA levels reflect myoblast proliferation and differentiation, respectively, which contribute to hypertrophic muscle growth. Allouh and Rosser [2] found that nandrolone administration induced an increase in the number of satellite cells per millimeter of fiber in the pectoralis muscle and was associated with muscle fiber hypertrophy. Almeida et al. [3] analyzed the quantitative expression of myogenic regulatory factors MyoD and myogenin with regard to hypertrophic and hyperplastic muscle growth mechanisms in pacu skeletal muscle and found that these transcription factors contribute to hypertrophic muscle growth. In contrast, Gentile et al. [14] found that treatment with testosterone or 5a-dihydrotestosterone (DHT) induced no significant changes in the RNA expression of MyoD, myogenin, monocyte nuclear factor or myostatin genes in aged castrated rats, but observed an increased expression of IGF1Ea and its splice variant MGF. However, the anabolic effect was evaluated in the soleus muscle without injury.

White et al. [36] also used ND during muscle repair, analyzing the expression of IGF-1, a transforming growth factor that acts by stimulating and regulating the growth of new immature fibers; the authors' findings revealed an increase in the mRNA expression of IGF-1 after 5 days of treatment with the steroid, demonstrating the stimulation of the repair process.

The results of the quantitative morphological analysis revealed edema, inflammatory infiltrate and myonecrosis one day following injury, which was expected. No edema or myonecrosis was detected after 7 days and no edema or inflammatory infiltration was detected after 14 days in the group treated with ND. The same did not occur in the other groups. Moreover, the group treated with the steroid exhibited an earlier emergence of and increase in new muscle fibers. Thus, the use of the anabolic steroid nandrolone seems to have beneficial effects on the resolution of the inflammatory process and the repair of muscle tissue. These data are consistent with the expression of myogenic regulatory factors. Furthermore, the results are in agreement with

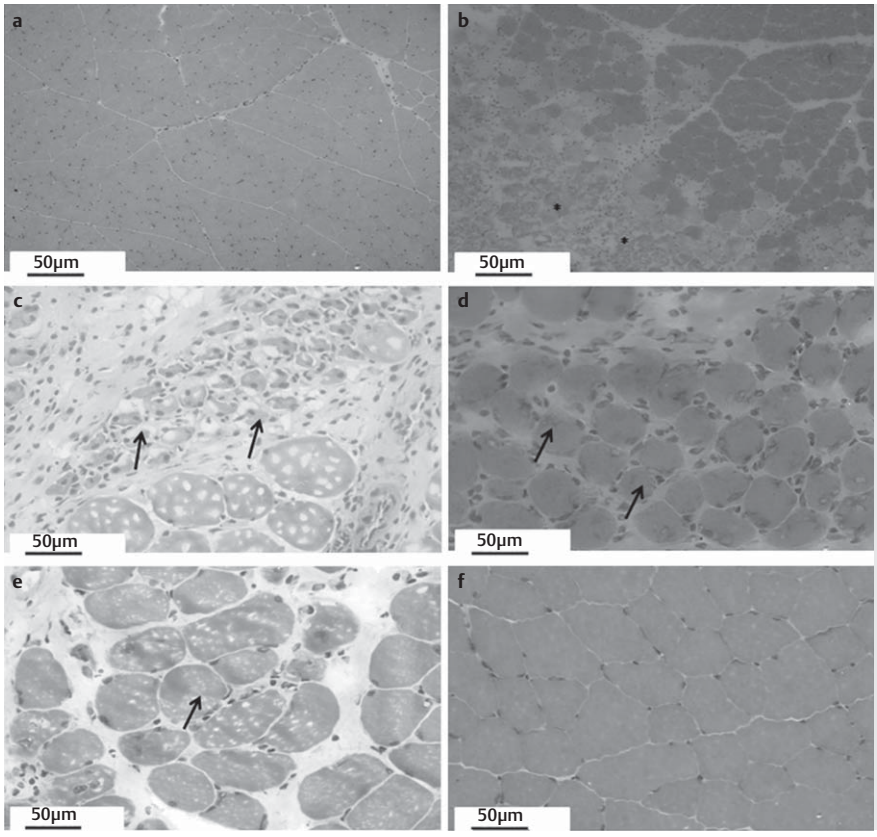

Fig. 3 Photomicrographs of histological sections of muscles stained with hematoxylin & eosin; **a** Control muscle showing normal morphology (original magnification, 100×); **b** Injured area after 1 day (original magnification, 400×); note presence of myonecrosis (*) and inflammatory infiltrate; **c** Injured area after 7 days showing lesser degrees of myonecrosis, inflammatory infiltration and edema; note early myogenesis (arrows) (original magnification, 200×); **d** Injured muscle treated with ND after 7 days; myogenesis showing myotubules (arrows) with greater degree of maturity in comparison to other groups (original magnification, 400×); **e** At 14 days, immature regenerated muscle cells (arrow) (original magnification, 400×); **f** At 21 days, larger, mature muscle cells with polygonal appearance (original magnification, 400×).

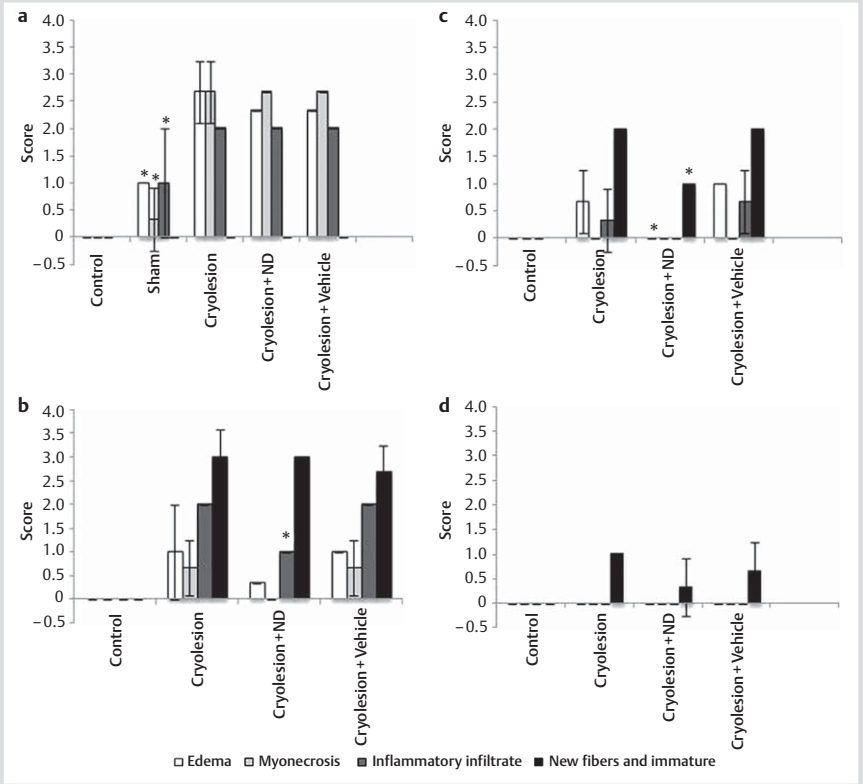

Fig. 4 Semi-quantitative morphological analysis of tissue components (edema, myonecrosis, inflammatory infiltrate and new muscle fibers) in different experimental groups; Grade 0: absent; Grade 1: mild; Grade 2: Moderate; Grade 3: intense.[35] **a** after 1 day; **b** after 7 days; **c** after 14 days; **d** after 21 days; * significant difference ($p \leq 0.05$, ANOVA/Tukey).

those described by Miyabara et al. [30] and Baptista et al. [4], who found complete muscle regeneration 3 weeks after the cryoinjury to the tibialis anterior muscle.

Based on these findings, we conclude that the anabolic steroid ND can modulate the muscle repair process in rats following cryoinjury by influencing the expression of regulatory myogenic factors and inducing a significant increase in MyoD mRNA after 7 days as well as an increase in myogenin mRNA after 21 days, with a decrease in edema, myonecrosis and inflammatory infiltrate during the muscle repair process.

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Conflict of Interest: The authors declare that there were no conflicting financial interests.

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