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The Nandrolone Effect on Cardiac Muscle of Adult Male Albino Rat and the Possible Role of *Nigella sativa*: Light and Electron Microscopic Studies

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Abstract

Aim: The current study was undertaken to evaluate the nandrolone effects on adult male albino rats cardiac muscle of and the possible protective role of *N. sativa*. Thirty Adult male albino rats were used and equally divided into three Groups: Group I: control, Group II (nandrolone treated), Group III (nandrolone and *N. sativa* treated). Left ventricular specimens were prepared for H and E, van Gieson staining, immuno-histochemical analysis for (caspase 3) and electron microscope examination.

Results: Cardiac muscles of nandrolone treated rats (Group II) showed histological, ultrastructural and histochemical changes such as dark stained nuclei, fragmentation, vacuolation, hemorrhage and dilated endomysium, pyknotic nuclei, loss of striations and dehiscent intercalated disc. Also, increased collagen fibers in the endomysium were found. Minimal changes were observed in Group III (nandrolone and *N. sativa* treated).

Conclusion: Nandrolone had a deleterious effect on rat cardiac muscle. Co-administration of *N. sativa* and nandrolone had a great protective effect.

Keywords: Cardiac muscle; Intercalated disc; Nandrolone; *N. sativa*

Abbreviations: ND: Nandrolone Decanoate; NS: Negella Sativa; TQ: Thymoquinone; BS: Black Seed; H and E: Hematoxylin and Eosin; AAS: Anabolic Androgenic Steroids; DAB: Diamiobenzidine; GPx: Glutathione Peroxidase; GSH: Growth Stimulating Hormone; ROS: Reactive Oxygen Species, SOD: Superoxide Dismutase

Introduction

Nandrolone decanoate (ND) is a synthetic derivative of testosterone. It is anabolic androgenic steroids (AAS) administrated by athletes and adolescents and considered as one of the most commonly AAS. ND is supposed to promote muscle mass increase and improves physical appearance as well as sporting performance [1].

ND abuse is often associated with serious adverse effects, interfering with many body systems such as the musculoskeletal system, the endocrine system and the reproductive system [2]. Also, ND may suppress the hypothalamic pituitary gonadal axis resulting in diminished endogenous testosterone production [3].

Nandrolone can be administrated by many routs such as oral pills, injectable steroid, creams and gel. Nandrolone was taken as a supportive therapy to increase body weight in some pathological conditions as cachexia that associated with some chronic diseases. Also, it was administrated to treat anaemia. But now, it is widely used mostly by young men for non-medical purposes, to improve their body building [4,5].

Nandrolone has many adverse effects especially when used by healthy people. Many serious adverse effects can be resulted from long-term intake of excessive doses of anabolic steroids. Hypercholestrolemia, hepatic dysfunction and hypertension are the most common adverse effects [6]. Cardiovascular diseases are the most important side effects, as they increased the incidence of young male body builder's death.

Nandrolone decanoate also reported to increase in atherosclerosis, tachycardia, cardiac dysfunction, arrhythmia, premature acute ischaemic heart diseases, myocardial infarction and even sudden death. Other effects as depression, nervousness, testicular atrophy and liver damage were also reported [7,8].

The use of medicinal plants for the prevention of cardiovascular diseases has been increasing recently. *N. sativa* (NS), also known as black cumin or black seed, has been proved to possess blood pressure-lowering effects in animals as well as humans [9]. NS has been used in medicinal purposes for centuries in the Middle East, India and Northern Africa [10]. Also, it has been widely used to treat nervous system diseases such as memory impairment, epilepsy, neurotoxicity and pain.

The *N. sativa* therapeutic properties are due to the presence of thymoquinone (TQ) and polyphenols which are the essential oil major bioactive components [11]. In traditional medicine, NS seeds are used in the treatment of different illnesses like obesity, hypertension, gastrointestinal problems, diarrhoea, back pain, cardiac diseases, bronchitis, asthma, sexual diseases, rheumatoid arthritis and skin disorders [12,13].

The aim of this work is to study the possible histological alterations that may occur in the cardiac muscle structure of adult male albino rats receiving nandrolone and the possible role of *N. sativa* supplementation.

Materials and Methods

Drugs and chemicals

Nandrolone decanoate (Deca-Durabolin): It was in the form of ampoules (25 mg/ ampoule). It was provided by Nile Company pharmaceuticals-Cairo.

N. sativa: Black seed (BS) was purchased from Kahira Pharm. and Chem. Ind. Co., Cairo, Egypt. Black seed is present as soft gelatin capsules (500 mg). The capsule was dissolved in 10 cm of vegetable oil and taken at dose of 50 mg/kg body weight *via* gastric gavage [14].

Animals

Thirty adult Wistar male albino rats weighing (200-250 gm.) were purchased from the Breading Animal House, Faculty of Medicine, Zagazig University, Zagazig, Egypt. They were housed at room temperature under standard laboratory conditions. They were maintained on standard laboratory food and water ad libitum during the period of the experiment.

All procedures of the experiment were approved and carried out according to the guidelines of the Institutional Animal Care and Use Committee accepted by Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Experimental design

The animals were classified into three equal Groups (each contains 10 rats):

Group I: control Group: Included 10 animals which subdivided into two equal subgroups:

Subgroup Ia: Received vegetable oil.

Subgroup Ib: (*N. sativa* treated): *N. sativa* was given at a dose of 50 mg/kg body weight per day *via* gastric gavage for four weeks [14].

Group II: Nandrolone treated Group: Included 10 rats received nandrolone by intra-muscular injection at a dose of 10 mg/kg/week for four weeks [15].

Group III: Nandrolone treated and *N. sativa* supplemented Group: Composed of 10 rats, each received nandrolone and *N. sativa* simultaneously at a dose similar to the previous Groups. 24 hour after the last injection, all the Groups were sacrificed.

At the end of the experiment, rats were anaesthetized by intraperitoneal injection of pentobarbitone sodium 60 mg/kg body weight. A midline incision was done on the anterior aspect of the chest, sternocostal junctions were cut.

Samples from the heart fixed in 10% neutral buffered formalin and processed for preparation of paraffin sections for histological: (Hematoxylin and Eosin) [16], van Geison stain [17]. For ultrastructural study, specimens were immediately fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.4). Thereafter, they were postfixed in 1% osmium tetroxide in the same buffer at 4°C, dehydrated, and embedded in epoxy resin [18].

Methods

Histological study

Paraffin sections (5 µm thick) stained with (H and E) for examination of overall morphology and Van Geison for examination of collagen fibers.

Immunohistochemical study: Immunohistochemical reaction for Caspase 3 protein was done by using streptavidin-biotin complex immunoperoxidase system. Serial sections of paraffin-embedded specimens were deparaffinized on charged slides. The sections were incubated in 0.1% hydrogen peroxide for 30 min to block the endogenous peroxidase, then incubated with the primary antibody. The primary antibody used for caspase-3 was ready-to-use rabbit polyclonal antibody (CAT-No: RB-3425-R2). The slides were incubated with the secondary anti-rabbit antibody versal kits (Zymed laboratories), diluted 1: 200 for 30 minutes, staining was completed by incubation with chromogen, called diamiobenzidine (DAB). Mayer's hematoxylin was used as a counterstain [19].

Ultrastructural study: Semithin sections 1 µm thick were stained with 1% toluidine blue for light microscopic examination. Ultrathin sections were stained with uranyl acetate and lead citrate [18], examined and photographed using (JEOL JEM -2100) Transmission Electron Microscope (Jeol Ltd, Tokyo, Japan) in Electron Microscope Research Unit, Faculty of Agriculture, Mansoura University, Egypt.

Histomorphometric analysis

The image analyzer computer system Leica Qwin 500 (Leica Ltd, Cambridge, UK) at the Image Analyzing Unit of Pathology Department, Faculty of Dentistry, Cairo University, Egypt, was used to measure the area percent of collagen fibers and caspase 3 immunoreaction. The area percent was measured using the interactive measure menu. The measuring frame of a standard area equal to 118476.6 mm^2 was chosen so that the brown positive immune reaction could be seen and masked by blue binary colour to be measured. Examination of ten readings from five non-overlapping sections from each rat of all Groups was done.

Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 13.00 (Chicago, Illinois, USA). Statistical significance was determined by one-way analysis of variance for differences between the means of different Groups. Further analysis was carried out using the post-hoc test to compare the parameters between the different Groups with each other. Probability of P less than 0.05 was considered statistically significant.

Histological Results

H and E stained sections of left ventricular myocardium of both control Groups Ia and Ib revealed nearly similar results. H and E stained sections of left ventricle of control Groups revealed cardiomyocytes with acidophilic sarcoplasm and central oval vesicular nuclei with clear perinuclear regions. The intercellular spaces are narrow containing blood capillaries (Figure 1 (a-d)). Nandrolone-Treated Group, sections revealed focal variable changes in the cardiac muscle. Some of them showed separated and degenerated muscle fibers with widening of the endomysium. The cytoplasm of the

cardiomyocytes appeared deeply acidophilic with condensation and flattening of some nuclei. Others affected areas revealed wavy muscle fibers with loss of their normal architecture and congested blood vessels. Massive cellular infiltration was noticed between the muscle fiber (Figure 1 (b-d)). Nandrolone treated and black seed supplemented Group, sections of the left ventricular myocardium revealed minimal changes in their histological features. Little intercellular spaces were observed in between muscle fibers. Some cardiomyocytes nuclei were vesicular and others appeared dark (Figure 2).

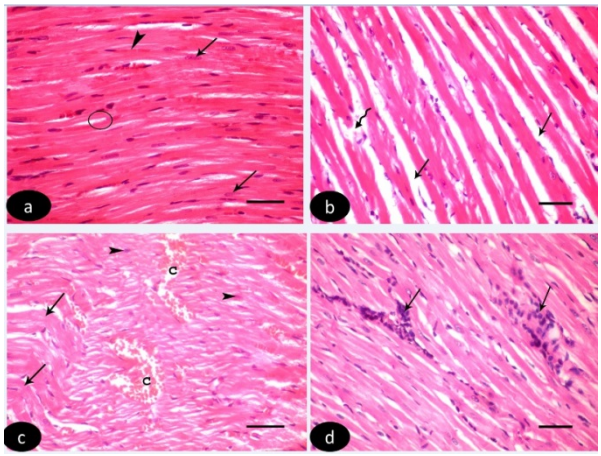

Figure 1: (a) H and E stained section in the left ventricular myocardium of a control rat showing long parallel and branched cardiac muscle fibers (circle). They have acidophilic cytoplasm and central, oval vesicular nuclei (arrow). The intercellular spaces are narrow containing blood capillaries (arrow head). (b) Nandrolone treated rat showing widely separated cardiomyocytes (arrow), fragmented muscle fibers (zigzag arrow). (c) Some sections of nandrolone treated showing loss of normal architecture of cardiomyocytes, wavy muscle fibers (arrow). Their nuclei are condensed (arrow head) and the blood vessels are congested (C). (d) Nandrolone treated Group showing massive cellular infiltration (arrow).

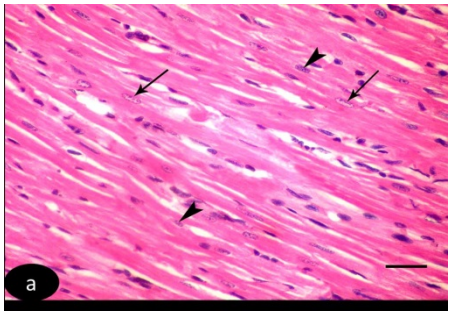

Figure 2: (a) Nandrolone treated and black seed-supplemented rat showing cardiomyocytes with minimal endomysium in between, some of them have vesicular nuclei (arrow) while others have dark nuclei (arrow head).

Van Geison stained sections of the control left ventricle revealed few fine collagen fibers between the cardiomyocytes (Figure 3 (a-c)). While in nandrolone treated rats, increased collagen fibers were noticed in the interstitium between cardiomyocytes and around blood vessels (Figure 3). In nandrolone treated and black seed supplemented rats, few fine collagen fibers between the cardiomyocytes and around blood vessels were noticed (Figure 3).

Immunohistochemical results: Immunohistochemical-stained sections of the control left ventricular myocardium revealed negative cytoplasmic reaction for caspase-3 in the cardiomyocytes (Figure 4 (a-c)). While, in nandrolone treated strong positive cytoplasmic reaction for caspase-3 in the cardiomyocytes (Figure 4). On the other hand in nandrolone treated and black seed-supplemented subgroup revealed weak positive cytoplasmic immunoreaction for caspase-3 in the cardiomyocyte (Figure 4).

Ultrastructural results: Examination of the ultrathin sections of the left ventricular cardiomyocytes of the control Groups revealed euchromatic nuclei with dispersed euchromatin. The cardiomyocytes joined by intact intercalated discs with their transverse and lateral portion. The sarcoplasm showed bundles of myofibrils with rows of mitochondria with closely packed cristae in between them. Their myofibrils had alternating dark A and light I bands. Z lines appeared bisecting I band. Glycogen granules could be detected in between myofibrils (Figure 5 (a and b)). In nandrolone treated Group.

The cardiac muscle exhibited fragmentation and lysis of some myofibrils and pyknotic nuclei with irregular nuclear envelope. Increased collagen fibers were noticed in the endomysium. Irregularly distributed intercalated discs were demonstrated in-between the adjacent cardiomyocytes (Figure 5 (c and d) and Figure 6a). Nandrolone treated and black seed supplemented Group, cardiomyocytes contained large euchromatic nuclei with dispersed euchromatin. Some myofibrils were well organized while others were degenerated. Numerous mitochondria were demonstrated in-between the myofibrils. The cardiomyocytes were joined by intact intercalated discs with their transverse and lateral portion (Figure 6 (b and c)).

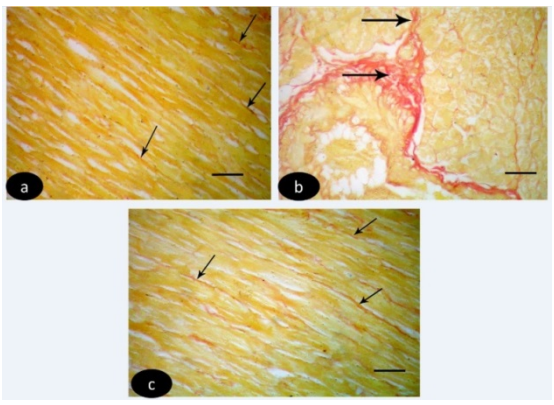

Figure 3: (a) van Geison stained sections of the control left ventricle reveal few fine collagen fibers (arrow) between the cardiomyocytes. (b) Nandrolone treated Group, increased collagen fibers (arrow) in the interstitium between cardiomyocytes and around blood vessels. (c) Nandrolone treated and black seed-supplemented rat, few fine collagen fibres (arrow) between the cardiomyocytes and around blood vessels are noticed.

Histomorphometric and statistical results

Highly statistically significant increase in the mean area % of collagen fibres was revealed in Group II when compared with Group I and Group III. No statistical significant difference was detected between Group I, when compared with III (Table 1). Highly statistically significant increase in the mean area % of caspase 3 immunoreaction was noticed in Group II when compared with Group I, Group III. No statistical significant difference was detected between Group I, when compared with III (Table 2).

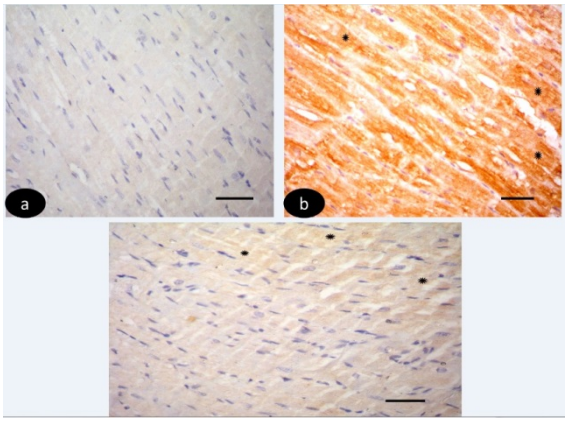

Figure 4: (a) Immunohistochemical reaction for caspase-3 in the control Group showing negative cytoplasmic immunoreaction for caspase-3 in the cardiomyocytes. (b) Nandrolone treated showing strong positive cytoplasmic immunoreaction for caspase-3 (asterisks) in the cardiomyocytes. (c) Nandrolone treated and black seed-supplemented showing weak positive cytoplasmic immunoreaction for caspase-3 (asterisks) in the cardiomyocytes.

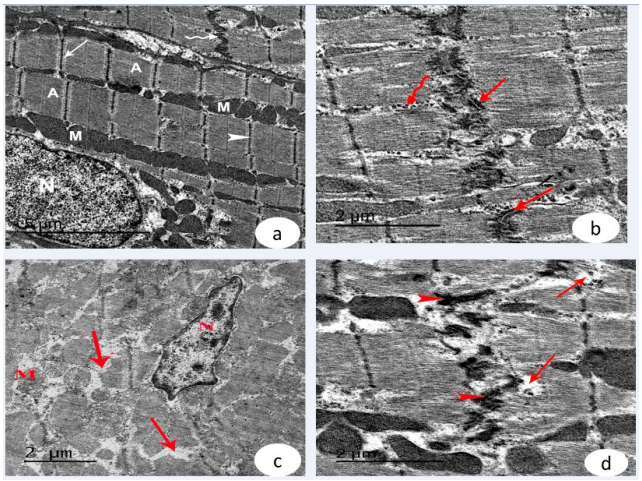

Figure 5: (a) An electron micrograph from the left ventricular cardiomyocytes of a control rat showing a cardiomyocyte with euchromatic nucleus (N). Bundles of myofibrils are seen with alternating dark (A) and light bands (arrow). Z lines (arrow head) appear bisecting I bands. Rows of mitochondria (M) with closely packed cristae in-between myofibrils and intact intercalated disc are also seen (zigzag arrow). (b) Cardiomyocytes of a control rat also showing portions of two cardiomyocytes joined at an intercalated disc (arrow) Glycogen particles (zigzag arrow) are seen in-between myofibrils, (c) Nandrolone treated Group cardiomyocytes revealed nucleus with irregular nuclear envelope (N), fragmented myofibrils (arrow) and mitochondria with disrupted cristae (M). (d) Nandrolone treated rat showing fragmented myofibrils (arrow) and irregularly distributed intercalated discs (arrow head).

Parameters	Mean ± SD	F	P-value
Group I	12.31 ± 7. 3	90	≤ 0.0001**
Group II	55.12 ± 10.5		
Group III	14.81 ± 5.2		

Table 1: Area % for collagen fibres in different studied Groups.

Parameters	Mean ± SD	F	P-value
Group I	17.03 ± 5.7	32	≤ 0.0001**
Group II	45 ± 13.9		
Group III	19.36 ± 0.31		

Table 2: Area % of caspase 3 immunoreaction in different studied Groups.

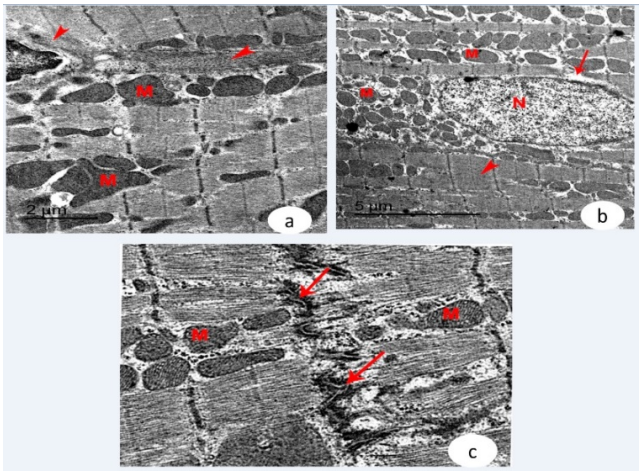

Figure 6: (a) Cardiomyocytes of nandrolone treated rat showing mitochondria with variable size and shape (M) and bundles of collagen fibrils (arrow head). (b) Nandrolone treated and black seed-supplemented rat showing a cardiomyocyte with euchromatic nucleus (N). Some myofibrils are well organized (arrow head) while others are fragmented (arrow). Numerous mitochondria (M) with variable size and shape are noticed. (c) Nandrolone treated and black seed-supplemented rat showing portions of two cardiomyocytes joined by apparently intact intercalated disc (arrow) and well organized mitochondria (M).

Discussion

AAS; nandrolone; although forbidden in sports, are still widely used by professional and recreational athletes who intend to quickly gain muscle mass and improve cardiovascular system performance [20]. Their anabolic actions are mainly due to increase synthesis and reduce the degradation of the muscle proteins as mentioned by [21,22]. In the present work, nandrolone abuse induces many cardiac changes as regard structure, ventricular thickness, size, as well as heart connective tissue content. These results are in accordance with previous findings which stated that, chronic nandrolone abuse had great effect on many organs function and structure [23-28]. Others added that the effect of nandrolone is dose dependant [29]. In the current work, nandrolone treated Group (Group II) showed marked histological, ultrastructural and histochemical changes in cardiac muscle when compared with control Group (Group I). Minimal changes were noticed when nandrolone and *N. sativa* are co-administered (Group III).

In the present study, H and E-stained sections of the left ventricular myocardium revealed focal variable changes in the cardiac muscle. The less apparently affected areas showed separated and degenerated muscle fibers with widening of the endomysium. Other focal changes revealed hypertrophied muscle fibers. The cytoplasm of the cardiomyocytes appeared deeply acidophilic with condensation and flattening of some nuclei. The more affected areas revealed wavy muscle fibers with loss of their normal architecture, condensed nuclei and congested blood vessels the same results were obtained by Soliman et al. [30]. The antioxidant system includes enzymes (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase) as well as non-enzymatic antioxidants (alpha-tocopherol and reduced glutathione (GSH)) are responsible for heart protection as they scavenge the unpaired electrons of free radicals and prevent damage propagation [31]. Nandrolone administration has cardiotoxic effects because it disturbs normal antioxidant and induce oxidative stress *via* significant reduction in heart antioxidant capacity (reducing GPx, glutathione reductase and super oxide dismutase) activities leading to free radicals liberation [32,33].

Oxidative stress is defined as a disruption of the prooxidant antioxidant balance in favor of the prooxidant, leading to potential damage which may alter all function through changes in intracellular calcium or intracellular pH and eventually can lead to cell death [34]. Rodrigo et al. [35] found that ROS which released as a result of oxidative stress activate some transcription factors. The unfavourable consequences of this activation include inflammation, fibrosis or apoptosis. Nitric oxide is considered one of the most important signalling molecules regulating cardiovascular function. It regulates blood pressure by dilating blood vessels and inhibits platelet aggregation and leukocyte adhesion. It also inhibits cell proliferation in vascular smooth muscle and subsequently regulates myocardial scar formation [36,37]. In the current work, Van Geison stained sections of the nandrolone treated Group (Group II) showed increased collagen fibers the interstitium between cardiomyocytes and around blood vessels. The same findings were obtained by Franquni et al. [38] and Soliman et al. [30] who found that nandrolone administration cause a 10 fold increase in heart collagen.

Parssinen et al. [39] added that this effect tended to be dose-dependent. These short-term changes in collagen metabolism may be explained by increased anabolic effects in muscle or secondary to increased working capacity. In the current work, electron microscope examination of the nandrolone treated Group confirmed the results observed by light microscope. The cardiac muscle exhibited destruction, fragmentation and lysis of some myofibrils and pyknotic nuclei with irregular nuclear envelope. Increased collagen fibers were noticed in the endomysium. Irregularly distributed intercalated discs were demonstrated in-between the adjacent cardiomyocytes. The same results were obtained by Soliman et al. [30]. Also, these findings are in accordance with Cavasin et al. [40] and Golestani et al. [41] who found anabolic steroids intake caused marked injuries in the rodent heart and led to sarcomere disruption, mitochondrial damage and apoptosis. Tokunaga et al. [42] stated that the free radicals facilitate the release of lysosomal enzymes into the cytosol with subsequent oxidation of the protein architecture of the cells causing their fragmentation. Swapnila et al. [43] added that the production of free radicals can damage DNA strands directly and induce apoptosis and expression of p53 in hepatocytes. Free radicals known to stimulate the peroxidation of membrane phospholipids causing severe damage [44].

In the present study, negative cytoplasmic immunoreaction for caspase -3 was noticed in the cardiomyocytes of control Group. While, in nandrolone treated Group strong positive cytoplasmic immunoreaction for caspase-3 was reaveled in the cardiomyocytes. On the other hand, in nandrolone treated and black seed-supplemented Group revealed weak positive cytoplasmic immunoreaction for caspase-3. Nandrolone abuse has been associated with thrombosis and arteriosclerosis, both of which predispose to myocardial ischemia and infarction. However, there are reports of sudden cardiac death in the absence of thrombus and atheroma following anabolic steroid use [45]. Programmed cell death (apoptosis) has been implicated in normal biological processes and in the pathogenesis of several diseases in humans. The characterization of genes incorporated in apoptosis has been pursued intensively and has been identified as two major classes

of genes, one represented by the Bcl2 family and the other by the Caspase family. Caspases are a family of proteases that cleave their target substrates at specific peptide sequences During apoptosis, activation of caspases, variably induced by nuclear, metabolic, or externally activated stimuli, takes place in a cascade fashion, leading to nuclear engulfment and cell death [46-50].

Light and electron stained sections of rat cardiac muscle fibers of Group III (*N. sativa* and nandrolone) revealed marked protection of myofibers against the deleterious changes induced by nandrolone, however, minimal changes in their histological features were noticed. Little intercellular spaces and mild congested blood vessels were observed in between muscle fibers. Some cardiomyocytes nuclei were vesicular and others appeared dark. Van Geison stained sections of nandrolone treated and black seeds supplemented rats revealed few fine collagen fibers between the cardiomyocytes and around blood vessels. Highly statistically significant increase in the mean area % of collagen fibers was detected in Group II when compared with the control (Group I) and NS-treated (Group III) Groups. Highly statistically significant increase in the mean area percent of caspase 3 immunoreaction was noticed in Group II when compared with Group I and Group III. No statistically significant difference was detected between Group I and Group III. In inflammation-induced fibrosis, NS reduced cardiac fibrosis and inflammatory markers (serum and tissue) possibly through its antioxidant activity. The cardiovascular health benefits of NS have been established in several studies. These include hypolipidemic, antidiabetic, hypotensive, antiplatelet, and bradycardiac effects and improving endothelial function [51-53].

Thymoquinone, the biologically active compound of NS seeds, reduces liver and pulmonary fibrosis and inflammation, and suggested it as a potential candidate for fibrosis therapy. NS had a dose-dependent antioxidative property. NS oil and thymoquinone protect gastric mucosa, which is partly attributed to their free-radical scavenging activity. Moreover, the ethanolic extract of NS protects radiation induced oxidative damage in mice. Several mechanisms for antioxidative properties of NS have been suggested. It is demonstrated that it preserves the activity of catalase, glutathione peroxidase, and glutathione-S-transferase. It also inhibits microsomal lipid peroxidation [54,30]. Verhagen et al. [55] proved that vitamin E and other antioxidants have a protective action against the damaging effects of free radicals. Free radicals destroy the cells and may contribute to the development of cardiovascular diseases and cancer.

Conclusion

In conclusion, this current work proved that the high dose of nandrolone not only elicits measurable increase in cardiac muscle performance, but also cause many adverse effects in cardiac muscle fibers at the histological, histochemical and ultrastructural levels. However, *N. sativa* supplementation has a great role in maintenance of muscle strength and doesn't cause any harmful effects to the cardiac muscle fibers. The application of *N. sativa* to the nandrolone treated rats may have a possible protective role against the deleterious effects of nandrolone injection. It seems that it acts through alteration of oxidative/antioxidative balance and raising antioxidative enzymes. More studies are guaranteed to clarify the beneficial effects and the underlying mechanisms of NS seed. So, nandrolone must be used under complete clinical supervision particularly in young misinformed athletes.

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