Morphometric and Histological Pattern of Recovery of Adult Rat Gastrocnemius Muscle Exposed to 8-Week Anabolic Steroid Administration and Similar Period of Drug Stoppage

Original Article

Doaa H. A. Abeed[1], Rasha I. Anwar[1,2], Adel K. Abdelmalek[1] and Tarik M. Mostafa[1]

[1]*Department of Anatomy and Embryology, Faculty of Medicine, Assiut University, Assiut, Egypt*

[2]*Department of Anatomy and Embryology, Deraya University, New Minya, Menia Governorate, Egypt*

ABSTRACT

Introduction: Anabolic androgenic steroids (AAS) were first used by athletes involved in pursuits of strength, young men interested in enhancing their appearance. There is a scarcity in studies that investigated long term effect of anabolic steroids on skeletal muscles.

Objectives: The current study elucidates morphometric and histological changes of the gastrocnemius (GAS) muscle of albino rats injected by supratheraputic dose of nandrolone and investigate the possible reversibility of these changes after drug stoppage.

Material and Methods: Rats were classified into 3 groups; group I (control group, 20 rats) received no treatments with 2 subgroups A and B: I A to be sacrificed after 8 weeks and I B to be sacrificed after 16 weeks. Group II (treated, 10 rats) received nandrolone by intramuscular injection of 10 mg/kg body weight twice weekly for 8 weeks. III (recovery group, 10 rats) received the same dose of nandrolone for 8 weeks and then sacrificed 8-weeks after stoppage of treatment. The GAS muscle was removed, inspected, weighed and processed for light and electron microscopic examinations, and evaluation of apoptosis by caspase- 3 immunohistochemistry.

Results: GAS muscle of the treated group II exhibit hypertrophy, fibrosis and apoptosis. Muscle fibers present sarcoplasmic degeneration. Dislocation of myonuclei is a conspicuous finding, they appear bizarre and clustering. Activation of satellite cells is presumed by nuclear lobulation and chromatin clumbings in preparation of cellular proliferation. Satellite cells detach from their positon toward the dislocated damaged myonuclei as a step leading to regeneration. Group III shows signs of regeneration in the form of decrease collagen fibers and decrease apoptosis.

Conclusion: There are signs of incomplete recovery skeletal muscles of group III after 8-week stoppage of AAS abuse. Dislocation and internalization of myonuclei and satellite cell proliferation and migration could be considered signs of regeneration after stoppage of the insulting drug.

Received: 16 July 2022, **Accepted:** 24 October 2022

Key Words: Gastrocnemius, hypertrophy, myonuclei, nandrolone, satellite cells.

Corresponding Author: Doaa Hamid Abdel-Hameed Abeed, PhD, Department of Anatomy and Embryology, Faculty of Medicine, Assiut University, Assiut, Egypt, **Tel.**: +20 10 1629 0908, **E-mail:** doaahamed@aun.edu.eg

ISSN: 1110-0559, Vol. 46, No. 4

INTRODUCTION

Athletes and youth are widely known by abusing anabolic androgenic steroids (AAS) aiming to obtain hypertrophied and strong muscles[1]. AAS have strong anabolic effect through increasing the protein synthesis which translates into muscular hypertrophy and hyperplasia. In spite of significant gain in performance, a large number of studies have documented discordant and often contradictory reports regarding the short and long term of AAS administration[2]. There is scarcity in the studies that have investigated the regenerative potentials that are induced after discontinuation of androgen administration.

The current study is a trial to elucidate implications of AAS administration on skeletal muscles and extent of late effects after discontinuation of the drug.

MATERIALS AND METHODS

Lab animals

The experimental protocol was approved by the Ethical Committee of the Faculty of Medicine, Assiut University, Egypt (Protocol 17200433, April 23, 2020) and followed the "Principles of Laboratory Animal Care" (NIH publication no.85-23, revised 1996). The current study was carried out on forty adult male albino rats (albino race of Rattus norvegicus), 3-month old, weighing 200-250 gm. Albino rats were obtained from Faculty of Medicine Animal House, Assiut University, Egypt. Albino rats are akin to a human model from a comparative physiological perspective. Food and water were provided ad libitum. Room temperature was kept at 23 ± 2 °C. 12-h light-dark

 DOI: 10.21608/ejh.2022.150295.1723

cycle was maintained throughout the experiment. Rats had one week of acclimatization before start of the experiment. Rats were classified into 3 groups; group I is the control group (20 rats), divided into two equal subgroups. Group I A (10 rats), received no treatment, to be sacrificed after 8 weeks. Group I B (10 rats), received no treatment, to be sacrificed after 16 weeks. Group II is the treated group (10 rats) received nandrolone by intramuscular injection of 10 mg/kg body weight twice weekly for 8 weeks. Group III is the reversibility group (10 rats) received nandrolone by intramuscular injection with a dose of 10 mg/kg body weight twice weekly for 8 weeks, and then sacrificed after another 8 weeks of discontinuation of the drug. Rats were sacrificed by using deep diethyl ether anesthesia. The gastrocnemius muscle was collected and freshly weighed. Tissue fixation and processing were performed for light and electron microscopic examinations.

Androgen administration

Nandrolone decanoate (Deca-Durabolin) was purchased from the Nile Company (under license of Organon, Roseland, USA) in the form of oily solution ampoules of one ml. Each ampoule contains 25mg/ml. The ampoule was diluted in 5 ml corn oil to get concentration of 5mg/ml. The drug was intramuscularly injected in the hind limb of the rats of the treated group (GII) and the reversibility group (GIII) at a dose of 10 mg/kg, twice weekly for 8 weeks[3]. 10 mg/kg body weight is considered to be a high dose (supra-physiological dose), commonly used by athletics and body builders.

Histological study

After sacrifice of rats' gastrocnemius muscles were extracted from the right hind limb and weighed. Middle piece fragments were dissected and processed for light and electron microscopic examinations. Samples for L/M were fixed in 10% formol saline and then embedded in paraffin blocks. Sections of 5-7 µ were cut in longitudinal and cross-sectional orientations and processed to be stained with H&E, and Masson trichrome. Sections were examined for general histological features and for muscle fiber diameters. Small pieces (2 X 2 mm) were taken from the middle portion of the gastrocnemius and fixed in buffered glutaraldehyde. Specimens were trimmed and cut into semithin sections (1-µm thick) to be stained with toluidine blue. The sections were examined using Olympus light microscope and photographed.

Immunohistochemistry for caspase-3

Random slides were taken for immunohistochemistry to investigate the possible activation of muscle apoptosis, using antibodies against caspase-3. Sections were de-paraffinized in xylene, rehydrated in descending grades of alcohol, then treated with trypsin solution for 15 minutes at 37 C for antigen retrieval and then were exposed to 0.2% hydrogen perioxide in phosphate buffered saline (PBS) to block endogenous perioxidase for 30 minutes. Sections were incubated overnight at 4 C with primary rabbit anti-rat caspase-3 antibody (New Markers, Fermont CA, and Lab Vision). The sections were washed three times in PBS and incubated with goat anti-rabbit perioxidase-conjugated secondary antibody (perioxidase-labelled streptavidin) for 1 hour at room temperature. After washing with PBS, sections were incubated with diaminobenzidine (DAB) chromogen to detect immunoreactivity. Counterstaining is performed by Mayer's hematoxylin. Positive reaction for caspase-3 appears as brown coloration[4].

Transmission electron microscopy

Selected specimens for electron microscopy were fixed in buffered glutaraldehyde 2% for 24 hours then post-fixed in 2% osmium tetroxide for 60 minutes. Specimens were dehydrated and embedded in epoxy resin. Ultrathin sections were cut on ultramicrotome, stained with uranyl acetate and lead citrate. Specimens were examined and photographed using transmission Joel electron microscope of Assiut University[5].

Statistical analysis of morphometric data

Data of gastrocnemius muscle weight, cross sectional area of muscle bundle/ specific area, number of myonuclei per mm of muscle fibers, area percentage of collagen fibers and caspase-3 immunoreaction were made from sections placed on the stage of computer aided microscopy based on image J analyzer displayed on a monitor screen. Five fields in each of five randomly selected slides for each animal in each of the studied groups were measured and recoded. Data were presented as mean ± standard error of the mean (X ± SE). All numerical data were input into a computer and analyzed using statistical analysis software (Statistical Package for Social Sciences, Version 12 SPSS Inc. Chicago, Illinois, USA). ANOVA was performed to evaluate significance of differences between the studied groups, followed by post-hoc Tukey test for multiple paired comparisons among the groups, with a significant level at $p \leq 0.05$ %.

RESULTS

It is worth to be noted that, a part from body weight, there are no detected differences in the microscopic features and morphometric measurements among the control subgroups. Therefore, the histological and morphometric measurements consider the control as one group.

Body Weight

(Table 1) presents mean body weights of the investigated groups. Rats of the control subgroups demonstrate time-dependent significant increase in their body weights after 8 weeks (group IA) and after 16 weeks (group IIA) when compared with the mean weight at the start. Mean weight of rats treated with AAS for 8 weeks (group II and group III) shows no significant difference when compared with the mean weight at the beginning of the study. After another 8 weeks of discontinuation of AAS, the mean body weight of the withdrawal group shows significant increase when compared with the mean weight at the start of the study and after 8 weeks of treatment.

Gastrocnemius muscle weight

Features of gastrocnemius muscles of the control subgroups are very similar. Gross features of the gastrocnemius muscle of rats injected with nandrolone show hypertrophy (Figure I). (Table 2) shows significant increase in weight of the muscle after 8-week of treatment compared with those of the control group. After 8 weeks of nandrolone discontinuation, the mean muscle weight is significantly heavier than that of the control and the treated groups, denoting sustained and even increased muscular hypertrophy in the recovery group.

Histological Results

Control group (group I): Examination of H&E stain of longitudinal sections of gastrocnemius muscle of the control group shows that the skeletal muscle is formed of bundles of fibers surrounded by connective tissue perimysium. The muscle fibers possess multiple ovoid nuclei that are located peripherally (Figures 1a,b). In transverse section, the muscle exhibits honeycomb appearance with peripheral nuclei and obvious fibrils. Connective tissue endomysium and perimysium invest muscle fibers and muscle bundles (Figure 1c).

Masson Trichrome stained specimens of gastrocnemius muscle fibers in the control group reveal scanty green colored collagen fibers between the gastrocnemius muscle fibers (Figure 1d).

Immunohistochemical staining of the gastrocnemius muscle for caspase-3 appear negative for the control group (Figure 2a).

Semithin sections of gastrocnemius muscle stained with toluidine blue reveal that the sarcoplasm contains longituidinal myofibrils with prominent transverse striations. The myofibrils are arranged parallel to the long axis of the myofiber, with peripheral ovoid multineuclei. There are satellite cells which are identified by their extrasarcolemmal location and with their huge nuclei to cytoplasm ratio. Sracoplasm shows regular alternating light and dark bands (Figure 2b).

Examination of longitudinal sections of the muscle fibers of the control group reveals the normal electron microscopic picture of skeletal muscle fibers (Figure 3a). The myofibrils are arranged parallel to the long axis of the myofiber and have regular alternating light and dark bands. Z line is seen bisecting the light band. There are mitochondria separating the myofibrils at the I-band level and few mitochondria are also observed at the subsarcolemmal area. The myocyte is limited by sarcolemma and adherent basal lamina. Sarcoplasmic vesicles are found between the myofibrils (Figure 3b).

The AAS treated group (group II): Examination of H&E stain of longitudinally cut sections of the gastrocnemius muscle of the 8-week AAS-treated group II reveal hypertrophy and irregularity of the skeletal muscle fibers with widening of the spaces in between the fibers. Splitting of muscle fibers is also observed, which appear as a transverse invaginations and separation of the sarcolemmal membrane (Figures 4a,b). Myonuclei exhibit frequent dislocation, disorientation, and internalization. Muscular degenerative changes are observed in the form of focal lysis with disrupted transverse striations (Figure 4c).

Masson Trichrome Examination of group II shows marked increase of greenish colored collagen fibers with areas of hypertrophied muscle fibers totally replaced by collagen (Figure 4d). The skeletal muscle tissue of the treated group exhibits high affinity of positive reaction for caspase-3, which indicates prominent apoptosis (Figure 5a).

Histological examination of the longitudinally cut gastrocnemius muscle fibers after treatment with nandrolone using semithin sections shows hypertrophy of muscle fibers with areas of degeneration. Some fibers show deeply stained sarcoplasm, while others show vacuolation, distorted nuclei with internalization. Interrupted sarcolemmal membrane can be detected. Migrating satellite cells are observed (Figure 5b).

The transmission electron microscopic changes observed in the AAS treated skeletal muscles show scattered areas of damages. The damaged cells are often present side by side with the normally appearing cells (Figure 6a). The muscle sections show focal disorganization and discontinuation of myofibrils together with focal areas of discontinuation and loss of Z lines.

Accumulations of the mitochondria in the sub-sarcolemmal space and in between myofibrils are commonly noticed. Swollen and damaged mitochondria with vacuolation of their matrix are occasionally seen. Dilatation of the sarcoplasmic reticulum is observed in the place of the degenerated muscle fibers. The nuclei appear in the center of some myocytes. Nuclear changes in the form of internalization with irregularity of its outline are frequently observed (Figure 6b). There are satellite cells with their characteristic large nucleus to cytoplasm ratio. Satellite cells show signs of proliferation in the form of bilobed nucleus and prominent nuclei with chromatin clumbing. Satellite cells are close to myonuclei in preparation of regeneration.

The withdrawal group (group III): H&E-stained sections of gastrocnemius of rats of the withdrawal group reveal increased hypertrophy of the skeletal muscle fibers. The muscle fibers regain their arrangement with some areas of splitting and focal degeneration (Figures 7a,b,c).

In Masson trichrome stain of the rat gastrocnemius muscle fibers in group III shows increased greenish colored collagen as compared with group I but decreased collagen fibers deposition as compared with group II denoting amelioration of fibrosis after stoppage of the drug (Figure 7d).

Withdrawal group shows decrease in positive reaction to caspase-3 in comparison with the treated group indicating partial regeneration (Figure 8a).

The semithin sections of gastrocnemius of this group show that the muscle fibers are still hypertrophied with patchy degenerated areas with distorted nuclei. Most of gastrocnemius muscle fibers appear restoring their normal appearance with ovoid peripheral nuclei. Some nuclei appear to have two nucleoli. There are some few areas that show degeneration (Figure 8b).

In transmission electron microscopic examination most of gastrocnemius muscle fibers have restored their normal appearance with ovoid peripherally located nuclei. There are some hypertrophied muscle fibers and some areas of degeneration with increased spacing. Numerous normal mitochondria are observed with few disrupted ones and dilated sarcoplasmic reticulum. Scattered glycogen granules are detected. Signs of regeneration are obvious but full recovery is still out of reach (Figures 9a,b).

Morphometric results

Gastrocnemius muscle weight

(Table 2) displays significant increase in the weight of the muscle after 8-week of treatment compared with those of the control group (IA). After 8 weeks of nandrolone discontinuation, the mean muscle weight appears significantly heavier than that of the control and the treated groups.

Cross sectional area of the skeletal muscle fiber

(Table 3) shows significant increase in the myocyte cross-sectional area in the treated group II as compared with the control. The withdrawal group III shows significant increase in the myocyte cross-sectional area as compared with the control and treated groups.

Number of myonuclei per muscle fiber

Nandrolone treatment causes significant increase in the number of the myonuclei per GAS muscle fiber on cross sections (CS) compared with that of the control group. The number of the myonuclei per fiber found on cross sections (CS) in the withdrawal group is not significantly reduced and remain higher than that of the control group (Table 4).

Collagen fibers analysis

(Table 5) presents the mean area percentage of collagen fibers in the gastrocnemius muscle of the studied groups. There is significant increase in the mean area % of collagen fibers in group II when compared with group I and group III. Collagen fibers in group III is significantly less than that of group II but significantly greater than that of group I, indicating incomplete recovery from fibrosis.

Immunoreaction of caspase 3

Highly significant increase in the mean area percentage of caspase-3 immunoreaction is noticed in group II when compared with group I and group III. There is significant increase in immunoreaction of group III compared with group I. There is significant decrease in immunoreaction of group III compared with group II, denoting decreased apoptosis as a sign of recovery (Table 6).

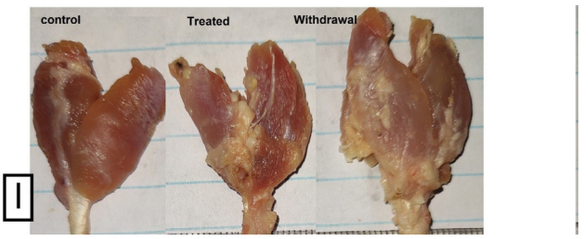

Fig. I: Gross picture of the gastrocnemius muscles of the control, treated and withdrawal groups, notice the hypertrophy of the muscle of the treated group and recognizable hypertrophy of the muscle of the withdrawal group.

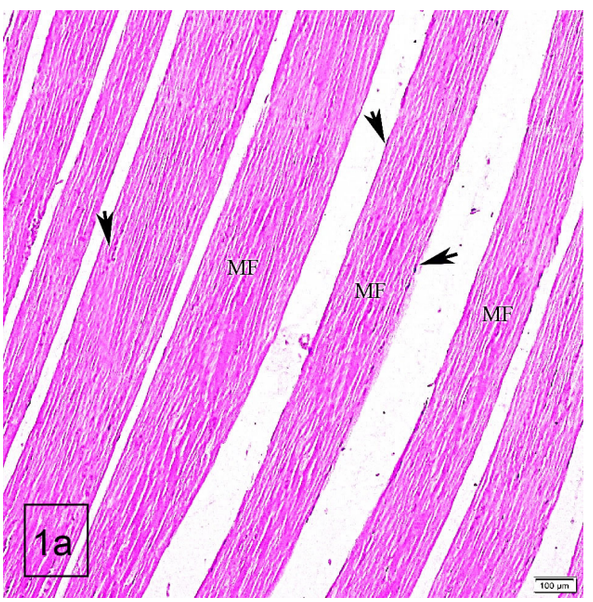

Fig. 1a: A photomicrograph of a longitudinally cut skeletal muscle fibers of the control group showing: parallel muscle bundles (MF) with parallel fibers that have peripherally located ovoid nuclei (arrows). H&E X100

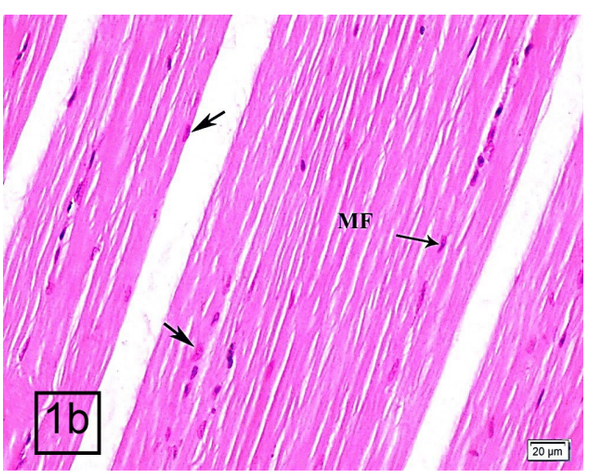

Fig. 1b: A photomicrograph of a longitudinally cut skeletal muscle fibers of the control group showing: parallel muscle bundles (MF) with parallel fibers that have peripherally located ovoid nuclei (arrows). H&E X400

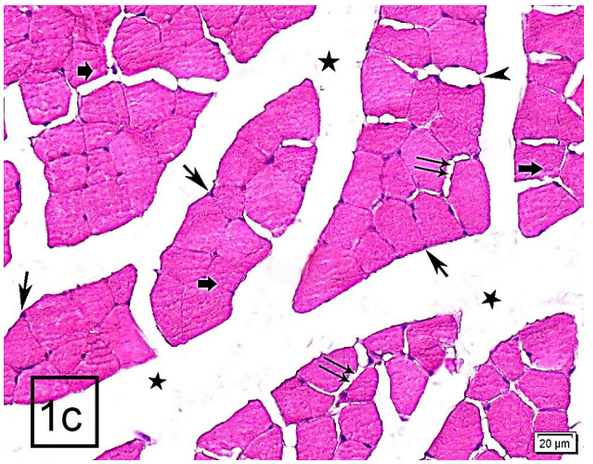

Fig. 1c: A photomicrograph of a transversely cut skeletal muscle bundles of the control group showing polygonal muscle fibers separated by endomysium (double arrow) with peripherally located nuclei (arrows). Muscle fibrils are noticed inside each muscle fiber (thick arrow) Muscle bundles are invested by connective tissue perimysium (*). Blood vessel is seen (arrowhead). H&E X400

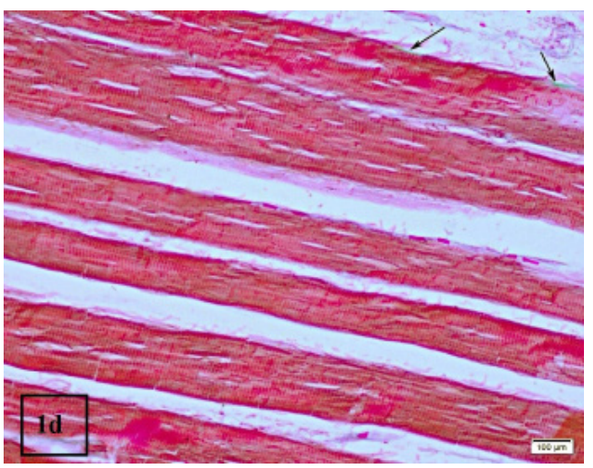

Fig. 1d: A photomicrograph of a longitudinally cut skeletal muscle fibers of the control group showing: scanty green colored collagen fibers between muscle fibers (arrows). Masson's trichrome X400

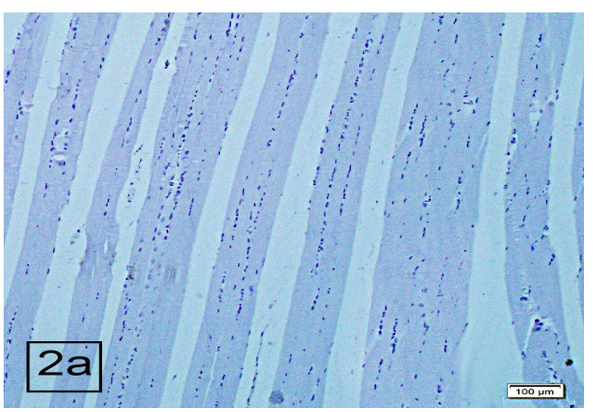

Fig. 2a: A photomicrograph of skeletal muscle fibers of the control group showing: negative caspase-3 immuno- reaction. Caspase-3 immune stain X 100

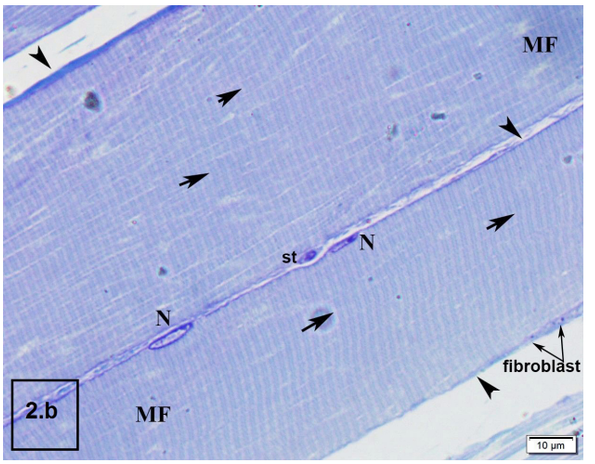

Fig. 2b: A photomicrograph of a longitudinally cut skeletal muscle fibers of the control group showing transverse striations of the skeletal muscle fibers (MF). The myofibrils are arranged parallel to the long axis of the myofiber with peripherally located ovoid myonuclei (N) and satellite cells (st). Sarcoplasm presents regular alternating light and dark bands (black arrows). Notice the intact sarcolemmal membrane (arrow head) formed of fibroblast cells (fibroblast). Toluidine blue x 1000

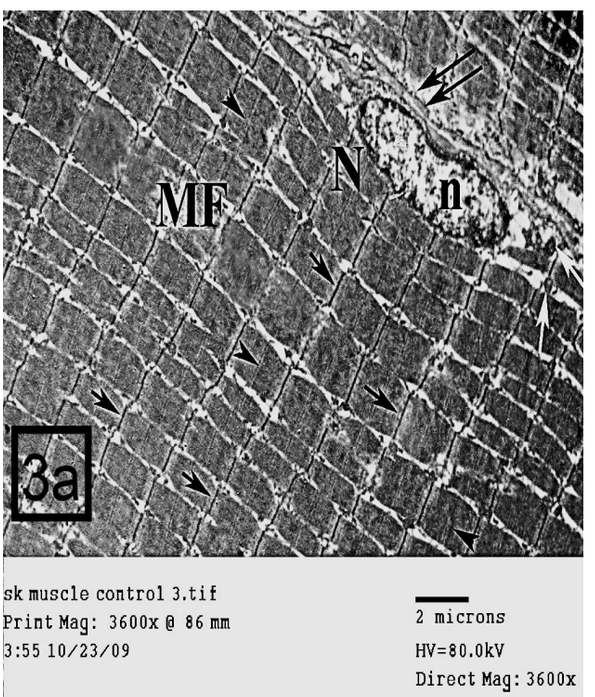

Fig. 3a: An Electron micrograph of the adult rat skeletal muscle of the control group showing peripherally located nucleus (N) with prominent nucleolus (n) and dispersed euchromatin. Sarcoplasm is composed of longitudinal myofibrils (MF). Notice intact Z lines (black arrow) with H band in the middle (arrowhead). Mitochondria (white arrow) could be seen just beneath the intact sarcolemmal membrane (double arrow). TEMX 3600

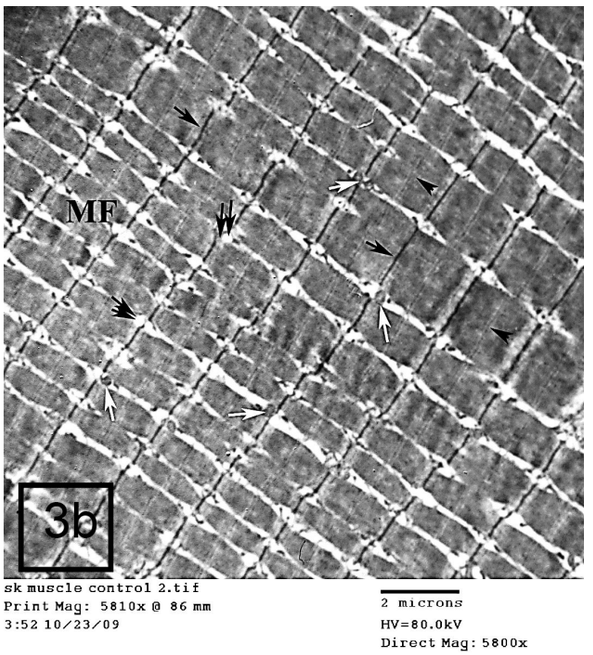

Fig. 3b: An Electron micrograph of the adult rat skeletal muscle of the control group showing Sarcoplasm is composed of longitudinal myofibrils (MF). Notice intact Z lines (black arrow) with H band in the middle (arrowhead). Mitochondria (white arrow) and sarcoplasmic reticulum (double arrow) can be seen. TEMX 5800

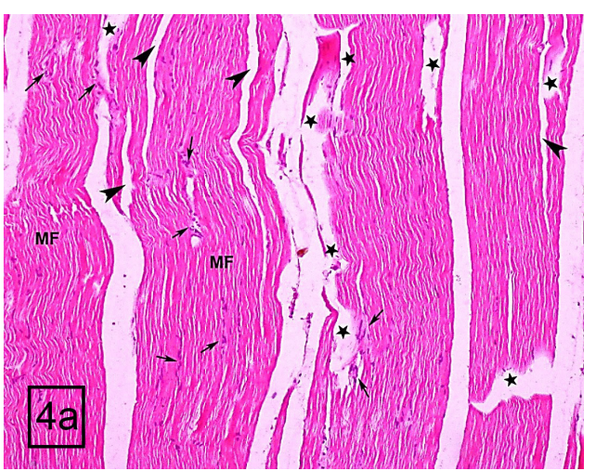

Fig. 4a: A photomicrograph of a longitudinally cut skeletal muscle fibers of the AAS treated group showing: hypertrophied muscle bundles and fibers (MF) with areas of degeneration (*) with areas of splitting (arrow heads) and dislocated crowded nuclei (arrows). H&E X100

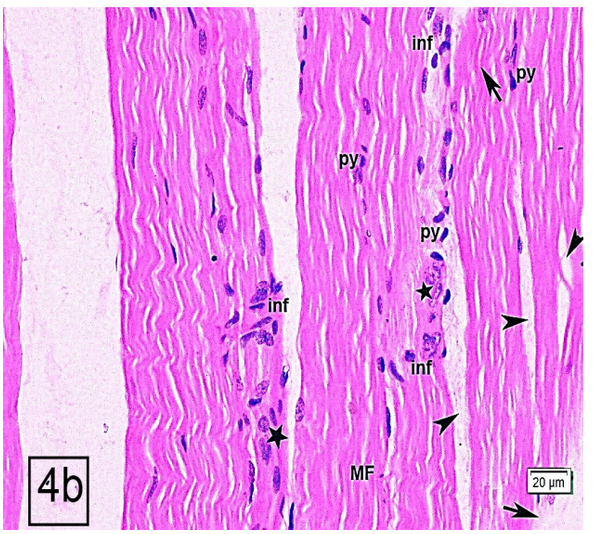

Fig. 4b: A photomicrograph of a longitudinally cut skeletal muscle fibers of the AAS treated groups showing hypertrophied muscle fibers (MF) with areas of degeneration (black arrow) and pyknotic nuclei (py), widened endomyesium (arrowhead). Notice the displaced crowded nuclei (*), and cellular infiltration (inf). H&E X400

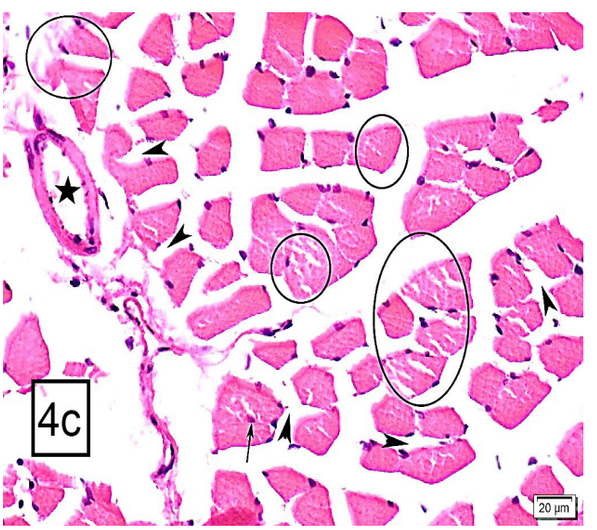

Fig. 4c: A photomicrograph of a transversely cut skeletal muscle fibers of the AAS treated group showing hypertrophy and splitting of many muscle fibers (circles) with wide separations between myofibers (arrow heads), and dilated blood vessel (*). Notice dislocation and internalization of nuclei (arrows). H&E X400

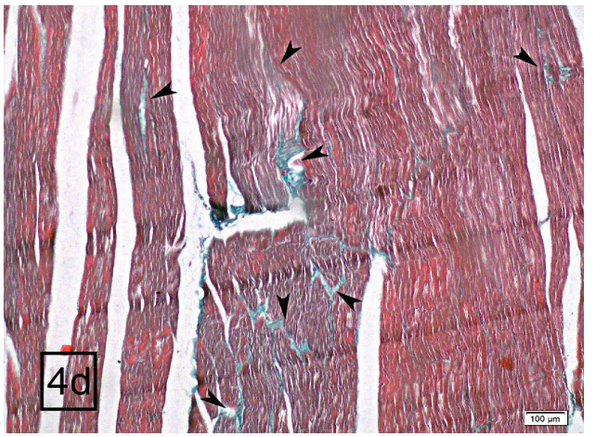

Fig. 4d: A Photomicrograph of longitudinally cut skeletal muscle the AAS treated group showing marked greenish coloration with areas totally replaced by collagen fibers (arrows). Masson's trichrome stain X100

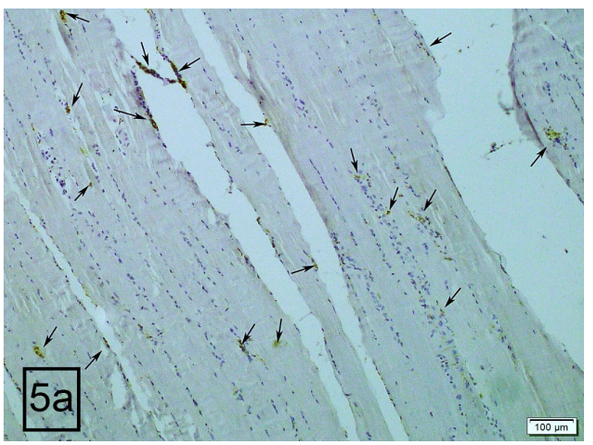

Fig. 5a: A photomicrograph of caspase-3-stained skeletal muscle fibers of the AAS treated group showing marked caspase-3 immunoreaction (arrows). Caspase-3 immune stain X 100

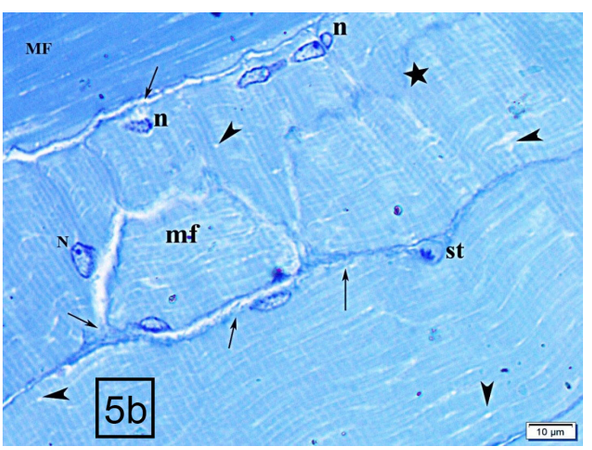

Fig. 5b: A photomicrograph of a longitudinally cut skeletal muscle fibers of the AAS treated group showing irregular hypertrophied muscle fibers (mf), areas of faint striations (MF), sarcoplasmic vacuolation (arrow head) and areas of degeneration (*). Some nuclei appear distorted and dislocated (n). Notice internalization of nuclei (N) and proliferating satellite cell (st), and Interrupted sarcolemmal membrane (black arrow). Toluidine blue X 100

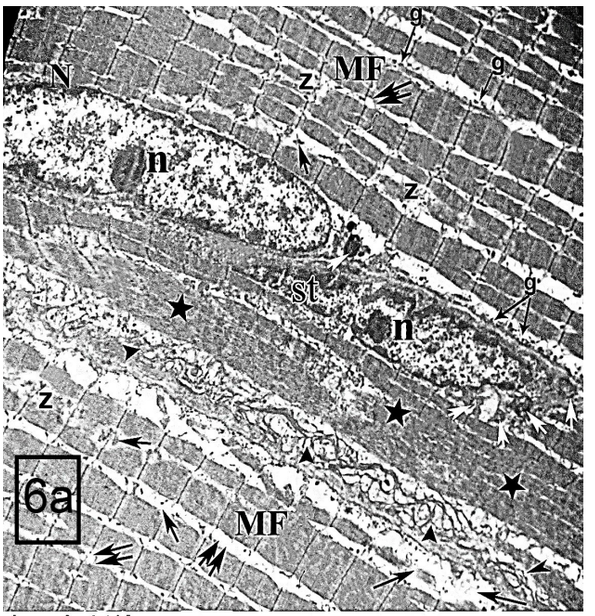

Fig. 6a: Electromicrograph of a longitudinally cut skeletal muscle fibers of the AAS treated group showing asymmetry of the muscle fiber appearance. In some areas there is hypertrophy of muscle fibers (MF) with distorted z line (z), while other areas with faint striations (*). Some other fibers show lysis and vacuolation (black arrows). Internally dislocated myocyte cell nucleus (N). Notice spindle shaped satellite cell (st) with bilobed nucleus and prominent nucleolus (n) and chromatin clumbing, seen inside the damaged muscle fibers close to myonucleus. Notice increased collagen fibers under the sarcolemmal membrane (arrow heads). Variable sized disrupted mitochondria (white arrows) dilated sarcoplasmic reticulum (double arrow) and scattered glycogen granules (g) can also be seen. TEMX 3600

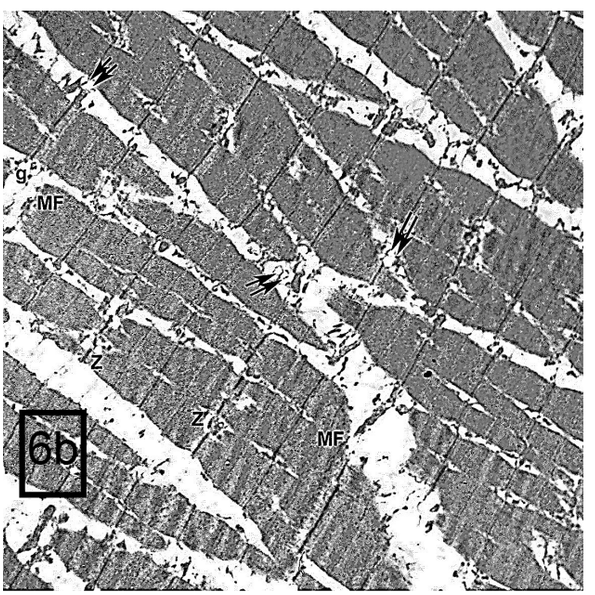

Fig. 6b: Electromicrograph of a longitudinally cut skeletal muscle fibers of the AAS treated group showing fibers with lysis and vacuolation (MF). Dilated sarcoplasmic reticulum (double arrow) and scattered glycogen granules (g) can be seen. TEMX 5800

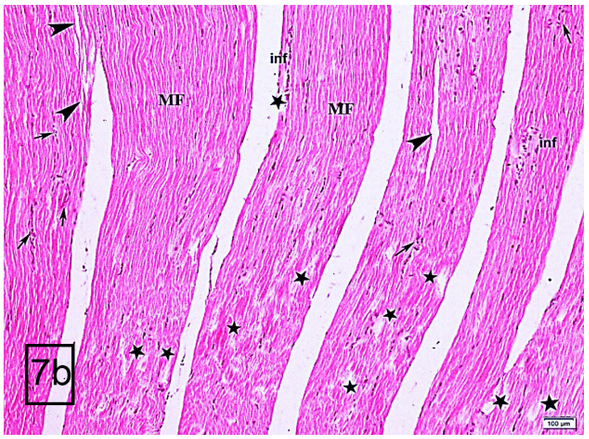

Fig. 7a: A photomicrograph of a longitudinally cut skeletal muscle fibers of the withdrawal group showing: hypertrophied muscle fibers (MF) with scattered areas of degeneration (*), splitting, widened perimysium (arrowhead) and disoriented crowded nuclei (arrows). Notice cellular infiltration (inf). H&E X100

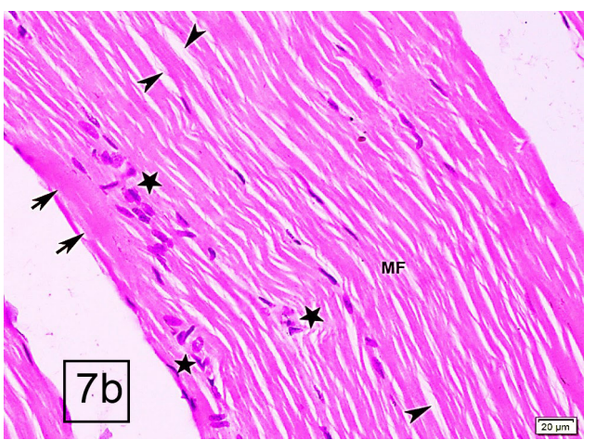

Fig. 7b: A photomicrograph of a longitudinally cut skeletal muscle fibers of the withdrawal group showing: hypertrophied muscle fibers (MF) with areas faint of striations (arrows), prominent splitting of muscle fibers (arrowhead) and crowded disoriented nuclei (*). H&E X400

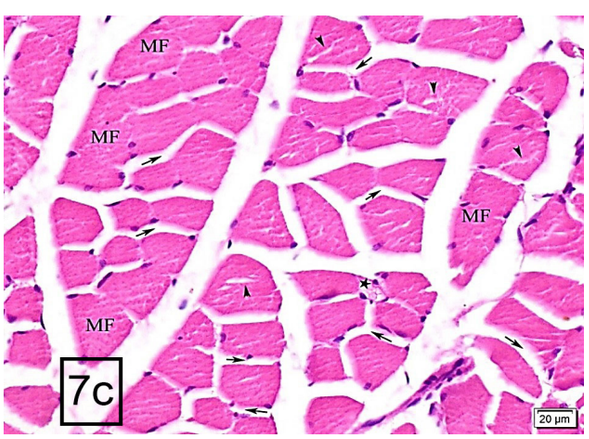

Fig. 7c: A photomicrograph of a transversely cut skeletal muscle fibers (mf) of the withdrawal group showing hypertrophy and splitting of many muscle fibers (arrow heads) with widened connective tissue spacing between the muscle fibers (arrow). Notice congested blood capillaries in between the muscle fibers (star). H&E x400

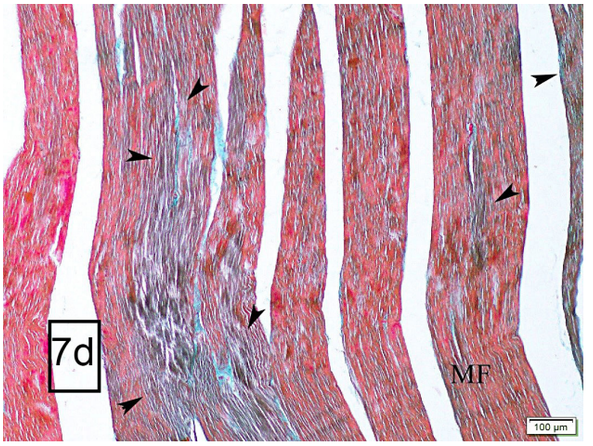

Fig. 7d: A Photomicrograph of longitudinally cut skeletal muscle of the withdrawal group showing moderate green collagen separating muscle fibers. Few muscle fibers are totally replaced by collagen (black arrows). Masson's trichrome X100

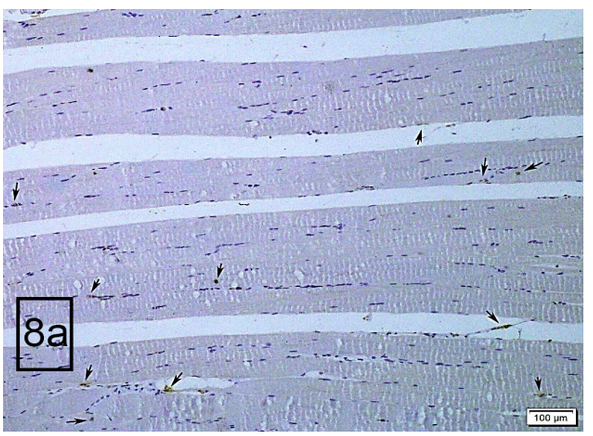

Fig. 8a: A photomicrograph of caspase-3 stained skeletal muscle fibers of the withdrawal group showing moderate caspase-3 immunoreaction (arrows). Caspase-3 immune stain X 100

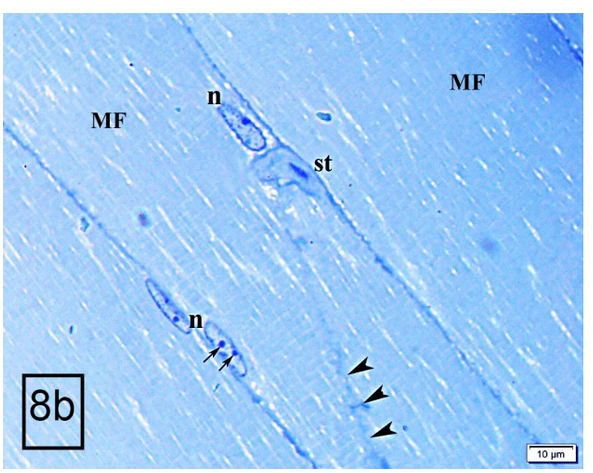

Fig. 8b: A photomicrograph of a longitudinally cut skeletal muscle fibers of withdrawal group showing: hypertrophy of muscle fibers (MF) which restored normal appearance with few areas of degeneration (arrow heads). Dislocated satellite cell can be seen (st). Notice double nucleoli (black arrows). Toluidine blue x 1000

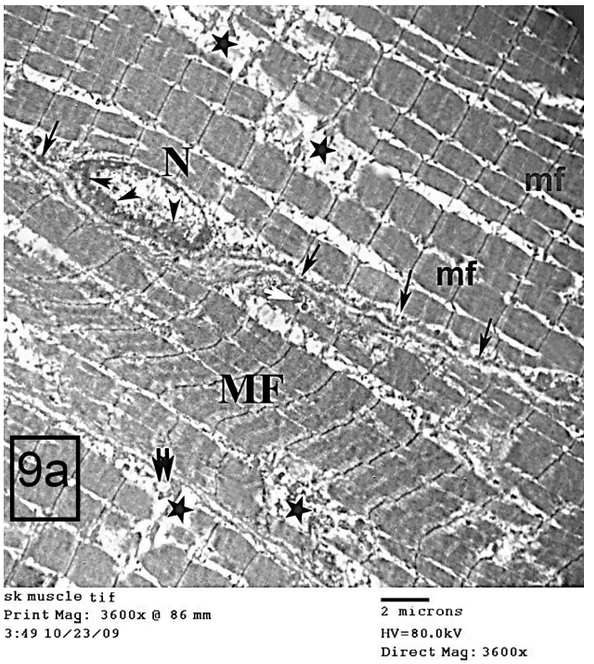

Fig. 9a: A photomicrograph of a longitudinally cut skeletal muscle fibers of withdrawal group showing hypertrophy of some muscle fibers (MF), some areas of lysis and vacuolation (*) while other fibers restore normal appearance (mf) with oval nucleus (N) with dispersed chromatin (black arrow heads). Notice dilated sarcoplasmic reticulum (double arrow). Mitochondria (white aarrow) beneath intact continuous sarcolemmal membrane are seen (black arrow). TEMX 3600

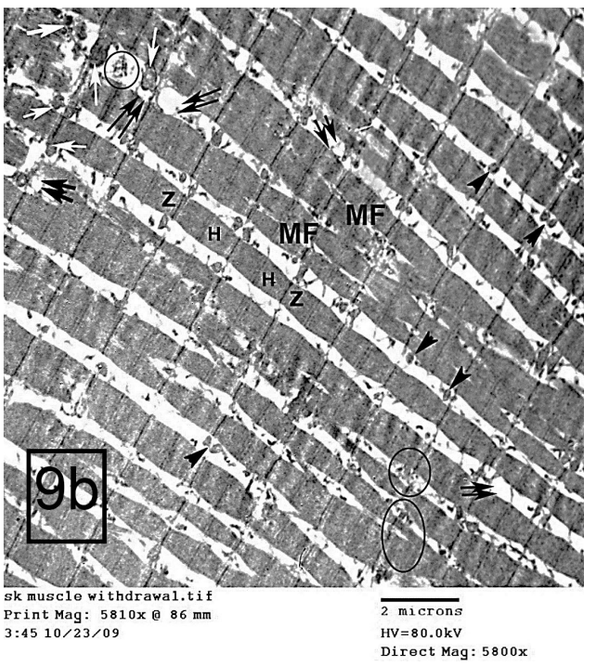

Fig. 9b: Electromicrograph of a longitudinally cut skeletal muscle fibers of withdrawal group showing that most fibers have restored their normal appearance (MF) with normal Z line (Z) and H band (H). Numerous normal mitochondria are observed (arrowheads) with few disrupted ones (white arrow) and dilated sarcoplasmic reticulum (double arrow). Notice scattered glycogen granules (black circles). TEMX 5800

Table 1: Mean body weight (BW) of the control, treated and withdrawal groups

Groups	Body weight (gm)		
	At the start	After 8 weeks	After 16 weeks
Group I (Control)	225±5.2	289±5.2#	325±5.5#$$
Group II (Treated)	227±3.1	235±4.6***#	
Group III (Withdrawal)	228±2.1	236±6.3***#	285±5.7$$

Data are presented as mean ± SEM. Compared with the control, * significant at $P<0.05$, ** highly significant at $P<0.01$, *** very highly significant at $P<0.001$. Compared with the start weight, # is significant at $P>0.05$. Compared with values at 8 weeks, $$ is highly significant difference at $P<0.001$.

Table 2: Mean gastrocnemius muscle weight of the studied groups

Groups	Gastrocnemius muscle weight in gm
Control	0.720 ± 0.052
Treated	1. 17 ± 0.51**
Withdrawal	2.35 ± 0.54***$$

Data are presented as mean ± SEM. As compared with the control, * significant difference at $P<0.05$, ** highly significant difference at $P<0.01$, *** very highly significant difference at $P<0.001$. As compared with the treated group, $ is significant difference at $P<0.05$.

Table 3: Mean cross sectional area of the gastrocnemius muscle fiber in control, treated and withdrawal groups

Groups	GAS muscle cross sectional area of the muscle fiber (μm²)
Control	1078 ± 27
Treated	2 103 ± 51**
Withdrawal	2 210 ± 54***$$

Data are presented as mean ± SEM. As compared with the control, * significant difference at $P<0.05$, ** highly significant difference at $P<0.01$, *** very highly significant difference at $P<0.001$. As compared with the treated group, $ is significant difference at $P<0.05$.

Table 4: Number of myonuclei per GAS muscle fiber in control, treated and withdrawal groups

Groups	Number of myonuclei per muscle fiber
Control	1.951 ±0. 083
Treated	2.521 ± 0.051**
Withdrawal	2.534 ± 0.062**

Data are presented as mean ± SEM. As compared with the control, * significant difference at $P<0.05$, ** highly significant difference at $P<0.01$, *** very highly significant difference at $P<0.001$. As compared with the treated group, $ is significant difference at $P<0.05$.

Table 5: Area percentage of collagen fibers in gastrocnemius muscle of the studied groups

Groups	Area % for collagen fibers
Control	8.21 ± 0. 3
Treated	43.7 ± 8.5***
Withdrawal	27.3 ± 4.2**$$

Data are presented as mean ± SEM. As compared with the control, * significant difference at $P<0.05$, ** highly significant difference at $P<0.01$, *** very highly significant difference at $P<0.001$. As compared with the treated group, $ is significant difference at $P<0.05$.

Table 6: Area % of caspase 3 immunoreaction in the in gastrocnemius muscle of studied groups

Groups	Area % for caspase fibers
Control	00.04 ± 0. 3
Treated	25.02 ± 1.5***
Withdrawal	11.01 ± 5.2***$$

Data are presented as mean ± SEM. As compared with the control, * significant difference at $P<0.05$, ** highly significant difference at $P<0.01$, *** very highly significant difference at $P<0.001$. As compared with the treated group, $ is significant difference at $P<0.05$.

DISCUSSION

Findings of the present study indicate, after 8-week of nandrolone administration to male albino rats, that there is no gain in body weight in the treated rats. The decreased appetite and increased fat metabolism induced by AAS might be the cause of this dormant growth in the young male rats[6].

In the present work the gastrocnemius muscles after 8 -week of nandrolone decanoate injection appeared grossly hypertrophied with significant weight increase in comparison with the control. These changes in skeletal muscle weights are in accordance with those of Sinha-Hikim *et al*.[7] on male rats given nandrolone decanoate (6 mg/kg bw/week) for 4 weeks. The authors attributed the increase in GAS muscle weight to the increase of synthesis of the skeletal muscle protein and to the amino acids provoked intracellular reutilization. In addition, the authors speculated that there is enhancement of the number of cells that differentiate into muscle cells following nandrolone administration. AAS are known to have receptors which is expressed over the satellite cells, differentiating myofibers and intramuscular fibroblasts[8].

In human male athletes, Hartgens *et al*.[9] documented similar findings entailing that the activation of the protein synthesis due to the intake of high dosages of AAS result in heavier GAS muscle. Quaglio *et al*.[10] explained the mechanism of action of decadurabolin in causing muscular hypertrophy that facilitates passage of the drug through the target skeletal muscle membrane to link with intracytoplasmic receptors. Then the hormone-receptor-complex attaches to the DNA of the skeletal muscle cell nucleus. After that the transcripted messenger RNA travels in the sarcoplasm to code for specific proteins synthesis that thereby eventually enhances myogenesis.

The present work indicates that the gastrocnemius remains excessively hypertrophied after 8 weeks of nandrolone decanoate withdrawal. There is a significant increase of the muscle weight of the recovery group III compared with the treated and control groups (group II and group I). This surprising finding is in harmony with the study of Yu *et al*.[11] on ten subjects admitted having taken AAS or AAS derivatives for the past 5 to 15 years (Doped). The authors found that, AAS administration for long period led to increases in the lean leg mass, muscle fiber size and a parallel improvement in the muscle strength. Despite of the discontinuation of AAS intake for long time the structure of the human skeletal muscles has preserved all these morphological changes and thus has kept improved physical performance.

In the present work, decadurabolin administration significantly increased the mean number of muscle fiber per cross sectional area, and myonuclear number per fiber. Coincident with these findings, Kadi *et al*.[12] pointed that in order to sustain a constant nuclear to cytoplasmic ratio, the anabolic-androgenic steroids enhances satellite cell proliferation and then incorporation into the preexisting fibers. This ratio is seemed to play a critical role in the increment of the myonuclear number, diameter, cross sectional area and growth of the muscle fiber.

In the same concern, Sinha-Hikim *et al*.[7] and McClung *et al*.[13] speculated that the increased number of satellite cells and their associated link to the muscle fibers lead to increase in muscle fiber diameter and cross sectional area due to the hyperplasia of the myonuclei and the resultant muscle hypertrophy.

The present study illustrated that decadurabolin caused myofibrous degeneration and inflammation in the treated skeletal muscles. In the same line, Egginton[14] reported that the administration of nandrolone in sedentary rats for 8 weeks resulted in increased mass, degenerative changes in the rat's skeletal muscle. McClung *et al*.[13] noticed that the treatment of male rats with nandrolone decanoate (6mg/kg/week) for 2 weeks caused mild muscular necrosis, slight inflammation, congested blood vessels, loss of striations and hypertrophy of skeletal muscle fibers. Furthermore, Kadi *et al*.[12] reported that athletes who abused anabolic-androgenic steroids especially in high doses showed necrosis, inflammation, and hypertrophy in their skeletal muscles.

In the present work, light, and electron microscopic examination of gastrocnemius skeletal muscle of rats after 8-week of administration of nandrolone showed markedly degenerated, hypertrophied skeletal muscle fibers widened endomysium. Fragmented and highly distorted muscles with increased collagen fibers were also detected. Moreover, many fibers were replaced by dense collagen fibers, a prominent sign of fibrosis. Variations in the mitochondrial size and shape were also observed. These results are in accordance with those of Hanan *et al*.[15] who stated that AAS given to male albino rats for 8-week induced severe ischemic degeneration of the skeletal muscle fibers and obvious inflammatory infiltrations. AAS might involve mechanisms that cause blood vessel and blood cell disorders. The disruption of the neuroendocrine and immune functions interactions induced by AAS is thought to be the cause of the pathologies that occur following the anabolic steroid abuse[16].

In the present work, the myocytes of nandrolone-treated rats showed dislocations and internalization of some myonuclei. It can be reported that the dislocated disoriented nuclei remain in the withdrawal group, a novel finding

worth to be highlighted. The repelling of the myonuclei to each other is normally vital to keep distances that help each nucleus to domain and assist the sarcomeric assembly which evidenced at the myonuclei proximity[17]. Short distances between myonuclei caused by their Clustering and dislocations may be the cause of muscle contractions disturbance. The cytoskeletal intermediate filaments, so-called desmin, has been described as the sarcoplasmic network diverging from the sarcolemma to the nuclear surface near to the nuclear pores. The concomitant activation of protease enzymes with nandrolone administration, and the resulting distortion of this cytoskeleton might be the cause of the nuclear displacement of the skeletal muscle myocytes[18].

The present work showed dilatation and congestion of skeletal muscle blood vessels. The study of Sadowska *et al*.[19] attributed that dilatation and congestion of blood vessels as well as the release of vasodilator materials which make the endothelial lining of the blood vessels more permeable to the blood elements into the extravascular tissue after AAS treatment in rat's skeletal muscle tissue to the oxidative stress caused by these agents in the muscle.

In the current study, pleomorphic, disrupted mitochondria amongst other cytoplasmic organelles were found in the skeletal muscles of the treated groups. Satoh *et al*.[20] observed that the diaphragm muscle fibers the after 4 weeks of exposure to nandrolone showed an elevated mean of their mitochondrial cross-sectional area. These structural changes of the mitochondria, their prominent proliferation and aggregation under the sarcolemmal membrane are referred by Cullen *et al*.[21] to the cell trial to overcome the respiratory chain deficiency, the reduction in the cytochrome C oxidase activity as well as the metabolic stress.

Myofibril destruction that was reported in the current study could be due to the action of the calcium activated proteinase (CAP) in the necrotic muscle. The degeneration of the plasmalemma that occur after the muscle necrosis is associated with elevation of the intracellular calcium ion concentration to mimic the extracellular one that elevation results in proteinase activation. Digestion out of the Z lines of the myofibrils and so that dissemination of the myofilaments are the signs of Myofibril destruction caused by proteinase activation[21].

The widening of the interstitial tissue of the skeletal muscles, which was detected in the current study could be due to the progressive increase in the connective tissue elements, especially collagen fibers. Damage of the skeletal muscles is associated with fibrosis. Increased collagen fibers in the present study was also noticed by Parssinen *et al*.[22] who elucidated that high doses of AAS were found to elucidate collagen synthesis, mainly in the soft connective tissues. This effect tended to be dose dependent. The increase in the anabolic and working capacity of the muscle could be the cause of these early changes in the collagen metabolism. Franquni *et al*.[23] noticed increase in the collagen in the extracellular matrix of the myocytes that exhibits hypertrophy.

Potentials of skeletal muscle recovery after stoppage of AAS

In the current investigation following 8 weeks cessation of treatment with nandrolones (as a recovery period) there was a remarkable regeneration in the skeletal muscle architecture as well as marked resumption and restoration of most of the histopathological damages.

The present study showed increase in the cross-sectional area of muscle fibers after nandrolone withdrawal. This finding is in agreement with Hartgens *et al*.[24] who investigated a cross-sectional diameter of muscle fiber characteristics in long term of AAS abuse by bodybuilders three months after drug withdrawal. The authors documented an increase in cross-sectional diameter of muscle fiber of the doped ones in comparison with the non-drug users. In the current study the sustained hypertrophy of the studied skeletal muscle fibers in the withdrawal group can be explained by the persistent increase in myonuclear number per fiber.

Von Maltzahn *et al*.[25] explained the persistent hypertrophy even after AAS withdrawal to that AAS enhances myognesis which is induced by the replication of the myonuclei. These new generations of the myonuclei are developed from the interstitial stem (satellite) cells activation. These extra myonuclei are sustained even drug removal. AAS are largely enhancing the skeletal muscles anabolism by their superimposed androgen receptors in the muscular tissue.

This is consistent with Egner *et al*.[26] who claimed that remote, may be permanent, improved physical activity can be obtained after exposure to anabolic steroids. The authors found greater muscle mass and more myonuclei, which are vital, for muscle fiber function, than in the control after AAS given to adult male rats. The addition of myonuclei accompany muscle hypertrophy and these nuclei seem to possess long-term memory to the cellular 'skeletal muscle memory'. Egner *et al*.[26] hypothesized the theory of existence of cellular memory residing in the skeletal muscles. AAS use may recruit a long-lasting pool of excess myonuclei with long-term memory that potentiates long lasting performance even after withdrawal of the drug.

There are signs of muscular regeneration in group III after 8-week stoppage of use of the AAS. These signs are in the form of decrease collagen deposition and decrease cellular apoptosis. Dislocation and internalization of myonuclei could be considered a sign of regeneration. Muscle cell regeneration is associated with nuclei that detach from peripheral anchorage to migrate to the cell center. In parallel, satellite cells proliferate and fuse to pre-existing myotubes that might recapitulate myogenesis giving rise to centrally located nuclei[25].

In between the basement membrane and sarcolemma of muscle fibers the satellite cells are populated[26]. They are

considered to be the primary stem cells which are essential for postnatal growth, hypertrophy and regeneration of skeletal muscles. They are the precursor unipotent for the myogenic cells development. The induced muscular hypertrophy after exercise, is thought to be due to the satellite cells activation and proliferation[27,28,29]. The skeletal muscle regeneration is critically depending on the satellite cells differentiaon and fusion to augment the existing degenerated muscle cells to form normal ones. In the elderly, satellite cells are not activated and undergo programmed cellular senescence, an irreversible non-dividing dormant state with the loss of the regeneration ability of the muscle. To mitigate that myogenic decline, the elderly are advised to do periodic exercise[30].

The histopathological lesions in the rat skeletal muscle in the current study were correlated with expression of caspase-3 which was increased in nandrolone treated rats compared to the control and withdrawal rats indicating apoptosis in the skeletal muscles. Apoptosis can be triggered by AAS in the vascular smooth muscle cells through the extrinsic pathway that involve androgen receptor activation and by the reactive oxygen species (ROS) which are developed due to mitochondrial stress[31]. AAS induced Apoptosis might be also referred to their ability to induce ROS generation which makes cells less viable through elevating the mediators of apoptosis and thereby initiating the programmed cell death. However, the withdrawal group in the current study showed marked decrease in caspase 3 reaction in comparison with the treated group, a prominent sign of recovery. Although sign of recovery are histologically and morphometrically observed, yet recovery is by no means complete and the mechanisms underlying it remains obscure, and encourage further investigations.

CONCLUSION

It could be concluded that, nandrolone deconate injection in male albino rats induced hypertrophy and degenerative changes in the gastrocnemius as a model of skeletal muscles which may disturb their functions. Signs of regeneration are observed in the withdrawal group in the form of decrease fibrosis and decrease apoptosis. However, it is worth mentioning that these regenerative changes observed in the recovery group are incompletely documented. Prolongation of the period of drug stoppage may promote the phenomenon of regeneration. There is element of repair and potentials of sustainability of skeletal muscle endurance most probably because hypertrophy is associated with excess myonuclei that have long- term memory for enhanced performance and increased muscle hypertrophy. This obtained muscle hypertrophy even after cessation of AAS seems to be cheater as it is associated with fibrosis, apoptosis and degeneration. Athletes, coaches, and physicians should be aware of AAS harmful side effects. Youth should take in their consideration that cessation of the drug is not enough to obtain full recovery. Drug-free aerobic exercise is recommended for the young in order to benefit at the old age.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

النمط المورفومتري والنسيجي لتعافي عضلة الساق للفئران البالغة التي تعرضت لإعطاء الاستيرويدات الابتنائيه لمدة ثمانية أسابيع بعد فترة مماثلة من وقف الدواء

دعاء عبيد[١]، رشا انور[١،٢]، عادل عبد الملك[١]، طارق مصطفي[١]

[١]قسم التشريح الادمي وعلم الاجنه، كلية الطب البشري، جامعة اسيوط، اسيوط، مصر

[٢]قسم التشريح الادمي وعلم الاجنه، كلية الطب البشري، جامعة درايه، المنيا، مصر

الخلفية: تم استخدام المنشطات الاندروجينية الابتنائيه لأول مرة من قبل الرياضيين المشاركين في مساعي القوة ، والشباب المهتمين بتعزيز مظهرهم. هناك ندرة في الدراسات التي بحثت في التأثير طويل المدى للستيرويدات الابتنائية على عضلات الهيكل العظمي.

الأهداف: توضح الدراسة الحالية التغيرات الشكلية والنسيجية للعضلة التوأميه للفئران البيضاء المحقونة بجرعة علاجية من الناندرولون وتحقق من إمكانية عكس هذه التغييرات بعد التوقف عن تناول الدواء.

المواد والطرق: تم تصنيف الفئران إلى ٣ مجموعات. المجموعة الأولى (المجموعة الضابطة ، ٢٠ فأرا) لم تتلق أي علاجات مع مجموعتين فرعيتين I A يتم التضحية بها بعد ٨ أسابيع و I B ليتم التضحية بها بعد ١٦ أسبوعًا. المجموعة الثانية (المعالجة ، ١٠ فئران) تلقت الناندرولون عن طريق الحقن العضلي بمقدار ١٠ مجم / كجم من وزن الجسم مرتين أسبوعياً لمدة ٨ أسابيع. تلقى III (مجموعة التعافي ، ١٠ فئران) نفس جرعة الناندرولون لمدة ٨ أسابيع ثم تم التضحية بها لمدة ٨ أسابيع بعد توقف العلاج. تمت إزالة العضلة التوأميه وفحصها ووزنها ومعالجتها لإجراء فحوصات مجهرية ضوئية وإلكترونية ، وتقييم موت الخلايا المبرمج عن طريق الكيمياء المناعية للكاسباس ٣.

النتائج: تظهر العضلة التوأميه من المجموعة الثانية المعالجة تضخمًا وتليفًا وموت الخلايا المبرمج. الألياف العضلية تظهر تنكس ساركوبلازمي. خلع النوى العضلية هو اكتشاف واضح ، فهي تبدو غريبة ومتجمعة. يُفترض تنشيط الخلايا الساتلية عن طريق الفصوص النووية و تكتل الكروماتين استعدادًا للتكاثر الخلوي. تنفصل الخلايا الساتلية عن موقعها باتجاه النوى العضلية التالفة المخلوعة كخطوة تؤدي إلى التجدد. تظهر المجموعة الثالثة علامات التجدد في شكل انخفاض ألياف الكولاجين وتقليل موت الخلايا المبرمج.

الخلاصة: هناك علامات تدل على عدم اكتمال شفاء عضلات الهيكل العظمي للمجموعة الثالثة بعد توقف لمدة ٨ أسابيع من تعاطي المنشطات الاندروجينيه. يمكن اعتبار خلع واستيعاب النوى العضلية وتكاثر الخلايا الساتلية والهجرة علامات على التجدد بعد توقف اعطاء الناندرولون.