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Nandrolone decanoate and physical activity affect quadriceps in peripubertal rats

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ABSTRACT

Anabolic androgenic steroids (AASs) are synthetic analogs of testosterone often used by athletes to increase the skeletal muscle mass. Our goal was to examine the effects of physical activity and physical activity combined with supraphysiological doses of nandrolone on functional morphology of the quadriceps muscle. The study included 32 peripubertal Wistar rats, divided into 4 groups: control (T-N-), nandrolone (T-N+), physical activity (T+N-) and physical activity plus nandrolone (T+N+) groups. The T+N- and T+N+ group swam for 4 weeks, 1 h/day, 5 days/week. The T-N+ and T+N+ groups received nandolone decanoate (20 mg/kg b.w.) once *per* week, subcutaneously. Subsequently, the rats were sacrificed and muscle specimens were prepared for the processing. Tissue sections were histochemically and immunohistochemically stained, while the image analysis was used for quantification. Longitudinal diameter of quadriceps muscle cells was increased for 21% in T-N+, for 57% in T+N- and for 64% in T+N+ group while cross section muscle cell area was increased in T-N+ for 19%, in T+N- for 47% and in T+N+ group for 59%, compared to the control. Collagen fibers covered area was increased in T-N+ group for 36%, in T+N- for 109% and in T+N+ group for 159%, compared to the control. Erythrocyte depots were decreased in T-N+ group and increased in T+N- and T+N+ group, in comparison with T-N-. VEGF depots were increased in all treated groups. Chronic administration of supraphysiological doses of AASs alone or in combination with physical activity induces hypertrophy and significant changes in the quadriceps muscle tissue structure.

1. Introduction

The anabolic androgenic steroids (AASs) include testosterone and its numerous synthetic analogs and they are frequently misused by athletes with intention of enhancing their physical performance (Riezzo et al., 2011; Hassan and Kamal, 2013; Nikolic et al., 2015; Piacentino et al., 2015). Nandrolone decanoate (ND) is an anabolic steroid and like the other AASs, was developed in order to maximize anabolic effects (such as muscle growth, protein synthesis and erythropoiesis) and minimize the androgenic ones (Tylicki et al., 2007; Frankenfeld et al., 2014; Piacentino et al., 2015; Frati et al., 2015). These substances can be administered either orally, parenterally, transdermally (by topical gels or patches) or subcutaneously (by implantable pellets) (Evans, 2004; Frati et al., 2015). Most often, athletes use nandrolone in oral or injectable form (Kohler and Lambert, 2002).

Administered androgens that bind to the nuclear androgen receptors (AR), are translocated into the nucleus and regulate the transcription of the group of genes which ultimately leads to increased muscle protein synthesis and muscle growth (Fragkaki et al., 2009; Frati et al., 2015). Studies show that nuclear ARs can be up-regulated when exposed to AASs, while the number and density of ARs is increased by strength training (Evans, 2004). The androgens and training combined complement each other in the mechanisms of the ARs up-regulation, and the strength of these effects is largely determined by the exercise program, sex and age of the athlete as well as the type of AASs misuse (short- or long-term) (Vingren et al., 2010).

Despite the fact that the International Olympic Committee (IOC) prohibited the use of nandrolone in 1976 (Kohler and Lambert, 2002; Evans, 2004), AASs are being abused by competitive and recreational athletes. This is especially related to the bodybuilders and power lifters

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who aim to improve physical appearance or enhance performance by increasing muscle mass and strength (Bhasin et al., 1996; Nieschlag and Vorona, 2015; Sretenovic et al., 2016). In praxis, the doses that they use are up to 10 and even 100 times higher than the therapeutic dose (TD) (Yesalis and Bahrke, 1995; Frankenfeld et al., 2014). Consequences of such AASs abuse are various and depend on dosage, type, frequency and model of use (Riezzo et al., 2011), but extremely high doses can cause acute or chronic adverse side effects in almost all major tissues and organs (Fragkaki et al., 2009; Karila et al., 2004).

It is reported that administration of AASs in puberty leads to early epiphyseal closure and deficit of growth, testicular atrophy, infertility, impotence, prostate hypertrophy, as well as prostate and liver tumors (Rodrigues et al., 2017). In adults, usage of supraphysiological doses of testosterone in combination with strength training leads to marked growth in triceps and quadriceps diameter (Bhasin et al., 1996), but long-term abuse of the AASs can lead to structural and functional alteration of the liver and sometimes even hepatocellular adenoma can occur (Ferrari et al., 2013). Hypertrophy of the left ventricle with disproportional accumulation of extracellular collagen and interstitial fibrosis (Takahashi et al., 2004; Tanno et al., 2011; Hassan and Kamal, 2013; Franquni et al., 2013; Sretenovic et al., 2016) is also detected, as well as higher risk of prostate cancer, impotence and morphological changes in testis (Ferrari et al., 2013). Specific mechanisms of all of these adverse effects are not yet clearly understood, but certain studies suggest that misuse of the AASs is followed by premature death rate (due to suicide, acute myocardial infarction or hepatic coma) in power lifters, that is almost 4 times higher than in normal population (Pärssinen and Seppälä, 2002).

Effects of high doses of AASs on the skeletal muscle hypertrophy, as mentioned above, have been known for a long time, but there are only a few reports in the available literature with comprehensive histomorphometric results concerning the side-effects of AASs and/or training on the skeletal muscle cells morphology, connective tissue composition and the changes of vascularization of the muscle. Most of the reports suggest that chronic use of AASs increases muscle diameter and muscle cell diameter (Venken et al., 2007), but the collagen dynamics in striated muscle is quite poorly described in the literature. In fact, couple of studies in which this matter was addressed had opposite results. Karpakka et al. (1992) reported that AASs significantly decreased the concentration of hydroxyproline *e.g.* collagen synthesis, while Pärssinen et al. (2000) reported the decrease of degradation with increase of production of collagen type I, in this context. On the other hand, investigators agree that training alone increases collagen type I production in skeletal muscle tissue (Hjorth et al., 2015; Carroll et al., 2015; Martinez-Huenchullan et al., 2017). When it comes to the changes of the vascularization in striated muscle after chronic AASs misuse, it should be noted that Paschoal et al. (2009) reported that AASs inhibit the vascular endothelial growth factor (VEGF) mRNA expression and impair the angiogenesis. VEGF is a potent mitogen of endothelial cells and it has been shown that endurance training induces capillary growth within angiogenic response to exercise (Prior et al., 2003; Waters et al., 2004). Also, Shikatani et al. (2012) suggested that corticosterone inhibits migration and proliferation of endothelial cells thus limiting the angiogenesis.

Although the amount of data regarding the effects of AASs on muscle tissue rapidly increases, there are still controversies and some unknown aspects in this field. Moreover, the synergistic impact of AASs and the physical load has been poorly investigated. Having in mind that the abuse of AASs has become more frequent among young sportsmen in recent decades, the aim of our study was to identify the effects of supraphysiologycal doses of nandrolone decanoate (DECA DURABOLIN®, Organon, Holland) alone, physical activity alone or their combination on the functional morphology of the quadriceps muscle in peripubertal rats.

2. Material and methods

2.1. Experimental animals, study design and organ extraction

Study included 32 peripubertal (5 weeks old) male Wistar albino rats, weighing 150–200 g, that were bred at the Faculty of Medical Sciences, University of Kragujevac, Serbia. Rats were housed in collective cages (four rats *per* cage). The room temperature was kept at 23 ± 1 °C with 12:12h light and dark cycles. Food and water are provided *ad libitum*.

The rats were randomly divided into four groups:

1 T-N-, sedentary rats with no administration of nandrolone decanoate and physical activity (control group),
2 T-N+, sedentary rats with *s.c.* administration of nandrolone decanoate depot (DECA DURABOLIN®, Organon, Holland; 20 mg/kg b.w.) during a period of 4 weeks (nandrolone group),
3 T+N-, physically active rats (swimming 1 h/day, 5 days *per* week, for 4 weeks) with no administration of nandrolone decanoate (the group that had physical activity),
4 T+N+, physically active rats (swimming 1 h/day, 5 days *per* week) with *s.c.* administration of nandrolone decanoate depot (DECA DURABOLIN®, Organon, Holland; 20 mg/kg b.w.) during a period of 4 weeks (the group that had physical activity and was treated with nandrolone).

The initial and final body weights (BW) were measured. During the experiment, the swimming was performed in a glass pool, measuring 120 × 50 × 80 cm (length/width/height), in which the depth of the water was 60 cm. The first week represented the period of adaptation to swimming in which the rats started with 10 min of continuous swimming. Afterwards, swimming time was increased for 10 min every day, until 60 min mark was reached at the end of the fifth day (Nakao et al., 2000). After a period of adaptation, the expermiental period started, during which rats were swimming 1 h *per* day, 5 days *per* week, for four weeks. The swimming was performed every day at 9 a.m. Water temperature was 37 °C. Upon expiry of the experimental period, the rats were sacrificed. Precisely, in order to avoid the effect of acute swimming, the rats were sacrificed 48 h after the last swimming exercise. After short-term ketamine (Ketamin 10%, CP-PHARMA, Burgdof, Germany; 100 mg/kg b.w.) and xilazid (Xyla, Interchemie, Holland; 10 mg/kg b.w.) anesthesia, the animals were premedicated with heparin as an anticoagulant and sacrificed by cervical dislocation (Schedule 1 of the Animals/Scientific Procedures, Act 1986 UK). Their quadriceps muscles were surgically removed for the further examination. All research procedures were carried out in accordance with the European Council Directive (86/609/EEC) as well as the principles of Good Laboratory Practice (2004/9/EC, 2004/10/EC), and were approved by the Ethics Committee for the Welfare of Experimental Animals, Faculty of Medical Sciences University of Kragujevac, Serbia (No. 01-14606, from 7. XII 2016).

2.2. Tissue processing, histochemistry, immunohistochemistry and image analysis

The samples of rat quadriceps muscles were fixed in 4% formalin for 24 h, dehydrated in a series of increasing concentrations of ethanol (50%–100%), enlightened in xylol and embedded in Histowax® (Histolab Product AB, Göteborg, Sweden). Molded blocks of skeletal muscle were cut on a rotational microtome (RM 2125RT Leica Microsystems, Wetzlar, Germany) and 5 µm thick sections were prepared for further quantitative and qualitative histomorphological analyses. The sections were stained with standard H&E (enabling visualization of the tissue structures and some rougher orientation), Masson-Trichrome dye that enables collagen detection, as well as with Novelli staining and VEGF immunostaining, appropriate for the vascularization

imprint display.

Masson trichrome staining is an adequate method when there is a need to label the collagen fibers in connective tissue. Although Picrosirius-red staining is more widely used, the final results of both methods are identical (Chen et al., 2011). Masson staining method results in dark brown to black stained nuclei, reddish muscle cell cytoplasm and blue stain of collagen. The procedure includes deparaffinization and rehydration (100%–70% ethanol, distilled water) of the tissue sections, followed by 5 min incubation in Weigert's hematoxylin. After washing in tap water, the sections were incubated in a mixture of 1% acid fuchsine and 1% ponceau dexylidine (1:2) for 5 min. The next step included multiple washing of muscle sections in distilled water and their incubation in 0.05% phosphomolybdic acid for 10 min. Finally, the sections were incubated in 2.5% aniline blue (3 min), washed several times in distilled water, dehydrated and mounted in DPX (Ajdžanović et al., 2017).

Novelli histochemical staining is used for visualization of tissue vascular profile, and after the deparaffinization and rehydration of quadriceps sections involved their incubation in hot 1 N HCl (60 °C, 3 min), 1% acid fuchsine (30 s) and 1% light green (30 s), respectively. Sections were then washed in distilled water, and after the dehydration, mounting in DPX was carried out. As a result, purple erythrocytes were clearly visible against the bright green background of the quadriceps muscle fibers (Ajdzanovic et al., 2015, 2017).

VEGF in our study was localized by immunohistochemistry. For immunohistochemical staining, 5 µm thick sections of quadriceps were deparaffinized and dehydrated, followed by heat-induced antigen retrieval in a microwave on high power (750 W), for 8 min in 0.1 M citrate buffer, pH 6.0. After washing with PBS, sections were incubated with 0.3% hydrogen peroxide in methanol during 15 min at the room temperature, to block endogenous peroxidase activity. Quadriceps sections were then treated with diluted normal swine serum (1:50) (DAKO, Glostrup, Denmark) and incubated with rabbit polyclonal primary antibody raised against VEGF (1:100; abcam®, ab46154, Lot No. GR247559-1; Cambridge, MA, USA) overnight at room temperature. It should be noted that the primary antibodies used are appropriate for immunocytochemistry/immunofluorescence, immunohistochemistry (frozen and paraffin sections) and Western blot, as previously validated by abcam®, while the species reactivity includes mouse, rat and human. After rinsing in PBS, sections were incubated with polyclonal swine anti-rabbit immunoglobulins/HRP − an affinity-isolated, peroxidase-conjugated secondary antibody (Code No. P0399, Lot No. 20011615; DAKO, Glostrup, Denmark) diluted in PBS (1:100) for 1 h at room temperature. Binding sites were visualized with 0.05% diaminobenzidine (DAB; Serva, Heidelberg, Germany), followed by counterstaining with hematoxylin and mounting in DPX (Sigma-Aldrich, Co., USA). Negative controls were obtained by replacing the primary antibody for PBS.

2.3. Morphometric analysis

Images of histochemically/immunohistochemically stained tissue sections were captured with digital camera attached to the Olympus BX51 microscope (Olympus Life and Material Science Europa GmbH, Hamburg, Germany). Morphometric analysis was performed by calibrated Axiovision software (Zeiss, USA), as well as with Image Pro-Plus (Media Cybernetics, USA). Calibrated software then measured the areas of the images that correspond to each specific cytomorphological entity. Cross section area and longitudinal section diameter measurement of the quadriceps muscle cells were performed on H&E stained sections. Both measurements were performed on at least 100 muscle cells of each tissue specimen and average values were presented in micrometers or micrometers squared. Longitudinal section diameter of each muscle fiber was measured on three spots and average value was taken in account. For the analysis of collagen fibers covered area, after labeling of desired areas of the sections, segmentation of the images was performed. Regions of interest were identified with the exclusion of the irrelevant areas. Identical procedure was conducted when erythrocyte depots and VEGF quantification were performed. During the collagen fibers covered area and VEGF quantification, blood vessel areas were excluded from the regions of interest. All measurements were defined compared to the control and were made in triplicate.

2.4. Statistical analysis

All data were tested and the normality of the data distribution was established using the Shapiro Wilk test. The non-parametric version of Anova test (Kruskall Wallis test) was used for statistical comparison of the data. Post-hoc test analysis was used by Mann–Whitney test for non-parametric variables. P values below 0.05 were considered statistically significant. All statistical calculations were made with the SPSS computer program, version 22.0 (SPSS Inc., Chicago, IL, USA). Data are presented as means ± standard deviation (SD).

3. Results

3.1. Body weight (BW)

The initial average values of body weight of peripubertal rats were 183.57 ± 13.40 g in T-N- group, 173.90 ± 12.18 g in T-N+ group, 176.32 ± 15.92 g T+N- group and 172.82 ± 9.32 g in T+N+ group. Final average body weight of rats were 329.85 ± 16.23 g for T-N- group, 290.00 ± 8.74 g for T-N+ group, 284.01 ± 18.04 g for T+N- group and 299.37 ± 16.25 g for T+N+ group. After four weeks of experimental period, our results have shown statistically significant increase in body weight in all groups compared to the initial weight. Increases ($p < 0.05$) in body weight were: in control group 76%, in T-N+ group 67%, in physically active group 61% and in T+N+ group 73%, all compared to the corresponding initial values. If we compare the final body weight after four weeks, the results show that all experimental animals were with significantly ($p < 0.05$) lower body weight compared to the control. For T-N+ group this reduction measured 13%, in T+N- it was 15% and in T+N+ group the reduction amounted 10%.

3.2. Quadriceps histomorphometry

Striated cell morphometry was performed in order to estimate the effect of physical activity alone or in combination with nandrolone administration on quadriceps muscle cell size. The mean longitudinal section diameter after four weeks of experiment was: 14.08 ± 1.82 µm in T-N- group, 17.64 ± 1.84 µm in T-N+ group, 22.20 ± 1.77 µm in T+N- group and 23.98 ± 2.85 µm in T+N+ group. Fig. 1A illustrates the approach when measuring the longitudinal section diameter (LD). LD of peripubertal rats was increased ($p < 0.05$) in all experimental groups compared to the control (Fig. 1B). Alone, nandrolone administration induced increase ($p < 0.05$) for 21%, physical activity alone induced increase ($p < 0.05$) for 57% and combination of nandrolone decanoate and physical activity induced increase ($p < 0.05$) of quadriceps muscle cell diameter for 64%, in comparison with the control values. The largest difference of cell diameter can be observed between control and T+N- or T+N+ group (64% or 57% of increase; $p < 0.05$), while the smallest difference was verified between T+N- and T+N+ group (only 4%; $p > 0.05$) (Fig. 1B). The average values of cross section cell area (CSCA) were: 1177.36 ± 324.17 µm^2 in T-N- group, 1407.05 ± 460.42 µm^2 in T-N+ group, 1732.95 ± 587.06 µm^2 in T+N- group and 1880.07 ± 547.83 µm^2 in T+N+ group. After four weeks CSCA was significantly increased in all groups (Fig. 1C). Nandrolone alone induced 19% increase, physical activity alone caused 47% of increase and nandrolone in combination with physical activity induced 59% increase of CSCA, all compared to the control. Comparison between the experimental groups showed that

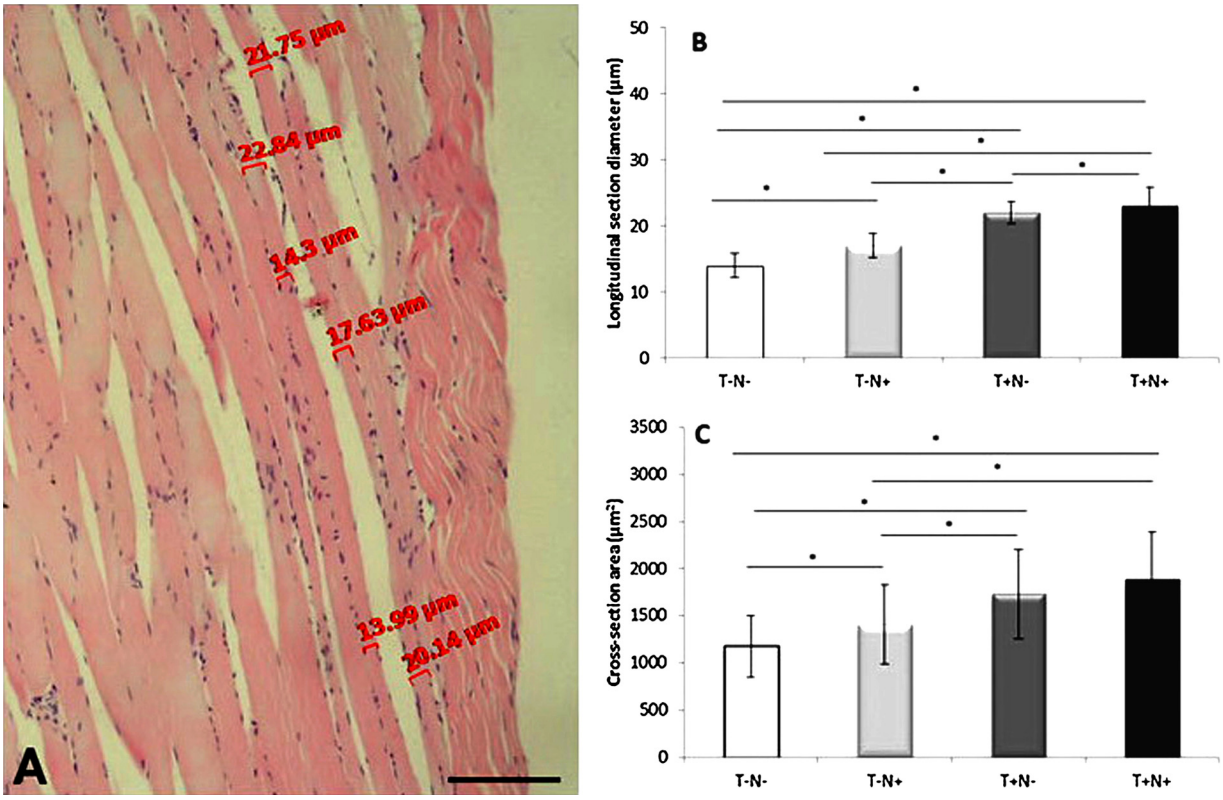


Fig. 1. A) Representative micrograph of H&E staining of the quadriceps muscle, which shows the approach when measuring the longitudinal section diameter; B) values of longitudinal section diameter and C) cross section area of quadriceps muscle cells in peripubertal rats after four weeks of experiment. All values are the means ± SD, n = 8 animals *per* group; *p < 0.05 *vs.* the adequate group, connected by a horizontal line.

the largest difference of CSCA can be observed between T-N+ and T+N+ (33%; p < 0.05) and the lowest, non-significant difference was found between T+N- and T+N+ (8%) (Fig. 1C).

3.3. Collagen fibers covered area analysis

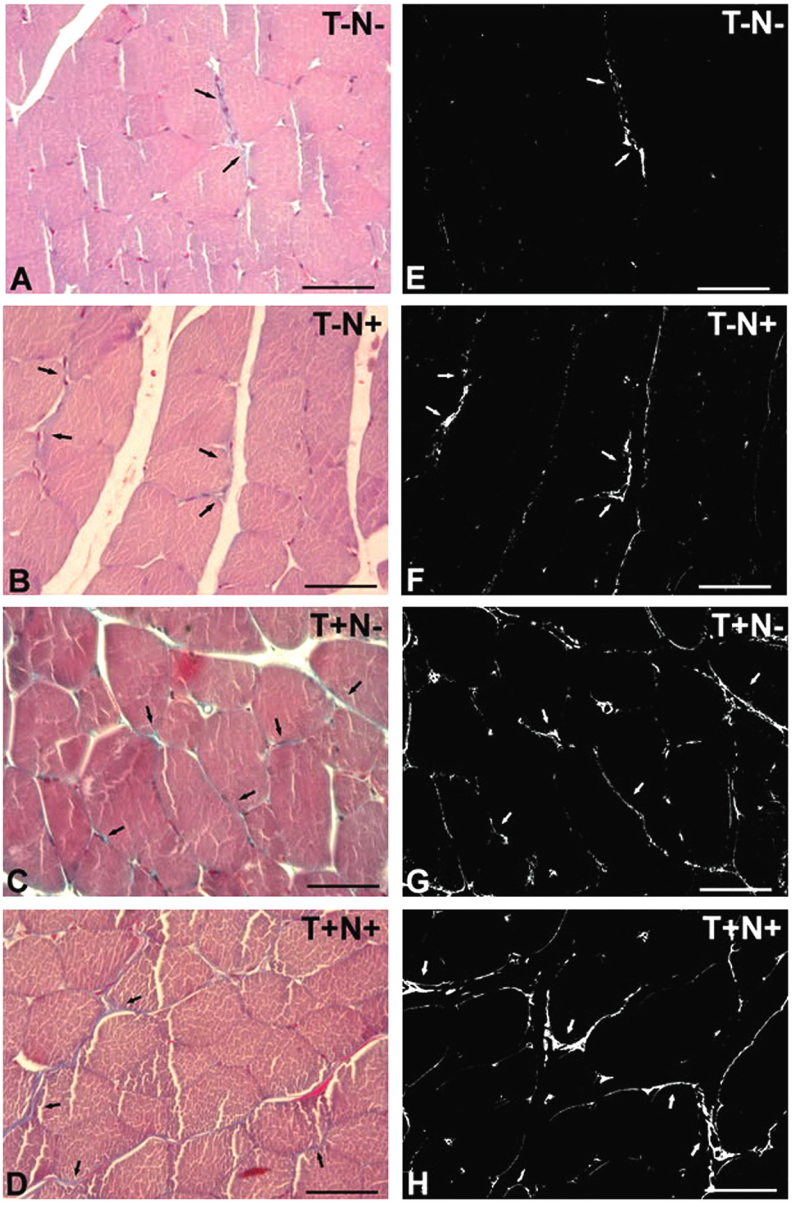
At the end of the experiment, collagen fibers covered area was increased in all experimental groups compared to the control (blue deposits between the reddish muscle fiber bundles at Masson trichrome stained sections (Fig. 2A–D) or white surfaces at segmented images; Fig. 2E–H)). Image analysis revealed significance of differences between the groups (Fig. 2I). In the control and T-N+ group, some delicate and thin collagen fibers were interspersed between the muscle cells. An 36% increase (p < 0.05) of collagen fibers covered area was verified in T-N+ group, compared to the control values (Fig. 2A–B, E–F, I). This endomysial collagen deposits were much thicker and more intense stained in the physically active animals. Quantification of the collagen covered area on the Masson trichrome stained tissue sections showed that the highest amount of collagen was present in T+N+ treated animals (159% increase (p < 0.05) compared to the controls), while physical activity alone induces 109% increase (p < 0.05) in comparison with the control group (Fig. 2A–D, E–H, I). Similar findings were observed when perimysial collagen was investigated. Comparison between experimental groups showed that the largest difference of collagen fibers covered area can be observed between T-N+ and T+N+ (90%; p < 0.05) (Fig. 2B–D, F–H, I) and the lowest difference was found between T+N- and T+N+ (24%; p < 0.05) (Fig. 2C–D, G–H, I). Comparison of collagen covered area between the experimental groups showed that physical activity alone when compared to nandrolone alone and their combination in comparison with nandrolone alone increased (p < 0.05) this parameter for 54% and 24%, respectively (Fig. 2B–C, F–G; C–D, G–H, I).

3.4. Erythrocyte depots

Erythrocyte depots in the quadriceps muscle tissue were identified and subjected to the quantification upon the Novelli histochemical staining (purple collections at the bright green background of the quadriceps muscle fibers; Fig. 3A–D). Results of quantification showed that T-N+ animals expressed the reduction of erythrocyte depots by 7% (p > 0.05) compared to the control group. Also, T+N+ rats showed diminished erythrocyte depots compared to T+N- group (17%; p > 0.05) (Fig. 3E). Animals exposed to physical activity and nandrolone showed increased erythrocyte depots by 112% (p < 0.05) compared to the controls. Largest difference (167%; p < 0.05) of erythrocyte depots can be observed in the muscle tissue between the rats from T+N- and T-N+ groups. Physical activity alone caused increase of these depots for 149% (p < 0.05) compared to the control value (Fig. 3E).

3.5. VEGF depots

Immunolocalization of VEGF depots within the quadriceps muscle tissue (brown fields at light blue background) of control and experimental groups of rats is shown in Fig. 4A–D. Very similar to the results trend related to erythrocyte depots, the VEGF expression in quadriceps tissue was the most prominent in T+N- animals (Fig. 4C, F). The administration of nandrolone when combined with physical activity decreased (p < 0.05) the VEGF amount for 16%, compared to physical activity alone (Fig. 4F). Although the trend of the results was quite similar to the erythrocyte depots appearance, the degree of differences between the experimental groups was much higher. Physical activity alone led to 339% VEGF expression increase (p < 0.05) compared to the control, while the combination of physical activity and nandrolone led to increase (p < 0.05) of 277% of the same parameter, in comparison with the controls (Fig. 4F). Also, physical activity alone



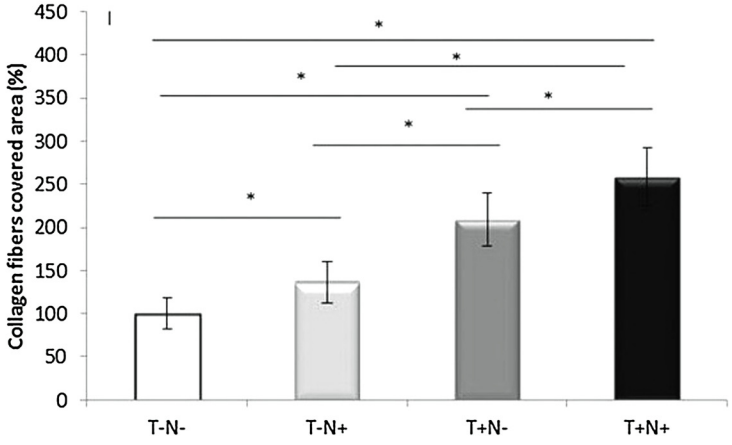


Fig. 2. Representative micrographs of Masson trichrome stained, rat quadriceps muscle sections A–D) and the corresponding segmented images E–H) (objective magnification 40×, bar = 25 μm; arrows are pointing out the collagen fibers). A, E) Control (T-N-) group, B, F) nandrolone (T-N+) group, C, G) physically active (T+N-) group and D, H) physically active, nandrolone-treated (T+N+) group. Graph (I) displays quantification of collagen fibers covered area (%) for the control and experimental groups. All values are the means ± SD, n = 8 animals *per* group; *p < 0.05 *vs.* control (T-N-) group. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

increased ($p < 0.05$) VEGF expression by 272% compared to nandrolone alone whereas their combination increased ($p < 0.05$) the same parameter by 219% in comparison with nandrolone alone (Fig. 4F).

4. Discussion

Anabolic androgenic steroids (AASs), for the past 40 years have been widely used among professional and amateur athletes with

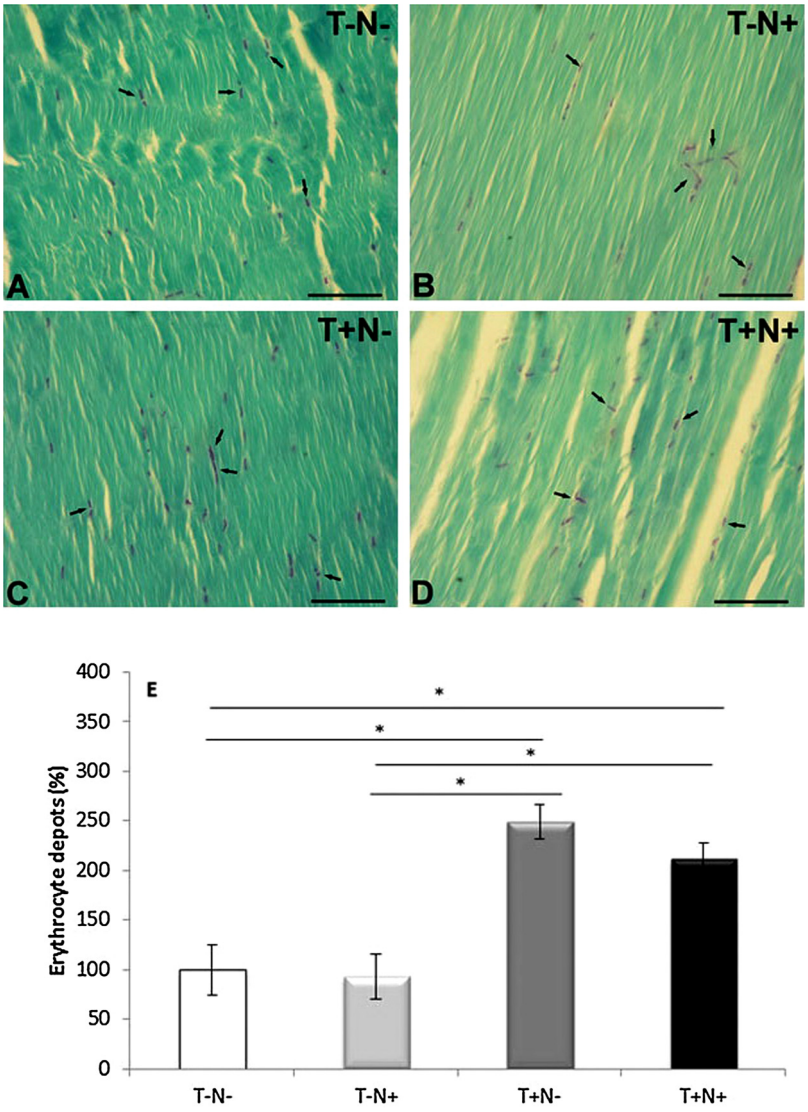


Fig. 3. Representative micrographs of Novelli stained, rat quadriceps muscle sections (objective magnification 40×, bar = 25 μm; arrows are pointing out the erythrocyte depots). A) Control (T-N-) group, B) nandrolone (T-N+) group, C) physically active (T+N-) group and D) physically active, nandrolone-treated (T+N+) group. Graph (E) displays erythrocyte depots (%) – related quantitative data for the control and experimental groups. All values are the means ± SD, n = 8 animals *per* group; *p < 0.05 *vs*. control (T-N-) group. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

intention to quickly gain muscle mass and improve physical performance. These drugs were primarily designed to promote growth of skeletal muscles. The degree of muscle growth depends on dose, type and duration of treatment (Nikolic et al., 2015). Nandrolone decanoate is a derivate of 19-nortestosterone and is one of the most popular AASs among the athletes. They use nandrolone alone or in combination with other AASs and the most frequent applied doses are in range of 200–400 mg, weekly in cycles of 4–6 weeks (Evans, 2004).

In our study, we investigated the effects of nandrolone decanoate and physical activity alone or in combination on functional morphology of the quadriceps muscle in peripubertal male rats. Physical activity that rats were exposed to in our experiment belongs to the endurance exercise training (Sugizaki et al., 2006), since swimming represents one of the common ways of rats movement in their natural habitat. Identical exercise pattern was selected by the authors of several similar studies that included animal training and steroid administration (Nakao et al., 2000; Sugizaki et al., 2006; Naraghi et al., 2010; de Almeida Chuffa et al., 2011; Nikolic et al., 2015; Selakovic et al., 2017).

In our study we showed that chronic administration of nandrolone decanoate and physical activity (swimming), alone or in combination, causes a significantly less body weight gain compared to the control group. This result is in line with the literature data (Rocha et al., 2007; Hassan and Kamal, 2013). Rocha et al. (2007) stated that the body weight decrease, upon anabolic steroids application along with training, was due to the reduction of intraperitoneal fat. Some other investigators reported that decrease in body weight of steroid-treated rats was maybe due to reduced appetite (Yu-Yahiro et al., 1989). Beutel et al. (2005), in the study in which they administered low and high doses of stanozolol for a period of eight weeks, showed that both, low and high doses of used synthetic steroid, will lead to a reduction of body weight in the animals.

It is well known that the AASs administration can increase a myofibril protein synthesis, which leads to increase of the muscle mass and strength (Schroeder et al., 2003; Venken et al., 2007). This is proven in our study also, because we detected that both CSCA and LD of the quadriceps muscle cells were increased in all experimental groups. Interestingly, nandrolone administration alone caused a significantly lower skeletal muscle hypertrophy than physical activity alone. It seems that the steroid action *via* nuclear androgenic receptor is not as effective as the physical stimuli for the muscle growth. Similar findings were

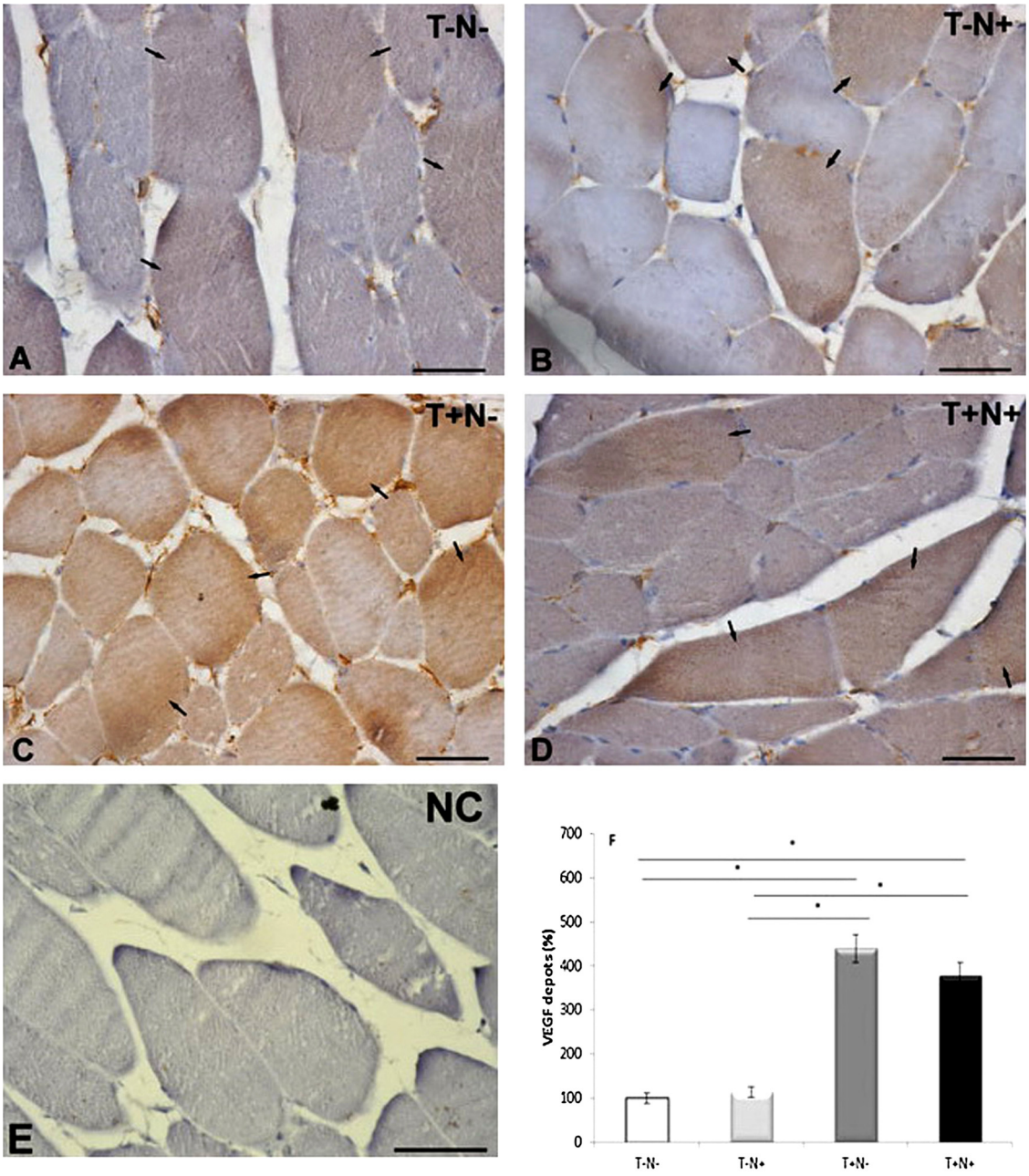


Fig. 4. Representative micrographs of VEGF immunostained, rat quadriceps muscle sections (objective magnification 40×, bar = 25 μm; arrows are pointing out the VEGF depots). A) Control (T-N-) group, B) nandrolone (T-N+) group, C) physically active (T+N-) group, D) physically active, nandrolone-treated (T+N+) group and E) negative control (NC). Graph F) displays VEGF depots (%) – related quantitative data for the control and experimental groups. All values are the means ± SD, n = 8 animals *per* group; *p < 0.05 *vs*. control (T-N-) group. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

reported in a recent study where significant difference in CSCA of the skeletal muscle cells between steroid alone and training alone group was verified (Krause Neto et al., 2017). Although there are some differences in the study design, general conclusion supports our findings on this matter.

Beside the structural role, the skeletal muscle endomysial, perimysial and fascial collagen is important for the distribution and transmission of the force which is created by muscle cell contraction. To a certain limit, increase of the collagen content, which can be readily seen in the muscle tissue of active athletes as a consequence of strenuous exercise (Miller et al., 2005), is attributed to generate the stronger and better aimed contraction forces (Purslow, 2010; Yucesoy, 2010; Findley et al., 2015), but excessive deposition of collagen shows the opposite effects. In our study, the epimysial deposition of collagen significantly rose with nandrolone treatment and even more after the physical activity, while the highest values were observed in the rats subjected to a combined approach. It is reasonable to believe that the endomysial fibrosis we observed, at least partly manifests the adverse effects of chronic high-dose nandrolone administration, given the reported, anabolic steroid-caused increase of muscle collagen content in cows (Cranwell et al., 1996). The concrete mechanism of nandrolone-induced fibrosis is not quite well understood, yet. Authors argue that oxidative stress and inflammatory cytokines may trigger proliferative and fibrotic pathways that can lead to increased collagen content in the cardiac muscle tissue (Vasilaki et al., 2016; Tofighi et al., 2017). Most probably, the same mechanisms contribute to the fibrosis in the skeletal muscle.

Regarding training alone, Hjorth et al. (2015) reported twofold up-regulation of both fibrillar and network collagens, while Carroll et al. (2015) found threefold higher collagen content in gastrocnemius of Wistar rats after 8 weeks of treadmill training, which is in correlation with our findings.

The degree of vascularization of the quadriceps muscle in our study was indirectly assessed by virtue of Novelli histochemical staining, while the angiogenesis potential was indicated with VEGF immunohistochemical detection. Pronounced capillary network and strong VEGF expression within quadriceps muscle tissue are observable after physical activity alone, or in combination with nandrolone application to our peripubertal rats. VEGF has been found to be the major proangiogenic factor in skeletal muscle, which is elevated in young men during the first weeks of aerobic exercise training, while returns to the baseline afterwards (Gavin et al., 2007). Actually, the mechanical stimulus induced by active muscle contraction leads to increased muscle interstitium VEGF levels (Hoffner et al., 2003; Gavin et al., 2007). It was also reported that training alone induces increase of the striated muscle capillary network (Soares, 1992). Some similar, but a bit stronger effect was observed upon the training combined with AASs (Neto and Gama 2017), all of which explain the realized scenario in our experimental setup. However, some data suggest that AASs can inhibit the VEGF expression and thus impair the angiogenesis (Paschoal et al., 2009; Shikatani et al., 2012). We proved that the exercise alone induced the most prominent erythrocyte depots as well as VEGF expression increase (2-fold and 3-fold respectively), but administration of nandrolone alone led to some lower values of these parameters, even compared to the controls. The latter is in line with the observation of Paschoal et al. (2009) pertinent to anabolic steroid-induced decrease of VEGF expression and impaired vasculature, which is undesirable in muscle remodeling and performance.

Generally, our study demonstrated that supraphysiological doses of nandrolone decanoate alone promote mild skeletal muscle hypertrophy, but only in combination with the physical activity the hypertrophy degree is significant. More importantly, we showed that the (ab)use of AASs, even in high doses, does not give a significant difference in muscle cell diameter compared to the training alone. On the other hand, increased collagen deposition and reduction of capillary network, as a result of chronic (ab)use of supraphysiological doses of nandrolone, may in a longer period lead to structural changes in skeletal muscle tissue, diminished performance and overall strength.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

Ajdžanović, V., Jarić, I., Živanović, J., Filipović, B., Ristić, N., Miler, M., Milošević, V., 2015. Testosterone application decreases the capacity for ACTH and corticosterone secretion in a rat model of the andropause. Acta Histochem. 117, 528–535.

Ajdžanović, V., Jarić, I., Miler, M., Filipović, B., Šošić-Jurjević, B., Ristić, N., Milenkovic, D., Milošević, V., 2017. Diosgenin-caused changes of the adrenal gland histological parameters in a rat model of the menopause. Acta Histochem. 119, 48–56.

Beutel, A., Bergamaschi, C.T., Campos, R.R., 2005. Effects of chronic anabolic steroid treatment on tonic and reflex cardiovascular control in male rats. J. Steroid Biochem. Mol. Biol. 93, 43–48.

Bhasin, S., Storer, T.W., Berman, N., Callegari, C., Clevenger, B., Phillips, J., Bunnell, T.J., Tricker, R., Shirazi, A., Casaburi, R., 1996. The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. N. Engl. J. Med. 335, 1–7.

Carroll, C.C., Martineau, K., Arthur, K.A., Huynh, R.T., Volper, B.D., Broderick, T.L., 2015. The effect of chronic treadmill exercise and acetaminophen on collagen and cross-linking in rat skeletal muscle and heart. Am. J. Physiol. Regul. Integr. Comp. Physiol. 308, R294–299.

Chen, J., Lee, S.K., Abd-Elgaliel, W.R., Liang, L., Galende, E.Y., Hajjar, R.J., Tung, C.H., 2011. Assessment of cardiovascular fibrosis using novel fluorescent probes. PLoS One 6, e19097.

Cranwell, C.D., Unruh, J.A., Brethour, J.R., Simms, D.D., 1996. Influence of steroid implants and concentrate feeding on carcass and longissimus muscle sensory and collagen characteristics of cull beef cows. J. Anim. Sci. 74, 1777–1783.

de Almeida Chuffa, L.G., de Souza, R.B., Frei, F., de Fátima Paccola Mesquita, S., Camargo, I.C., 2011. Nandrolone decanoate and physical effort: histological and morphometrical assessment in adult rat uterus. Anat. Rec. (Hoboken) 294, 335–341.

Evans, N.A., 2004. Current concepts in anabolic-androgenic steroids. Am. J. Sports Med. 32, 534–542.

Ferrari, F., de Paiva Foletto, M., Franzói de Moraes, S.M., Peres, S.B., Segatelli, T.M., Mareze da Costa, C.E., 2013. Testis morphophysiology of rats treated with nandrolone decanoate and submitted to physical training. Acta Sci. Health Sci. 35, 161–167.

Findley, T., Chaudhry, H., Dhar, S., 2015. Transmission of muscle force to fascia during exercise. J. Bodyw. Mov. Ther. 19, 119–123.

Fragkaki, A.G., Angelis, Y.S., Koupparis, M., Tsantili-Kakoulidou, A., Kokotos, G., Georgakopoulos, C., 2009. Structural characteristics of anabolic androgenic steroids contributing to binding to the androgen receptor and to their anabolic and androgenic activities. Applied modifications in the steroidal structure. Steroids 74, 172–197.

Frankenfeld, S.P., de Oliveira, L.P., Ignacio, D.L., Coelho, R.G., Mattos, M.N., Ferreira, A.C., Carvalho, D.P., Fortunato, R.S., 2014. Nandrolone decanoate inhibits gluconeogenesis and decreases fasting glucose in Wistar male rats. J. Endocrinol. 220, 143–153.

Franquni, J.V., do Nascimento, A.M., de Lima, E.M., Brasil, G.A., Heringer, O.A., Cassaro, K.O., da Cunha, T.V., Musso, C., Silva Santos, M.C., Kalil, I.C., Endringer, D.C., Boëchat, G.A., Bissoli, N.S., de Andrade, T.U., 2013. Nandrolone decanoate determines cardiac remodelling and injury by an imbalance in cardiac inflammatory cytokines and ACE activity, blunting of the Bezold-Jarisch reflex, resulting in the development of hypertension. Steroids 78, 379–385.

Frati, P., Busardo, F.P., Cipolloni, L., Dominicis, E.D., Fineschi, V., 2015. Anabolic androgenic steroids (AAS) related deaths: autoptic, histopathologycal and toxicologycal findings. Curr. Neuropharmacol. 13, 146–159.

Gavin, T.P., Ruster, R.S., Carrithers, J.A., Zwetsloot, K.A., Kraus, R.M., Evans, C.A., Knapp, D.J., Drew, J.L., Mc Cartney, J.S., Garry, J.P., Hickner, R.C., 2007. No difference in the skeletal muscle angiogenic response to aerobic exercise training between young and aged men. J. Physiol. 585, 231–239.

Hassan, A.F., Kamal, M.M., 2013. Effect of exercise training and anabolic androgenic steroids on hemodynamics, glycogen content, angiogenesis and apoptosis of cardiac muscle in adult male rats. Int. J. Health Sci. (Qassim) 7, 47–60.

Hoffner, L., Nielsen, J.J., Langberg, H., Hellsten, Y., 2003. Exercise but not prostanoids enhance levels of vascular endothelial growth factor and other proliferative agents in human skeletal muscle interstitium. J. Physiol. 550, 217–225.

Hjorth, M., Norheim, F., Meen, A.J., Pourteymour, S., Lee, S., Holen, T., Jensen, J., Birkeland, K.I., Martinov, V.N., Langleite, T.M., Eckardt, K., Drevon, C.A., Kolset, S.O., 2015. The effect of acute and long-term physical activity on extracellular matrix and serglycin in human skeletal muscle. Physiol. Rep. 3, e12473.

Karila, T., Hovatta, O., Seppälä, T., 2004. Concomitant abuse of anabolic androgenic steroids and human chorionic gonadotrophin impairs spermatogenesis in power athletes. Int. J. Sports Med. 25, 257–263.

Karpakka, J.A., Pesola, M.K., Takala, T.E., 1992. The effects of anabolic steroids on collagen synthesis in rat skeletal muscle and tendon. A preliminary report. Am. J. Sports Med. 20, 262–266.

Kohler, R.M., Lambert, M.I., 2002. Urine nandrolone metabolites: false positive doping test? Br. J. Sports Med. 36, 325–329.

Krause Neto, W., Silva, W.A., Ciena, A.P., Nucci, R.A.B., Anaruma, C.A., Gama, E.F., 2017. Effects of strength training and anabolic steroid in the peripheral nerve and skeletal muscle morphology of aged rats. Front. Aging Neurosci. 9, 205.

Martinez-Huenchullan, S., McLennan, S.V., Verhoeven, A., Twigg, S.M., Tam, C.S., 2017. The emerging role of skeletal muscle extracellular matrix remodelling in obesity and exercise. Obes. Rev. 18, 776–790.

Miller, B.F., Olesen, J.L., Hansen, M., Døssing, S., Crameri, R.M., Welling, R.J., Langberg, H., Flyvbjerg, A., Kjaer, M., Babraj, J.A., Smith, K., Rennie, M.J., 2005. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. J. Physiol. 567, 1021–1033.

Nakao, C., Ookawara, T., Kizaki, T., Oh-Ishi, S., Miyazaki, H., Haga, S., Sato, Y., Ji, L.L., Ohno, H., 2000. Effects of swimming training on three superoxide dismutase isoenzymes in mouse tissues. J. Appl. Physiol. 88, 649–654.

Naraghi, M.A., Abolhasani, F., Kashani, I., Anarkooli, I.J., Hemadi, M., Azami, A., Barbarestani, M., Aitken, R.J., Shokri, S., 2010. The effects of swimming exercise and supraphysiological doses of nandrolone decanoate on the testis in adult male rats: a transmission electron microscope study. Folia Morphol. (Warsz) 69, 138–146.

Neto, W.K., Gama, E.F., 2017. Strength training and anabolic steroid do not affect muscle capillarization of middle-aged rats. Rev. Bras. Med. Esporte 23. http://dx.doi.org/10.1590/1517-869220172302157902.

Nieschlag, E., Vorona, E., 2015. Doping with AAS: adverse effects on non-reproductive organs and function. Rev. Endocr. Metab. Disord. 16, 199–211.

Nikolic, T., Zivkovic, V., Jevdjevic, M., Djuric, M., Srejovic, I., Djuric, D., Jeremic, N., Djuric, D., Bolevich, S., Jakovljevic, V., 2015. The effets of chronic administration of nandrolone decanoate on redox status on exercised rats. Mol. Cell Biochem. 411, 95–105.

Pärssinen, M., Karila, T., Kovanen, V., Seppälä, T., 2000. The effect of supraphysiological

doses of anabolic androgenic steroids on collagen metabolism. Int. J. Sports Med. 21, 406–411.
Pärssinen, M., Seppälä, T., 2002. Steroid use and long-term health risks in former athletes. Sports Med. 32, 83–94.
Paschoal, M., de Cássia Marqueti, R., Perez, S., Selistre-de-Araujo, H.S., 2009. Nandrolone inhibits VEGF mRNA in rat muscle. Int. J. Sports Med. 30, 775–778.
Piacentino, D., Kotzalidis, G.D., Del Casale, A., Aromatario, M.R., Pomara, C., Girardi, P., Sani, G., 2015. Anabolic-androgenic steroid use and psychopathology in athletes. A systematic review. Curr. Neuropharmacol. 13, 101–121.
Prior, B.M., Lloyd, P.G., Yang, H.T., Terjung, R.L., 2003. Exercise-induced vascular remodeling. Exerc. Sport Sci. Rev. 31, 26–33.
Purslow, P.P., 2010. Muscle fascia and force transmission. J. Bodyw. Mov. Ther. 14, 411–417.
Riezzo, I., De Carlo, D., Neri, M., Nieddu, A., Turillazzi, E., Fineschi, V., 2011. Heart disease induced by AAS abuse, using experimental mice/rats models and the role of exercise-induced cardiotoxicity. Med. Chem. 11, 409–424.
Rocha, F.L., Carmo, E.C., Roque, F.R., Hashimoto, N.Y., Rossini, L.V., Frimm, C., Aneas, I., Neagro, C.E., Krieger, J.E., Oliveira, E.M., 2007. Anabolic steroids induce cardiac renin-angiotensin system and impair the beneficial effects of aerobic training in rats. Am. J. Physiol. Heart Circ. Physiol. 293, H3575–H3583.
Rodrigues, J.M., Oliveira, V.P.P., Furlan, J., Munhoz, A.C., Rempel, M.R.S., Brito, M.N., Brito, N.A., Pedrosa, M.M., Costa, C.E.M., 2017. Immediate and residual effects of low-dose nandrolone decanoate and treadmill training on adipose and reproductive tissues of male Wistar rats. Arch. Physiol. Biochem. 123, 68–77.
Schroeder, E.T., Terk, M., Sattler, F.R., 2003. Androgen therapy improves muscle mass and strength but not muscle quality: results from two studies. Am. J. Physiol. Endocrinol. Metab. 285, E16–E24.
Selakovic, D., Joksimovic, J., Zaletel, I., Puskas, N., Matovic, M., Rosic, G., 2017. The opposite effects of nandrolone decanoate and exercise on anxiety levels in rats may involve alterations in hippocampal parvalbumin-positive interneurons. PLoS One 12, e0189595.
Shikatani, E.A., Trifonova, A., Mandel, E.R., Liu, S.T., Roudier, E., Krylova, A., Szigiato, A., Beaudry, J., Riddell, M.C., Haas, T.L., 2012. Inhibition of proliferation, migration and proteolysis contribute to corticosterone-mediated inhibition of angiogenesis. PLoS One 7, e46625.
Soares, J.M., 1992. Effects of training on muscle capillary pattern: intermittent vs continuous exercise. J. Sports Med. Phys. Fit. 32, 123–127.
Sretenovic, J., Zivkovic, V., Srejovic, I., Milosavljevic, Z., 2016. The effects of high doses of nandrolone decanoate on cardiac muscle tissue. Ser. J. Exp. Clin. Res. 17, 303–308.
Sugizaki, M.M., Dal Pai-Silva, M., Carvalho, R.F., Padovani, C.R., Bruno, A., Nascimento, A.F., Aragon, F.F., Novelli, E.L., Cicogna, A.C., 2006. Exercise training increases myocardial inotropic response in food restricted rats. Int. J. Cardiol. 112, 191–201.
Takahashi, M., Tatsugi, T., Kohno, T., 2004. Endocrinological and pathological effects of anabolic-androgenic steroid in male rat. Endocr. J. 51, 425–434.
Tanno, A.P., das Neves, V.J., Rosa, K.T., Cunha, T.S., Giordano, F.C., Calil, C.M., Guzzoni, V., Fenandes, T., de Oliveira, E.M., Novaes, P.D., Irigoyen, M.C., Moura, M.J., Marcondes, F.K., 2011. Nandrolone and resistance training induce heart remodeling: role of fetal genes and implications for cardiac pathophysiology. Life Sci. 24, 631–637.
Tylicki, A., Kawalko, A., Sokolska, J., Strumilo, S., 2007. Effect of anabolic steroid nandrolone deaconate on enzymes in the heart, liver and muscle of rats and rat's electrophysiology. Horm. Metab. Res. 39, 268–272.
Tofighi, A., Shirpoor, M., Ansari, M.H.K., Shirpoor, A., Zerehpoosh, M., 2017. The effect of nandrolone treatment with and without enforced swimming on histological and biochemical changes in the heart and coronary artery of male rats. Anatol. J. Cardiol. 3, 176–183.
Vasilaki, F., Tsitsimpikou, C., Tsarouhas, K., Germanakis, I., Tzardi, M., Kavvalakis, M., Ozcagli, E., Kouretas, D., Tsatsakis, A.M., 2016. Cardiotoxicity in rabbits after long-term nandrolone decanoate administration. Toxicol. Lett. 241, 143–151.
Venken, K., Movérare-Skrtic, S., Kopchick, J.J., Coschigano, K.T., Ohlsson, C., Boonen, S., Bouillon, R., Vanderschueren, D., 2007. Impact of androgens, growth hormone, and IGF-I on bone and muscle in male mice during puberty. J. Bone Miner. Res. 22, 72–82.
Vingren, J.L., Kraemer, W.J., Ratamess, N.A., Anderson, J.M., Volek, J.S., Maresh, C.M., 2010. Testosterone physiology in resistance exercise and training: the up-stream regulatory elements. Sports Med. 40, 1037–1053.
Waters, R.E., Rotevatn, S., Li, P., Annex, B.H., Yan, Z., 2004. Voluntary running induces fiber type-specific angiogenesis in mouse skeletal muscle. Am. J. Physiol. 287, C1342–C1348.
Yucesoy, C.A., 2010. Epimuscular myofascial force transmission implies novel principles for muscular mechanics. Exerc. Sport Sci. Rev. 38, 128–134.
Yu-Yahiro, J.A., Michael, R.H., Nasrallah, D.V., Schofield, B., 1989. Morphologic and histologic abnormalities in female and male rats treated with anabolic steroids. Am. J. Sports Med. 17, 686–689.
Yesalis, C.E., Bahrke, M.S., 1995. Anabolic-androgenic steroids. Curr. Issues Sports Med. 19, 326–340.