ISSN NO. 2320-5407

Journal homepage:http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Histological, histochemical and ultrastructural studies on the effect of Deca-Durabolin and whey protein isolate on cardiac muscle in adult male albino rats

Hemmat M. Abdelhafez
Faculty of Science (Girls), Zoology Department, Al-Azhar University, Egypt.

Manuscript Info

Manuscript History:
Received: 15 August 2014
Final Accepted: 26 September 2014

Published Online: October 2014

Key words:

Nandrolone, whey protein, cardiac muscle,histopathology, ultrastructure**.**

****Corresponding Author***

Hemmat M. Abdelhafez

Abstract

The abuse of anabolic androgenic steroids (AAS) is under constant debate world-wide. A large number of young adolescents abuse AAS to improve their physical fitness and appearance. The abuses of AAS have been associated with cardiovascular disorders and have a negative impact on exercise performance, since it disturbs heart function. Whey protein is often described as a "nutritionally perfect protein" in the sense that it contains all the essential and non-essential amino acids required by the human body. Whey's amino acid profile is closely related to the optimal physiological needs of the human body. In addition, whey protein may be the best candidate for maximizing muscle growth especially whey protein isolate. It contains an optimal balance of amino acids for muscle growth, especially glutamine and taurine. Today, whey is a popular dietary protein supplement purported to provide antimicrobial activity, immune modulation, improved muscle strength, body composition and prevents cardiovascular diseases and osteoporosis. The effects of whey protein on human health are of great interest and are currently being investigated as a way of reducing disease risk.

So the present study was conducted to evaluate the effects of Deca-Durabolin "nandrolone decanoate" the most widely used AAS and whey protein as a natural anabolic product on the histological, histochemical and ultrastructural pattern of cardiac muscle and also to investigate whether whey protein can ameliorate the deleterious effects induced by nandrolone injection.

Material and methods:140 male albino rats were used in this study. They were divided into four groups, Group 1: Untreated control rats. Group II: Rats treated with nandrolone (intramuscular injected) for 3months .Group III: Rats treated with whey protein (orally administered daily) for 3 months. Group IV: Rats treated with whey protein and nandrolone, whey protein extract was orally administered daily for 6 weeks and then, they were intramuscularly injected by nandrolone extract for 6 weeks. The experimental rats were sacrificed after 1 and 30 days post treatment. Then the cardiac muscle specimens were removed for light and electron microscopic studies. **Results:** treatment of rats with nandrolone after 1 &30 days post treatment, revealed many histopathological and ultrastructure changes in the myocardium varying from hypertrophy, intramuscular haemorrhagic areas, lymphocytic infiltration, hyalinization,vacuolation to myocytolysis and loss of myofibrils. The nuclei exhibited different histopathological signs varying from pyknosis to karyolysis. Ultrastructural observations showed extensive degeneration of the muscle fibers with marked destruction of myofibrils. Increased in collagen fibers was detected and mitochondriosis with degeneration and

electron dense appearance of many mitochondria. The nuclei appeared hyperchromatic with peripherally clumped chromatin. In whey group no distinctive changes were observed in the architecture of the cardiac muscle after 1 &30 days of treatment .Whey and nandrolone group, revealed marked improvement of myofibers against the deleterious changes induced by nandrolone, however, mild widened endomysium, some pyknotyic and karyolytic nuclei and partial increase in collagen fibers were still present. The quantitative histochemical measurements in the current study recorded a significant increase in carbohydrates, total protein and DNA content allover the experimental period **Conclusion:**Nandrolone injection in male albino rats induced degenerative changes in the cardiac muscle which may led to loss of heart function .The whey protein did not cause any damaging effects to the cardiac muscle and ameliorated the toxic effects of nandrolone to a large extent.

Introduction

Scientific investigations have shown that the use of certain androgenic anabolic steroids (AAS) namely nandrolone decanoate and testosterone increased athletic performance by building muscle mass and strength **(Van *et al.*, 2004)**. The AAS (nandrolone) was indicated as supportive therapy in pathological conditions characterized by a negative nitrogen balance to increase body weight as cachexia associated with chronic diseases and long-term use of glucocorticoids. Also it was used for treatment of osteoporosis and anemia **(Bagchus*et al.*, 2005)**. Now it is widely abused in the community for non medical purposes, mostly by young men to improve their appearance by building up their body muscles (**Corrigan, 1988)**, increase self confidence, energy and motivation **(Lombardo and Sickles, 1992)**.Anabolic steroid abuse has become a major public health problem around the world. The adverse effects of AAS were numerous, especially when they were abused by otherwise healthy people. Serious health risks can be produced by long-term use or excessive doses of anabolic steroids. These effects include harmful changes in cholesterol levels (increased low-density lipoprotein and decreased high-density lipoprotein), acne, high blood pressure, liver damage and dangerous changes in the structure of the left ventricle of the heart **(Yesalis, 2000)**. **Shokri*et al.* (2014)** reported that high doses of AAS associated with male infertility so the combination of exercise and high doses of nandrolone decanoate negatively influenced the DNA integrity and protamine content resulting in lower sperm quality and reduced pregnancy rate. Cardiovascular effects are of particular concern, as they have led to the death of several young male body builders**(Fineschi *et al.*, 2001).Dos Santos *et al.* (2014)** recorded a reduction in HDL cholesterol, increased inflammatory markers and oxidative stress in AAS users. Strong evidence associating AAS used with blood pressure at hypertensive levels, as well as hypertrophy, cardiac dysfunction and early mortality has also been reported. The most commonly reported side effects of AAS were previously recorded as an increase in atherosclerosis, tachycardia, cardiac hypertrophy, defected cardiac function, sudden death, depression, nervousness, behavior disorder, muscles pains, nausea, vomiting, testicular atrophy and liver injury **(Casavant *et al.*, 2007; Marshall-Grandisnik *et al.*, 2009).**

Different arrhythmias have been reported to be associated with abuse of AAS, including atrial fibrillation and ventricular tachycardia **(Sullivan *et al.*, 1999)**. High-dose androgenic steroid administration results in a reduction in left ventricle (LV) weight in the sedentary rats (**Trifunovic *et al.*, 1998**). In medical literature, many case reports recorded premature acute ischemic heart disease and myocardial infarction related to AAS abuse **(Huie, 1994)**. Supraphysiological doses of AAS increased myocardial mass, this growth was likely having pathological features and oxygen consumption was also increased **(Deligiannis *et al.*, 1992)**. Some case reports have linked anabolic steroids to atherosclerosis **(Mewis *et al.*, 1996)** and thrombosis **(Nieminen *et al.*, 1996)** both of which significantly increase the risk of cardiac ischemia.

Milk contains two primary sources of protein, the casein and whey. After processing casein the protein responsible for making curds, whey remains in an aqueous state **(Marshall, 2004).** Whey contains components that not only

provide nutrition, but can also prevent and attenuate diseases. Whey proteins have been reported to have utility in many different applications ranging from effects on bone, muscle, blood, brain, pancreas, immune, cancer, infection, metabolism, wound healing, learning and aging enhanced liver and heart glutathione concentrations in aging mice and increased longevity **(Bounous *et al.*, 1989)**. Whey protein is traditionally used amongst bodybuilders and athletes for its ability to promote muscle growth. But, whey is being used in other applications. Some of these include: weight loss, cancer treatment, infant health, wound healing and the elderly. Practically everyone can benefit in some way from whey protein supplementation **(Antonio *et al.*, 2002*)*.**The components of whey include beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lactoperoxidase enzymes, glycomacropeptides, lactose and minerals **(Marshal, 2008).** High levels of constituent proteins including beta-lactoglobulin, alplactalbumin, lactoferrin, lactoperoxidase and glycomacropeptide have demonstrated a range of immune-enhancing properties **.**In addition, whey has the ability to act as an antioxidant, antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial and chelating agent **(Kanwar *et al.*, 2007).**Whey proteins have been reported to enhance digestion and gut function **(Marshall, 2004**), as well as glutathione production and immune function, hence increasing dietary availability may promote general health in a variety of ways **(Bounous, 2000).**Since limited studies on animal models were existed to correlate and establish the negative effects of nandrolone (as an AAS) with the myocardium structure therefore, the aim of the current study was to investigate the histological, histochemicals and ultrastructural alterations produced in myocardium of adult male albino rats treated with nandrolone and /or whey protein and the possible protective role of whey protein isolate against the injury induced by nandrolone injection.

Material and methods:

Experimental animals, feeding and maintenance:

A total of 140 male albino rats (*Rattus –norvegicus*) weighing about150-180 g were used. The animals were housed in especially designed cages, 7 rats per cage, with controlled air ventilation, temperature and relative humidity. The animals were fed standard rodent pellets. Food and water were made available *ad-libitum* throughout the whole experimental period. All animals procedures were performed after approval from the Ethics Committee of the National Research Center-Egypt and in accordance with the recommendations of the proper care and use of laboratory animals.

Nandrolone injection:

Nandrolone decanoate (Deca-Durabolin) oily solution is manufactured by the Nile Co. Pharmaceuticals-Cairo, under license of N.V.Organon-OSS-Holland. Each ampoule contains 25mg/ml. The drug was intramuscularly injected at a dose of 10mg/kg b.wt./week for 3 months **(Joumaa and Leoty, 2001).**

Whey protein administration:

Gold standard whey protein isolates (100%) primary source powder was used 0.8 g/kg/day /human according to **Press (2003).** Whey protein is manufactured in the U.S.A. by ON Company and dissolved in distilled water. The drug was administered orally by gastric tube at a dose of 5 g/kg b.wt./day for 3 months. The dose for rats was calculated according to the Paget's formula on the basis of the human dose (**Paget and Barnes, 1964).**

Experimental design:

The experimental animals were divided into 4 groups. The whole experimental setting was repeated three times as triplicate.

Group 1: Untreated control rats (C)
Group II : Rats injected intramuscularly with nandrolone (10 mg/kg body weight/week) for 3 months (N).
Group III: Rats orally administrated with whey protein extract (5 g/kg body weight/day) for 3 months (W).
Group IV: Rats treated with whey protein and nandrolone: 5 g/kg body weight of whey protein extract was orally administered daily for 6 weeks and then they were injected intramuscularly with 10 mg/kg body weight/week of nandrolone for 6 weeks (W+N).

The experimental rats were sacrificed at days 1 post treatment. For testing the recovery response the experimental animals were sacrificed after 30 days post –treatment in all previous groups.

Samples collection:

After sacrifice samples of cardiac muscles were rapidly removed, weighed and processed for light and electron microscopic studies.

For histological and histochemical purposes:

Small pieces of the myocardium of the left ventricle were removed, fixed in neutral buffer formol for 24 hours, dehydrated, cleared and embeded in paraffin wax. Sections were cut at 5µ and stained with hematoxylin and eosin according to the method of **Drury and Wallington (1980),** Periodic Acid Schiff reaction (PAS)for carbohydrates materials and Mallory's trichrome stain for collagen fibers **(Humason, 1972),** mercuric bromophenol blue method for total protein (**Mazia*et al.*, 1953)** and Feulgen reaction for DNA (**Drury and Wallington, 1980).**

Tissue preparation for electron microscopy:

A small portion of the left ventricle near the apex was excised and minced into 2x2 mm^2 pieces, primary fixed in 3 % glutaraldehyde and 0.1 M phosphate buffer at pH 7.4, post fixed in osmium tetra oxide, processed and embedded in epon. Semithin sections (1 µm thick) stained with toluidine blue and examined by light microscope. Ultrathin sections (50-80 nm thick) were contrasted with uranyl acetate and lead citrate and examined by transmission electron microscope in the National Cancer Institute. **(Hayat, 1989).**

For Quantitative morphometric analysis:

The optical density of histochemically stained sections of cardiac muscles for carbohydrates, total protein and nucleic acid DNA of the control and treated groups was recorded using IPWIN 32 image analysis software. The mean optical density was used to compare the positive content of these materials in the different groups. The comparison was established as the percentage of the treated group value with the control group.

Statistical analysis:

Statistical analyses were performed using analysis of variance (ANOVA) according to **Snedecor and Cochran (1980)**. The data were processed and analyzed using the SPSS software (Statistical Analysis for Social Science, Version 8). Significant differences between treatment means were determined by student T-test. Data were presented as mean ± SE, mean ±SD and $P \leq 0.05$ was considered statistically significant.

Results

Heart weight:

Rats injected with nandrolone exhibited a marked significant decrease in the weight of heart amounted -28.57 after one day post treatment and this decrease in the weight of myocardium was slightly improved after 30 days post-treatment of recovery to reach -21.91% (table 1)

Drenching whey protein to rats induced a mild significant decrease in weight of heart recorded -11.43% while non significant decrease in weight of heart was observed after 30^{th} day post-treatment of recovery.

Treatment of rats with whey protein and then with nandrolone resulted in a significant decrease in the level of weight of heart which reached -14.29 % on day one post-treatment, while non significant decrease in weight of heart could be observed on the 30^{th} day post-treatment, recording the possible ameliorative effect of whey protein against the destructive effect of nandrolone.

Table (1): Effect of nandrolone and/ or whey protein on weight of heart (g) in male adult albino rats.

Parameter	Heart weight			
Time	One day		One month	
Groups	Mean± S.E	% chang	Mean± S.E	% chang
Control	***1.05±0.08*** [b]	0.0%	***1.05±0.08*** [b]	0.0 %
Nandrolone	***0.75±0.02***^{acd}	-28.57%	***0.82±0.06***^{ad}	-21.91%
Whey protein	***0.93±0.02*** [b]	-11.43%	***0.97±0.04***	-7.62%
Whey protein +nandrolone	***0.90±0.04*** [b]	-14.29%	***1.03±0.05*** [b]	-1.91%

a: Significant difference from control at P ≤ 0.05
b: Significant difference from nandrolone at P ≤ 0.05
c: Significant difference from whey protein at P ≤ 0.05
d: Significant difference from whey protein + nandrolone at P ≤ 0.05

1-Light microscopic results

a-Histological observation:
The Control Group

Cardiac muscle fibers of rats of the control group appeared elongated and branched with oval central nuclei and pale acidophilic cytoplasm which containing parallel myofibrils (Figs. 1A&B).With Mallory's trichrome stain the muscle fibers appeared elongated ,branched and the spaces between them being occupied by small amounts of endomysial supporting tissue. The endomysium consists mainly of small amounts of collagen fibers (stained blue) (Fig. 1C).

TheExperimental Groups
i-Nandrolone-treated group:

Histological examination of the rat cardiac muscle fibers one day post –treatment with nandrolone showed highly degenerated muscle fibers with intramuscular haemorrhagic areas and widened endomysium. Numerous pyknotic and karyolytic nuclei were obviously seen (Figs.2 A& E).Cardiac muscle fibers lost their normal architecture and take a wavy appearance and some muscle fibers were distorted(Fig.2B).Hypertrophied muscle fibers were noticed and the myocardial fibers were disrupted by a prominent interstitial infiltrate of lymphocytes (Fig.2 C). Fragmented and highly distorted muscle fibers with numerous blood corpuscle,obvious vacuolation,odema and hyalinization were also detected (Fig.2 D). Increased collagen fibers could be detected and many cardiac muscle fibers were replaced by bundles of collagen fibers (Fig.2 E).

Following thirty days of nandrolone treatment (as a recovery period), the cardiac muscle exhibited myocardial necrosis with focal infiltrates of lymphocytes (Fig.3 A) .Most of the cardiac muscle fibers lost their normal architecture with numerous blood corpuscle and numerous vacuoles with variable sizes. Abundance of necrotic areas with pyknotic and karyolytic nuclei .widened endomysium was also markedly seen (Figs.3 B, C&D).Many hypertrophied muscle fibers were totally replaced by collagen fibers (Fig. 3E).

ii-Whey protein-treated group:

Cardiac muscle of rats administered whey protein and examined one day post- administration showed myocardium which appeared more or less like the normal pattern with highly increased number of nuclei (Figs.4 A,B) . Normal distribution of collagen fibers in the endomysium was clearly seen (Fig.4C). No detectable changes were observed after thirty days of treatment with whey protein (recovery interval).

iii-Whey protein and nandrolone - treated group:

Examination of the rat cardiac muscle fibers one day post-treatment with whey protein and nandrolone revealed a noticeable protection of myofibers against the deleterious changes induced by nandrolone, however mild widened endomysium and some pyknotyic and karyolytic nuclei were still present (Figs. 5A& B) with partial increase in the deposition of collagen fibers (Fig. 5C).

Thirty days following treatment with both whey protein and nandrolone showed an obvious recovery from the hazardous effects of nandrolone, while muscle fibers with some pyknoyic and karyolytic nuclei were still observed in the matrix of myocardium (figs 6A& B). Mallory's trichrome revealed partial increase in collagen fibers (Fig. 6C).

Figure (1): Photomicrographs in the rat cardiac muscle of the control group showing:

A-All the muscle fibers are branched with elongated, centrally located nuclei and intercalated discs connect them. **(L.S., Hx& E X100).**

B- Branching muscle fibers with centrally located oval nuclei **(N)**. Note: flat dark nuclei of the fibroblasts **(arrow heads)** in the endomysium**.** **(Hx&E X400)**

C -Red stained muscle fibers with blue stained collagen fibers supporting the sarcolemma and scattered in the endomysium **(arrow).** **(L.S.,Mallory'strichrome stain X100)**

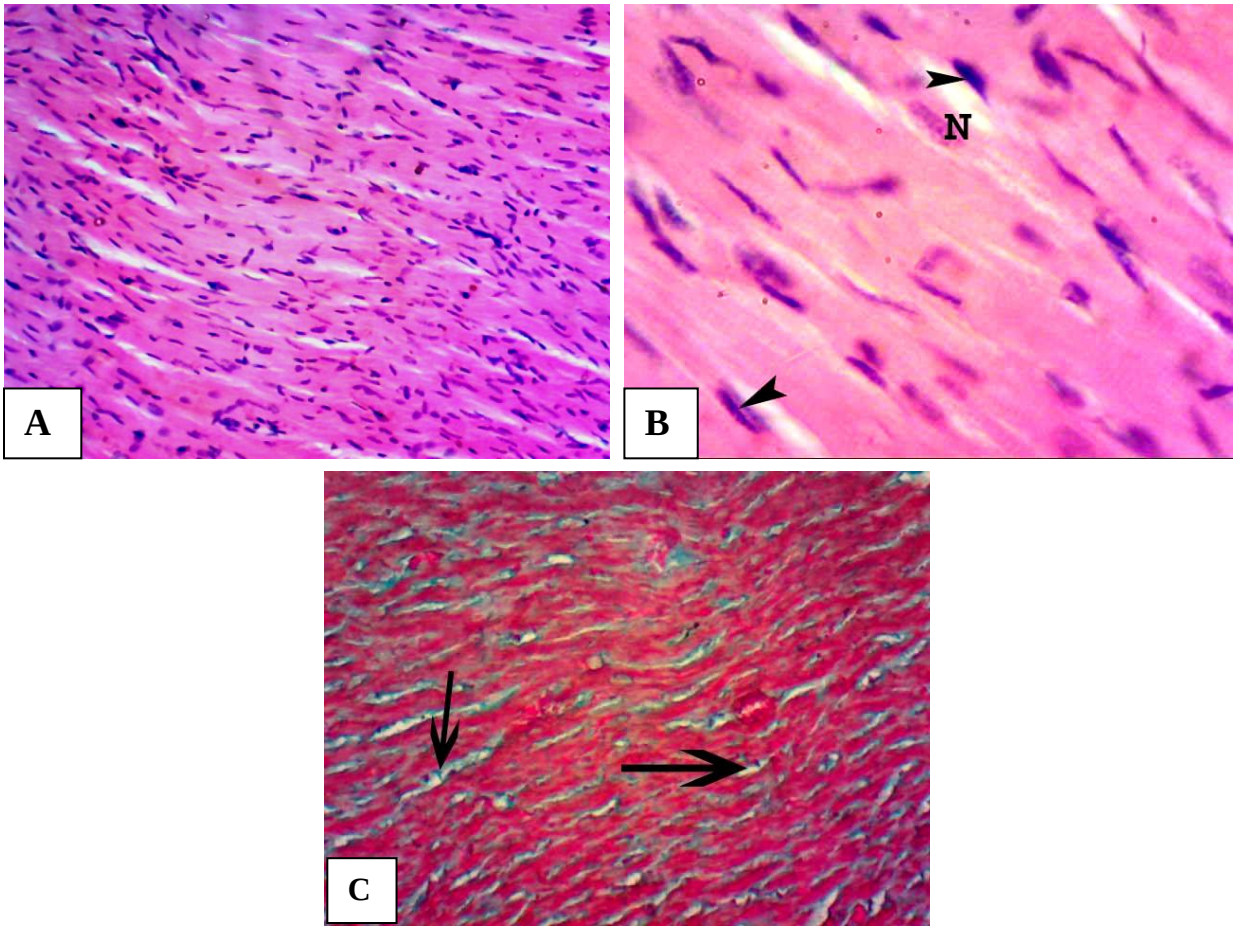


Figure (2) - Photomicrographs in the rat cardiac muscle one day post-treatment with nandrolone showing:

A- Areas of myocytolysis **(m) ,** extensive degeneration **(d),** intramuscular haemorrhagic areas**(h)** and highly thickened atrial wall with narrow lumen**(→)** ,numerous pyknotic nuclei (**py**) and others showed karyolysis **(kr)** and widened endomysium**(w) .** **(L.S., Hx&E X100)**

B- Cardiac muscle fibers lost their normal architecture and take a wavy shape,abundant necrotic nuclei with pyknosis**(Py)** ,karyorrhexis**(K)**and karyolysis**(kr)**some muscle fibers are distorted**(→).** **(L.S., Hx&E X250)**

C-Magnified portion of (B) illustrating hypertrophied muscle fibers, the myocardial fibers are disrupted by a prominent interstitial infiltrate of lymphocytes **(L)**. **Notice**: karyolitic nuclei **(kr).** **(L.S., Hx&E X400)**

D- Fragmented and Highly distorted muscle fibers with numerous blood corpuscle (b), obvious vacuolation (V), odema **(arrow)**and hyalinization **(h)** Notice: most of nuclei in the myocardium are pyknotic **(py).**
(L.S., Hx&E X250)

E- Abundance of necrotic areas with nuclear changes such as pyknosis**(Py)**, karyorrhexis**(K)** and karyolysis**(kr)** Notice: internal haemorrhage**(h)**in the widened endomysium. **(L.S., Hx&E X250)**
F- Hypertrophy of the muscle fibers, many muscle fibers are replaced by collagen fibers **(F).** **(L.S., Hx&E X250)**

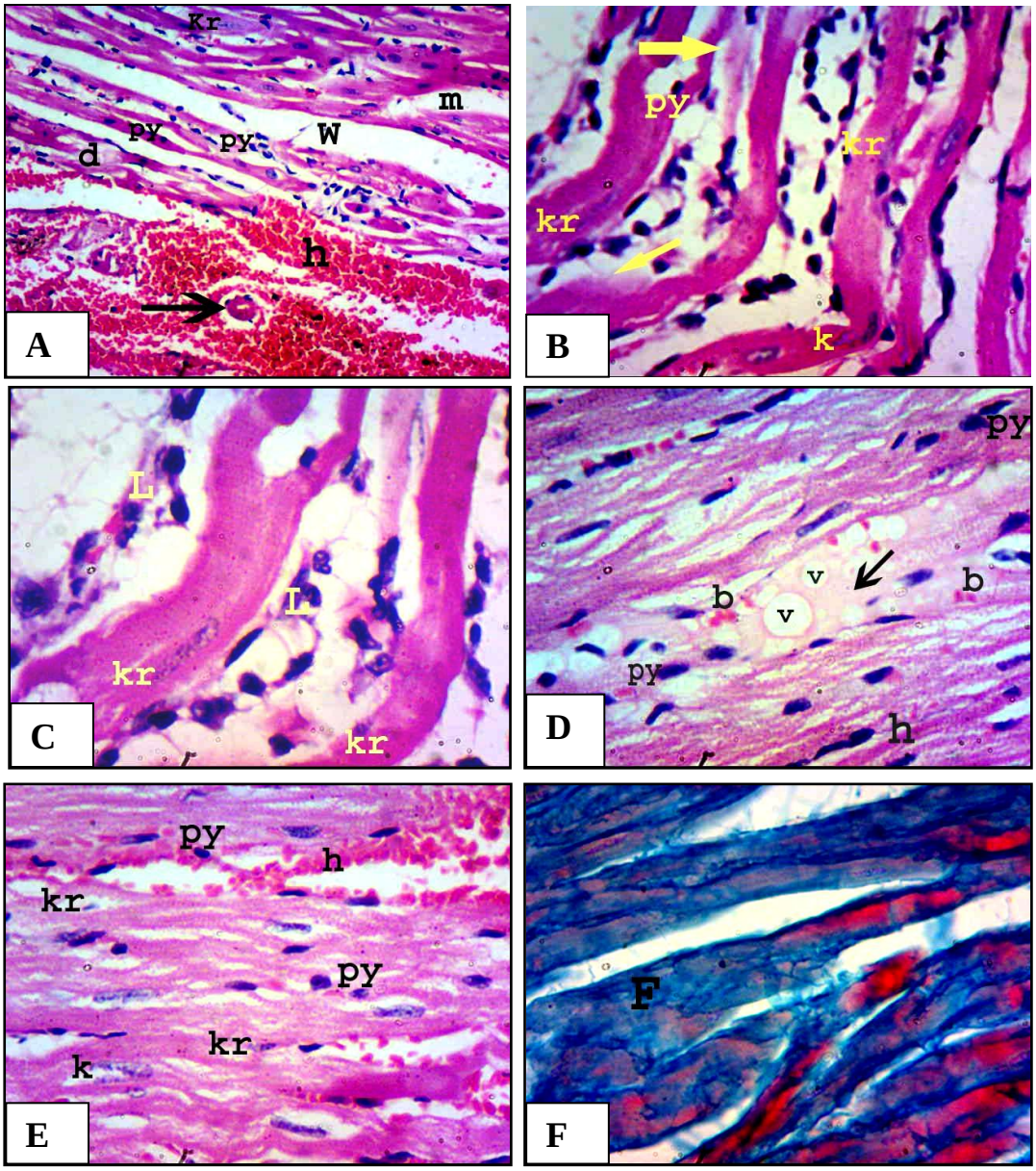


Figure (3): Photomicrographs in the rat cardiac muscle thirty days post-treatment with nandrolone showing:
A- Areas of diffused myocardial necrosis with focal infiltrates of lymphocytes **(black arrow).** **(L.S., Hx&EX100)**

B- Complete loss of striation, haemorrhagic areas (h) and highly widened endomysium (w). **(L.S., Hx&EX250)**
C- Most of the cardiac muscle fibers lost their normal architecture, with numerous blood corpuscle**(b)**, numerous vacuoles with different sizes are also detected. Notice: pyknotic (py) and karyolytic **(kr)** nuclei. **(L.S., Hx&EX250)**
D- Abundance of necrotic areas with pyknosis **(py)** karyorrhexis**(K)** and karyolysis**(kr)** of nuclei and internal bleeding **(arrow)**. **(L.S.,Hx&E X400)**
E- Many hypertrophied muscle fibers totally replaced by collagen fibers **(F)**.Notice: wide endomysium **(w).**
(L.S., Mallory's trichrome stainX100)

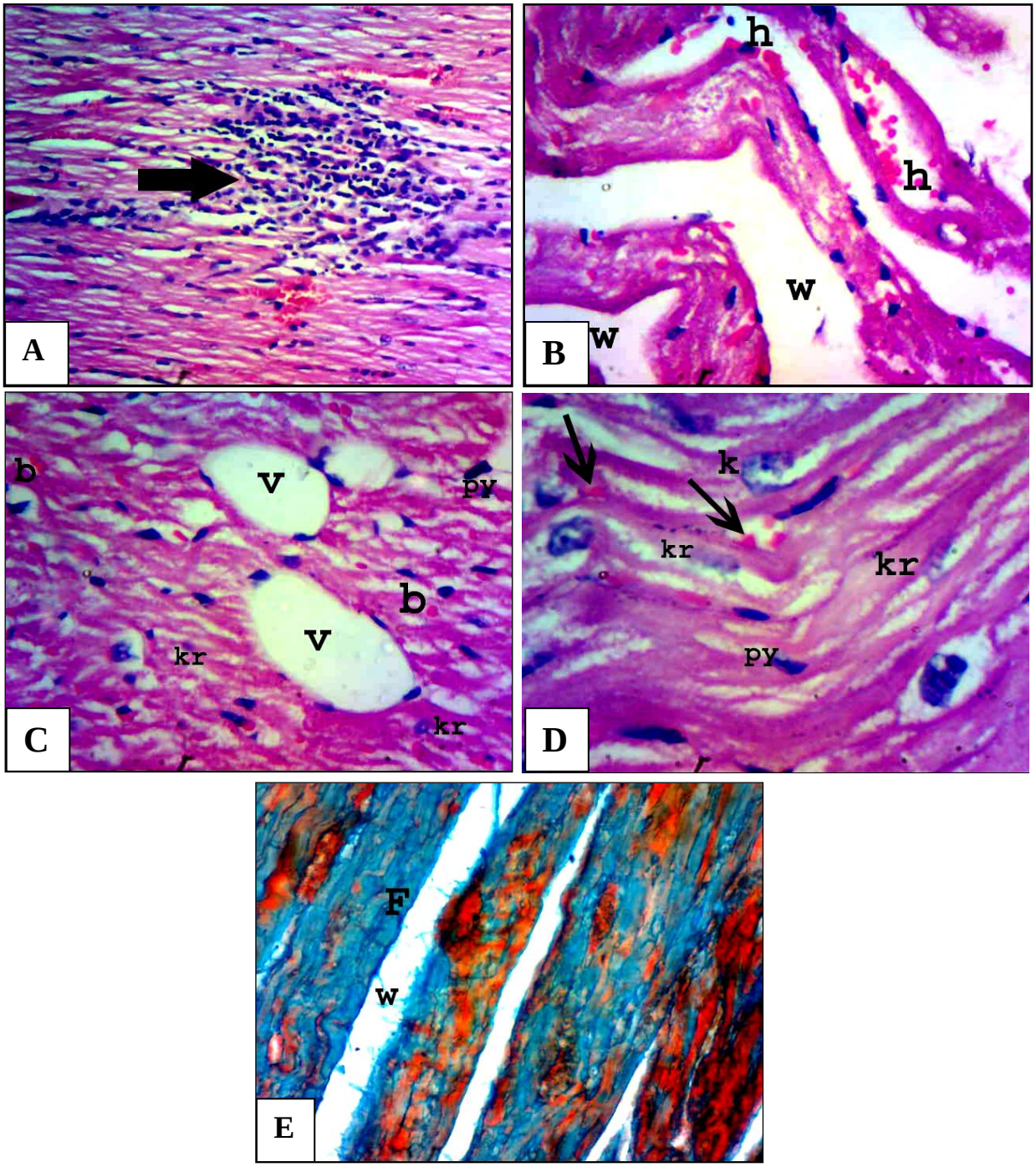


Figure (4): Photomicrographs in the rat cardiac muscle one day post-treatment with whey protein showing:
A, B-Somewhat normal appearance of the myocardium with increased number of nuclei**. A-(L.S., Hx&EX100**).
B-(L.S., Hx&EX400)

C- Normal distribution of collagen fibers**. (Mallory's trichrome stain X100)**

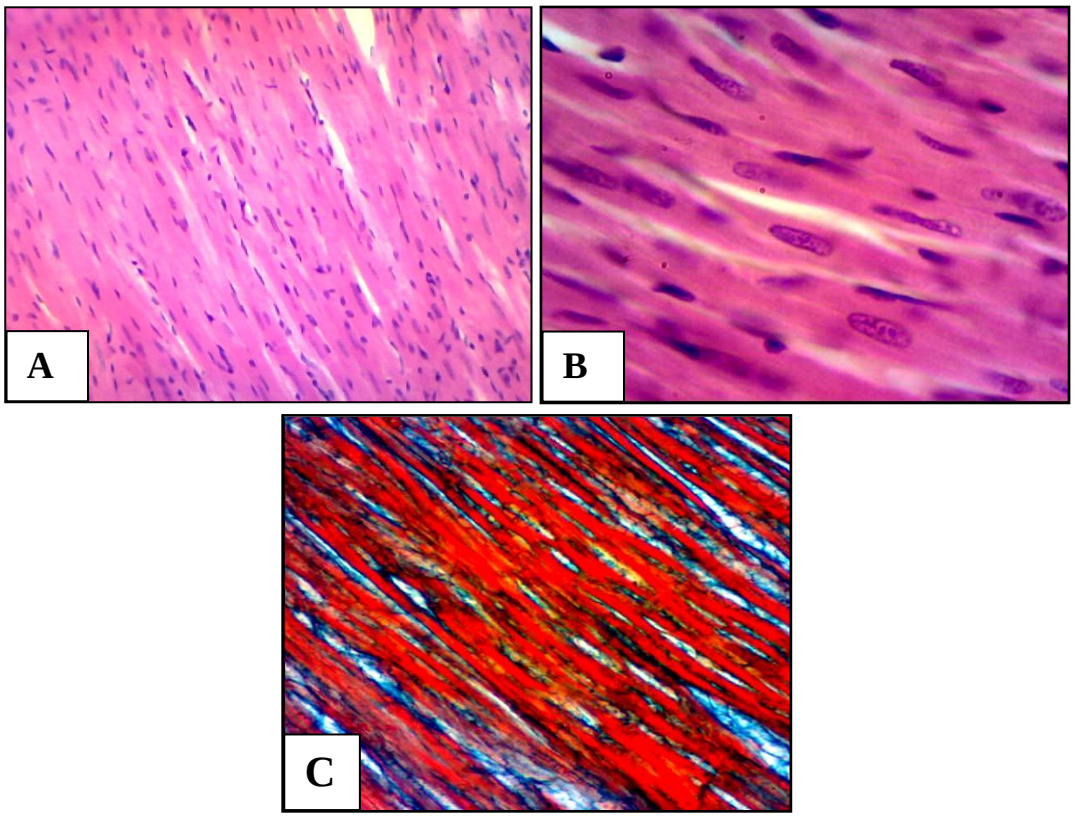


Figure (5): Photomicrographs in the rat cardiac muscle one day post-treatment with whey protein and nandrolone showing:

A- Improved myocardial architecture, mild widened endomysium and some pyknotic nuclei are still detected.

(L.S.,Hx&E X100)

B- Few muscle fibres containing different types of inflammatory cells, numerous large and small haemorrhagic areas (h) in, between the muscle fibres, most nuclei show pyknosis (py) or karyolysis (kr**). (L.S.,Hx&E X250)**

c- Partial increase in collagen fibers (**arrow). (L.S., Mallory's trichrome stain X100)**

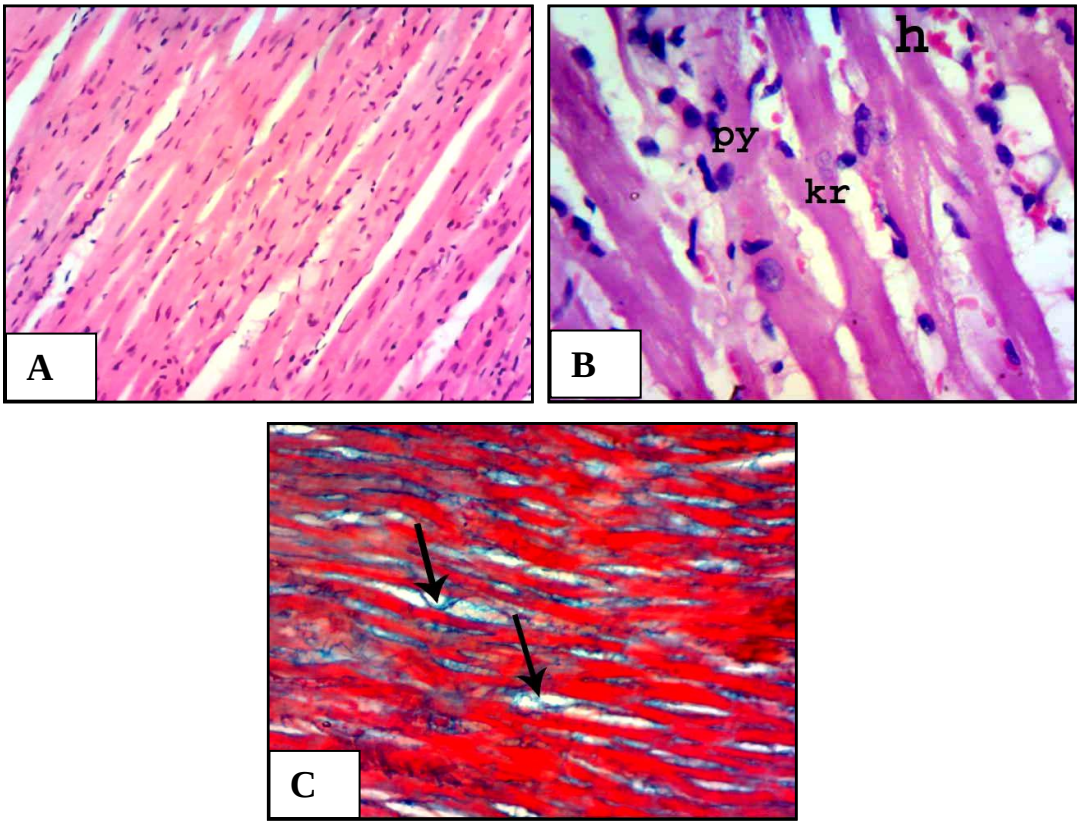
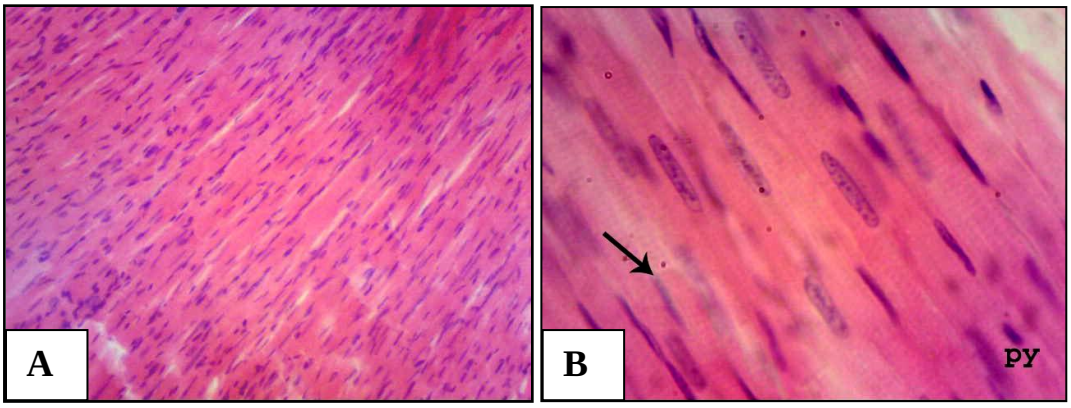
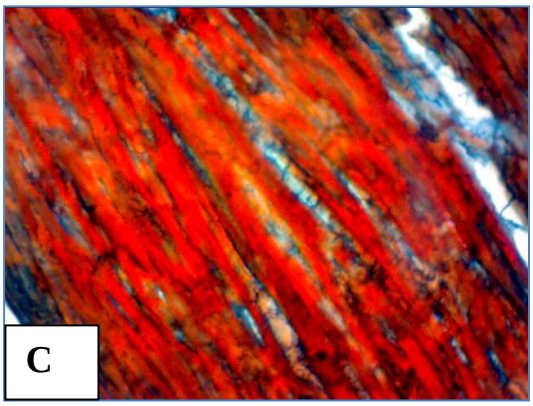


Figure (6): Photomicrographs in the rat cardiac muscle thirty days post-treatment with whey protein and nandrolone showing:
A, B-Normal appearance of the muscle fibers but some pyknotyic **(py)** and karyolytic nuclei **(arrow)** are still present. **A-(L.S., Hx&EX100)**
B-(L.S.,Hx&E X100)
C- A degree of improvement, however there are partial increase in collagen fibers**.**
(L.S.,Mallory's trichrome stain X100)





b-Quantitative histochemical measurements

PAS- positive materials

The changes in PAS positive materials in sections of the cardiac muscles of the control and treated groups after one and thirty days of treatment illustrated in table (2). All the treated groups exhibited a significant increase in PAS positive materials allover the experimental periods.

Table (2): Effect of nandrolone and/ or whey protein on PAS positive materials of the cardiac muscles in adult male albino rats.

Parameter	**PAS positive materials**			
Time	**One day**		**One month**	
Groups	**Mean± S.E**	**% chang**	**Mean± S.E**	**% chang**
Control	***0.418±0.062***	0.0%	***0.418±0.062***	0.0 %
Nandrolone	***0.568±0.172****	35.89%	***0.636*±0.11***	52.15%
Whey protein	***0.460±0.087****	10.05%	***0.451±0.086****	7.89%
Whey protein +nandrolone	***0.513±0.093****	22.73%	***0.458±0.112****	9.57%

*** Significant difference from control at P ≤ 0.05**

Total protein content

All the treated groups exhibited a significant increase in the total protein content relative to the control value allover the experimental periods except the group of whey protein which showed a significant decrease after one month of the treatment (Table 3).

Table (3): Effect of nandrolone and/ or whey protein on total protein content of the cardiac muscles in the adult male albino rats.

Parameter	total protein content			
Time	One day		One month	
Groups	Mean± S.E	% chang	Mean± S.E	% chang
Control	***0.43±0.02***	0.0%	***0.43±0.02***	0.0 %
Nandrolone	***0.75±0.07****	74.42%	***0.645±0.06****	51.16%
Whey protein	***0.46±0.02****	6.98%	***0.408±0.02****	-4.65%
Whey protein +nandrolone	***0.45±0.02****	4.65%	***0.44±0.06****	2.79%

***Significant difference from control at P ≤ 0.05**

Total DNA content

All the treated groups exhibited a significant increase in the total DNA content relative to the control value all over the experimental periods (Table 4).

Table (4): Effect of nandrolone and/ or whey protein on total DNA content of the cardiac muscles in adult male albino rats.

Parameter	total DNA content			
Time	One day		One month	
Groups	Mean± S.E	% chang	Mean± S.E	% chang
Control	***0.37±0.02***	0.0%	***0.37±0.02***	0.0 %
Nandrolone	***0.51±0.06****	37.84%	***0.49±0.05****	32.43%
Whey protein	***0.38±0.03****	2.70%	***0.41±0.03****	10.81%
Whey protein +nandrolone	***0.498±0.06****	34.595%	***0.43±0.02****	16.22%

*, Significant difference from control at P ≤ 0.05

2-Electron Microscopic Results:

The control group

The sarcomeres of the cardiac muscle fibers form a branching myofibrillar network continous in three dimensions throughout the cytoplasm. The branching columns of sarcomeres are separated by sarcoplasm containing rows of mitochondria and sarcoplasmic reticulum. The T- tubules ramify throughout the cardiac muscle cytoplasm at the Z line. The elongated nuclei are mainly centrally located, the glycogen granules in the endomysium could be detected (Figs. 7A, B&C)

The Experimental Groups

i-Nandrolone-treated group:

The rat cardiac muscle fibers, one day post –treatment with nandrolone, showed dilated endomysium**,** dystrophic changes in the wall of blood vessels which contained RBCs with abnormal shapes. Nuclei of the cardiac myocytes exhibited several degenerative changes where some of them showed peripherally condensed marginated chromatin with destructed nucleolus. Pronounced variation in the mitochondrial size was detected with ruptured cristae and ruptured outer and inner membranes. Numerous degenerated areas and increased collagen fibers and glycogen granules in the endomysium**(**Figs. 8A, B, C, D &E).

Following thirty days of nandrolone treatment, the cardiac muscle exhibited destruction, fragmentation and lysis of some myofibrils, numerous electron – dense mitochondria with degenerated and pyknotic nuclei(Figs.9 A,B). Some glycogen granules were dispersed around many mitochondria inbetween the contractile myofibrils, but most of the glycogen flaks and granules were observed near the edge of the cell (Fig.9 C).

ii-Whey protein -treated group

One and thirty days post treatment, the cardiac muscle fibers exhibited normal appearance with normal blood vessels (Figs. 10A&B).

iii-Whey protein and nandrolone - treated group

One day post-treatment with whey protein and nandrolone revealed that most of myocardial fibers restored their normal appearance, but few areas of myofibrils were still degenerated with increased collagen fibers in the widened endomysium (Fig.11A)

Thirty days following the treatment, normal architecture of myocardium could be observed with increasing the numbers of mitochondria, some of them appeared degenerated (Figs.12, A&B)**.**

Figure (7): Electron micrographs of the myocardium of the control adult male albino rat showing:

A -Part of cardiomyocyte, which contains an euchromatic nucleus (N) with dispersed chromatin and distinct nucleolus (arrow), strands of myofibrils (f) with mitochondria (m) lying between them and near the nucleus**. (X 3000)**

B-Cardiomyocyte containing basal lamina (BL), strands of myofibrils (f) formed of light bands, Z lines, dark bands, H zone and T- tubules (arrow). **(X 4000)**

C- Rows of mitochondrial (m) inbetween the myofibrils. Notice: the glycogen granules (arrow). **(X 4000)**

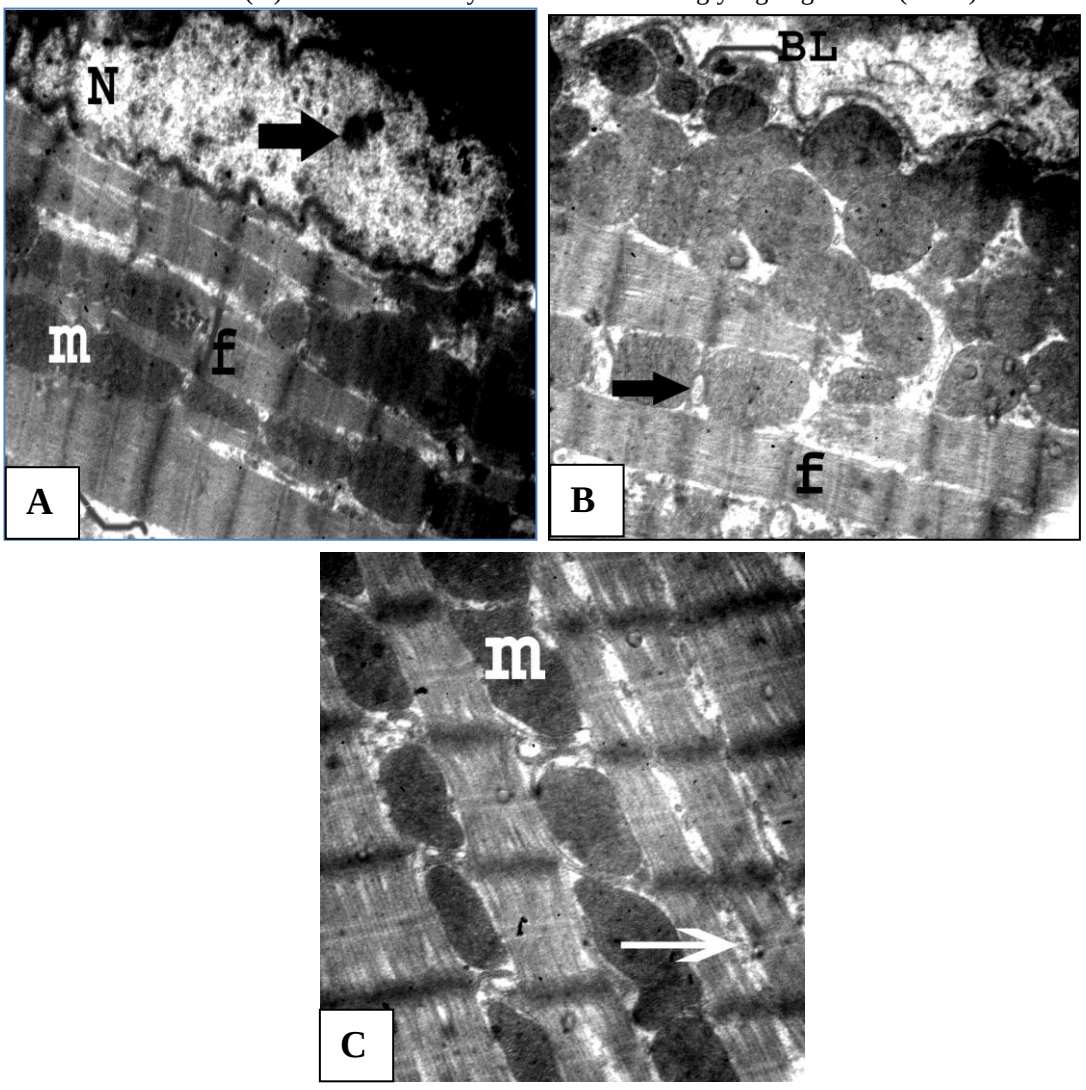


Figure (8): Electron micrographs of the myocardium of adult male albino rat one day post-treatment with nandrolone showing:
A- Dilated endomysium which is occupied by collagen fibers **(F),** dystrophic changes in the wall of blood vessels which contain RBC **(→)** with abnormal shape. Nuclei of the cardiomyocytes**(N)** appeared abnormal with peripherally condensed marginated chromatin . **(X1000)**
B- Destruction of sarcolemma **(arrow head**) and myofibrils .Pronounced variation in the mitochondrial size with ruptured cristae and some of them with ruptured outer and inner membranes **(→)**. **(X 4000)**
C- Most of muscles fibers lost their normal architecture with abnormal distribution of mitochondria **(m)**, disorganization of intercalated disc **(→)** and numerous degenerated areas **(d)**. **(x3000)**
D- Ruptured elements of sarcolemma,endomysium and myofibrils **(→)** , many degenerated areas**(d)**, bilobed nucleus **(N)** with peripherally condensed marginated chromatin. Some muscle fibers lost their striation. **(x1000)**
E - Increased glycogen flaks and granules in the endomysium between the muscle fibers **(arrow**) and abnormal shape of the myocardial nucleus. **(x3000)**

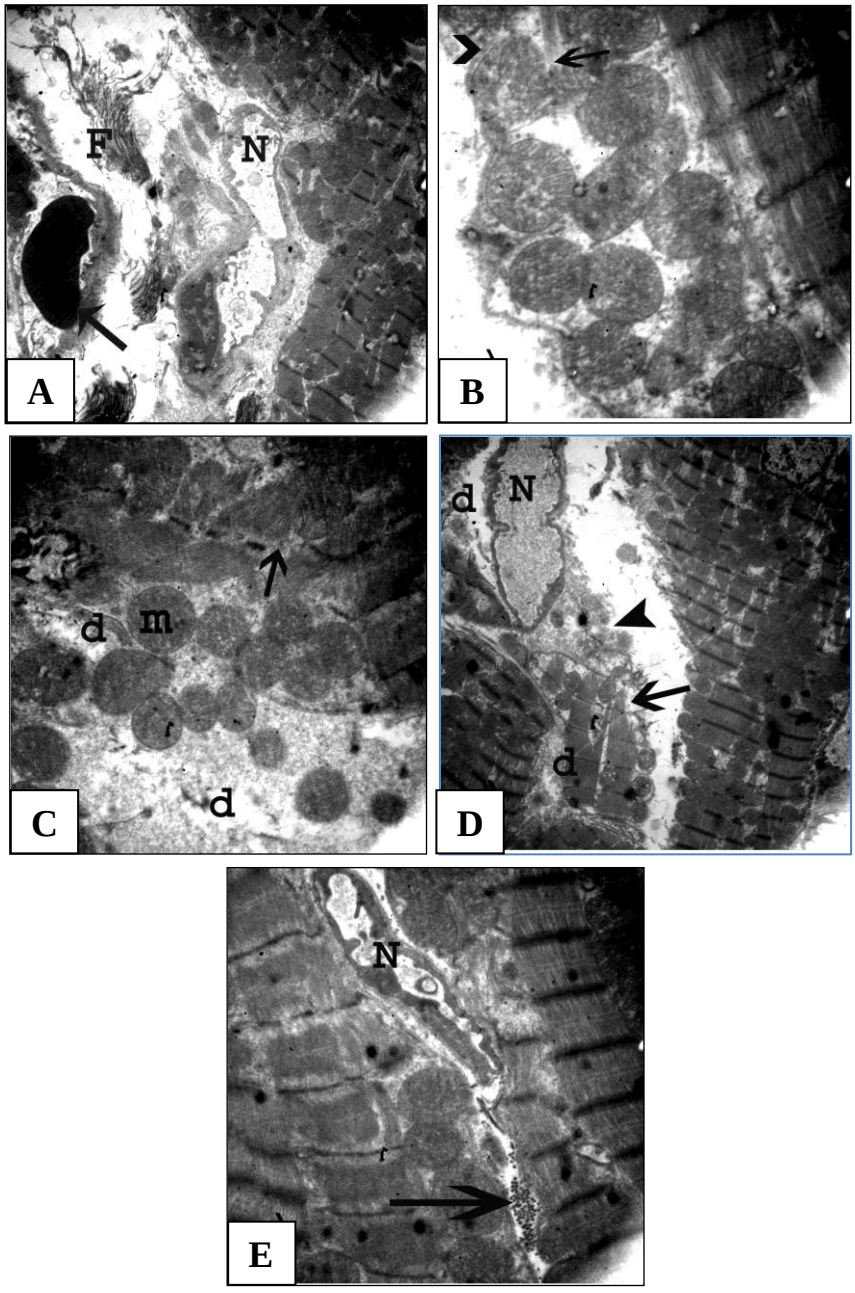


Figure (9): Electron micrographs of the myocardium of adult male rat thirty days post-treatment with nandrolone showing:
A- Destruction, fragmentation and lysis of some myofibrils **(arrow)** Note: electron – dense mitochondria **(m)** with variable sizes among the myofibrils. **(x1000)**
B- Numerous electron – dense mitochondria (**m)** They are crowded together, with many of them touching one another. Notice: degenerated **(d)** and pyknotic**(→)** nuclei . **(x1000)**
C-Some muscle fibers with wide pale segment banding and interrupted Z line **(→)**. **(x 2000)**

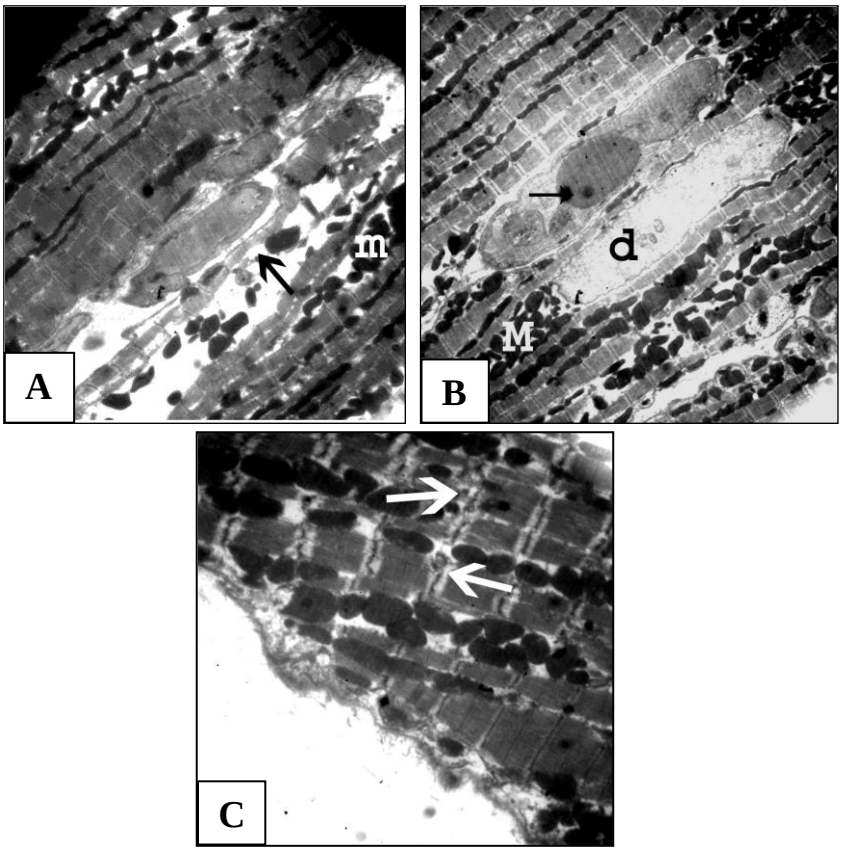
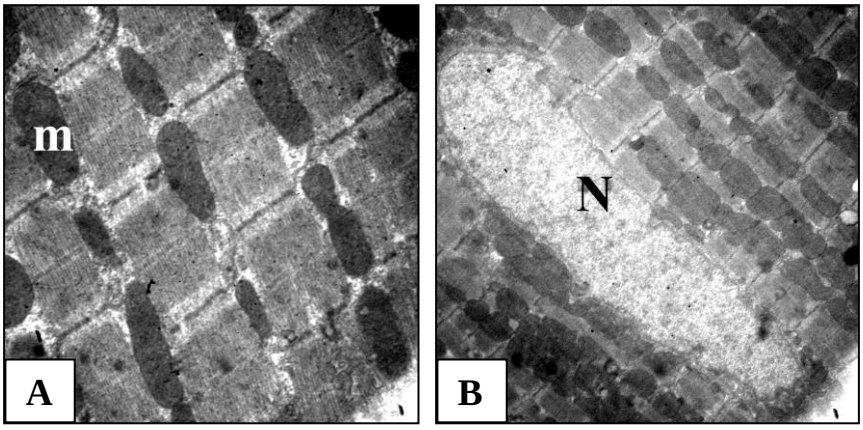


Figure (10): Electron micrographs of rat cardiac muscle one day post-treatment with whey protein showing:
A- An almost normal appearance of the cardomyocytes with apparently normal mitochondria **(m)** in between muscle fibers. **(x 4000)**
B-Normal architecture of myocardium with its nucleus **(N).** **(x 2000)**
C- Normal appearance of cardomyocyte which contains normal RBC (arrow). **(X1000)**



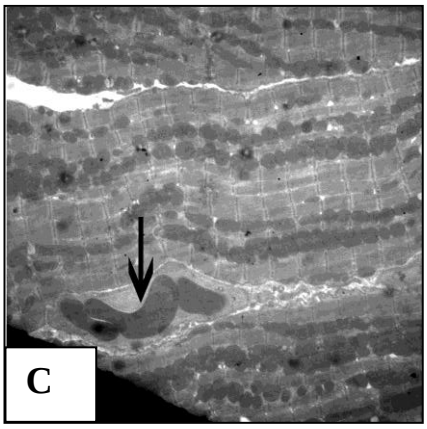


Figure (11): Electron micrographs of rat cardiac muscle one day post-treatment with whey protein and nandrolone showing:

A-Most of myocardial fibers restored their normal appearance, but few areas were still degenerated **(d)**. Notice: increased collagen fibers **(arrow)** in the widened endomysium. **(x2000)**

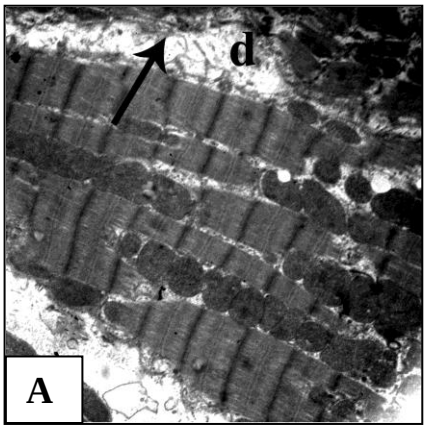
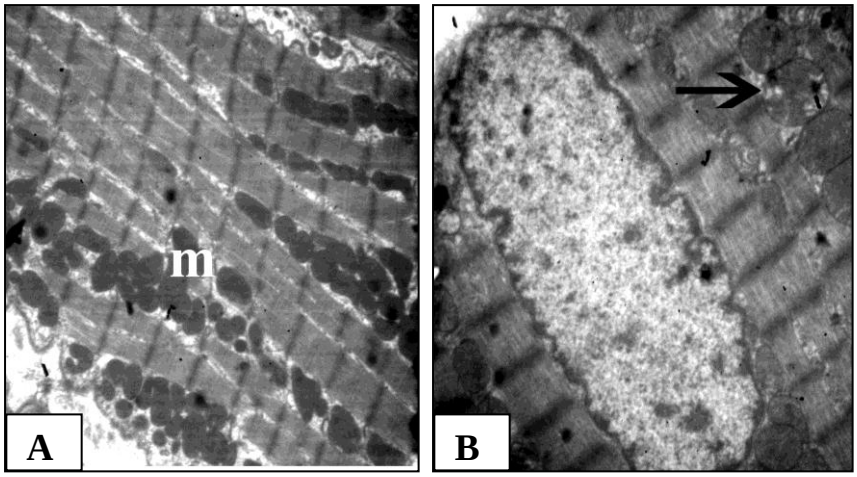


Figure (12): Electron micrographs of rat cardiac muscle thirty days post-treatment with whey protein and nandrolone showing:

A–Nearly normal architecture of myocytes Notice: - increased number of mitochondria **(m).** **(x2000)**

B -Normal nucleus, but one of mitochondria having destructed outer membrane (arrow)**.** **(x3000)**



Discussion

Anabolic androgenic steroid (AAS) were first used by weight lifters and others involved in pursuits of strength, but are now taken, often in large doses, by young men interested in enhancing their appearance (**Corrigan, 1996).**Many AAS-induced adverse effects, of particular concern is the increased risk of cardiovascular adverse effects associated with AAS abuse, especially among persons predisposed to such events or diseases. Increased numbers of premature cardiac events were caused by AAS abuse (**Lukas, 1993**). **Franquni *et al.* (2013)** concluded that high doses of nandrolone elicited cardio toxic effects with cardiac remodeling and injury. Many studies demonstrated that milk and milk products intake lower blood pressure and reduce the risk of hypertension **(Svetkey *et al.*, 1999).** Whey protein enhanced protein levels within the muscle which can enhance muscle regeneration from injury **(Andersen *et al.*, 2005).**

In the current study, administration of nandrolone caused a significant decrease in the heart weight during the experimental period in comparison with the control group. **Trifunovic *et al.* (1998**) reported that high-dose androgenic steroid administration resulted in a reduction in the left ventricle weight in sedentary rats .In contrast **Pereira *et al*.(2006)** reported that no significant alteration in the heart weight of rats treated with nandrolone. In addition to that, **Nahrendorf *et al*. (2003)** reported an increased in the left ventricle mass in rats treated with testosterone during 10 weeks. This difference in results may be due to the dose or time dependent .This is a reasonable interpretation and the loss in heart weight in this study may be attributed to increased toxicity and autolysis in cardiomyocytes.

Administration of whey or whey + nandrolone resulted in mild significant decrease in the heart weight of rats only after one day of treatment, while non significant decrease in weight of heart was observed on day 30th post-treatment as compared to the control group. Such decrease was lower than that in rats treated with nandrolone alone reflecting the possible protective role of whey protein and these observations are in agreement with those of **Wolfe** (**2002**) who recorded that the efficacy of higher protein intakes yielded greater indices of strength and enhanced lean body mass

In the present work, light and electron microscopic investigations for nandrolone treated rats after one day of treatment showed highly degenerated, hypertrophied muscle fibers with intramuscular haemorrhagic areas, vacuolation**,** odema , hyalinization and widened endomysium. Numerous pyknotic and karyolytic nuclei were obviously detected. The myocardial fibers were disrupted by a prominent interstitial infiltrate of lymphocytes. Fragmented and highly distorted muscles with increased collagen fibers were also detected. Moreover, many fibers were replaced by dense collagen fibers .Pronounced variation in the mitochondrial size with ruptured cristae and ruptured outer and inner membranes. Following thirty days of treatment (as a recovery period) there is no any improvement in cardiac muscle architecture. These findings support and confirm those of **Marsh *et al.(*1998**) who reported that supraphysiological dose of AAS and not the physiological amounts of natural testosterone could induce pathophysiological cardiac hypertrophy due to presence of androgen receptors in the cardiac myocytes can directly mediate a significant hypertrophy.**Karhunen *et al*. (1988**) reported that all major tissues, including the brain, having androgen receptors. AAS possess large systemic and psychological effects **(Haupt, 1993).** Androgen actions were mediated by binding to its androgen receptor, which is localized in the cytoplasmic compartment of target cells (**Tyagi*et al.*, 2000).Karhunen*et al*. (1988**) showed that prolonged treatment with anabolic steroids led to increased peripheral vascular resistance and dose dependent cardiac hypertrophy together with depressed contractility of the heart. **Golestani *et al*. (2012)** reported that AAS indirectly led to mitochondrial damage, apoptosis and sarcomere disruption. Experimental studies have shown that prolonged treatment with AAS led to dose-dependent reversible myocardial hypertrophy together with irreversibly reduced compliance of the left ventricle and decreased inotropic capacity of the myocardium **(Karhunen, 1988).** Nevertheless, similar findings without altered contractility of the heart have also been demonstrated **(Trifunovic *et al.*, 1995).**AAS induced cardiac hypertrophy was associated with similar histopathological changes as those encountered in the dilated cardiomyopathy (**Ferrera *et al.*, 1997)**. In autopsy samples and myocardial biopsies taken after AAS exposure, myocardial fibrosis and inflammation have been observed **(Nieminen *et al*. 1996**). Deleterious effects of AAS on the myocardial cells depend on the dose administered and the length of exposure **(Melchert *et al*. 1992**). Experimental studies with myocardial cell cultures revealed cell destruction associated with depressed contractile activity, increased lysosomal fragility and depressed mitochondrial activity (**Melchert *et al*. 1992**). Further, **Tagarakis *et al.* (2000)** demonstrated that muscular exercise combined with AAS impaired the cardiac microvascular adaptation to physical conditioning. These findings support the direct toxic effect of AAS on the myocardium (**Melchert *et al*., 1995).**

Androgens might involve either indirect mechanisms involving blood vessel disorders, notably atherosclerosis, as well as increased erythropoiesis, haematocrit, hyperviscosity and hypertension, but in addition androgens may have direct effects on the cardiac muscle fibers and its function, since the androgen receptor is expressed in the cardiac

muscle fibers and *in vitro* study of nonhuman cardiac myocytes showed that testosterone can decrease action potential duration (thereby altering repolarization) and peak shortening times**(Kimura *et al.*, 1993).**Use of an anabolic steroid caused serious injuries in the rodent heart **(Cavasin *et al.*, 2006)** .Androgens decrease elastic and increase fibrous proteins in the arterial vascular tissue **(Ferenchick, 1991).**Some of the pathologies attributed to anabolic steroid abuse point to disturbances in the intimate connection between neuroendocrine and immune function and interaction (**Thomas *et al.*, 1998).**Increased collagen fibers in the present study was also noticed by **Parssinen *et al.* (2000)** who elucidated that high doses of AAS were found to enhance collagen synthesis, especially in soft connective tissues. This effect tended to be dose-dependent. These short-term changes in collagen metabolism may be due to increased anabolic effects in muscle or may be secondary effects of increased working capacity.

In this study light and electron microscopic investigations in the cardiac muscle fibers of rats examined one and thirty days after whey protein administration exhibited normal cardomyocytes with increased nuclei as compared to control group, this increase come in parallel with increased DNA content in the quantitative studies in the present work.

Normal distribution of collagen fibers in the endomysium was clearly seen after one and thirty days of treatment with whey protein. These findings were considerably supported by the results of **Krissansen(2007)**who reported that the use of whey protein as a source of amino acids and its effect on reducing the risks of diseases such as heart disease and cancer is the focus of ongoing research. **Byron (2008)** stated that eating a higher protein diet lead to many benefits such as improve your fitness. As you build strengthed, you will be far healthier, pH will be better. **Mercola (2011)** reported that whey protein prevented the decrease in cell viability and also inhibited markers associated with DNA oxidative damage.Based on research conducted with trained and untrained individuals, there seems to be no basis for fear of supra- physiological protein consumption**(Lemon,1994).**As further evidence of the relative safety of high-protein diets, murine study revealed decreased oxidative stress in rats consuming a high-protein diet as compared to an adequate-protein diet **(Petzke *et al.*,2000)** .The National Research Council essentially supported this view on safety, writing, habitual intakes of protein in the United States were substantially above the requirement and although there is no firm evidence that these intake levels are harmful, it has been deemed prudent to maintain an upper bound of no more than twice the recommended dietary allowance (RDA) for protein [1.6 g per kg per day] **(N A S N R C ,1989).**

In the current investigation light and electron microscopic examination of rat cardiac muscle fibers one and thirty days post-treatment with whey protein and then treated with nandrolone revealed marked protection of myofibers against the deleterious changes induced by nandrolone, however, mild widened endomysium, some pyknotyic and karyolytic nuclei and few degenerated mitochondria were still present and there was a partial increase in the collagen fibers.

In myocardial cell cultures, testosterone cypionate caused a significant release of lactate dehydrogenase (LDH) indicating cellular injury (**Brilla *et al.*, 1993)**. The beneficial effect of whey protein supplement was likely due to its amino acid content, in particular branched-chain amino acids which could reduce the release of LDH and consequently reduce the degree of muscle damage **(Koba *et al.*, 2007)** and inflammation **(Matsumoto *et al.*, 2009).**As reported by **Mahe *et al.* (1996)** whey isolate was more effective at increasing blood amino acids and protein synthesis due to its different absorption kinetics and amino acid profile**.**

Kimball and Jefferson (2006) reported that leucine plays a key role in initiating the transcription pathway that fires up protein synthesis. When leucine is ingested in high amounts, such as with whey protein supplementation, there is a greater stimulation of protein synthesis which may speed recovery and adaptation to stress (exercise) **(Ha and Zemel, 2003).Morr and Ha (1993)** mentioned that most whey proteins were cysteine rich. Cysteine was known as an amino acid that regulates *in vivo* concentrations of GSH. The supplementation of the diet with whey protein high in cysteine may promote GSH biosynthesis. The latter has been reported to be an antioxidant and anticarcinogenic tripeptide, thus improving protection against oxidant-induced cell damage (**Bounous *et al.*, 1989; Bounous ,2000)**.Whey proteins have been reported to enhance immune function and the ability of whey protein extract to enhance the generation of superoxide anion and the release of primary granules content could serve as a mechanism to accelerate the destruction of foreign pathogens and to enhance the healing process**(Bounous, 2000). Bounous*et al.* (1989**) indicated that the observed immunoenhancing effect of the whey protein mixture is dependent on the overall amino acid pattern resulting from the contribution of all its protein components. Whey protein contains substantially more cysteine than casein. Dietary cysteine is considered to be a rate limiting substrate for the synthesis of glutathione which is necessary for lymphocyte proliferation. Enhancement of host humoral immune response is associated with Lactalbumin which is found in whey protein as an immunostimulator. It stimulates the proliferation of lymphocytes in culture **(Kayser and Meisel, 1996).** The immunostimulating peptide derived from

Lactalbumin, binds to specific sites on human neutrophils and monocytes **(Jaziri*et al*., 1992)**, stimulates superoxide anion production by neutrophils **(Migliore-Samour *et al*., 1992).** Besides,Lactoferrin (Lf) as an iron binding-protein could increase the output of neutrophil precursors and attenuated the spontaneous production of TNF-α and IL-6 by peripheral blood cells of human volunteers **(Zimecki *et al*., 1999).** Lf also can attenuate stress induced suppression of cellular and humoral immune responses in mice **(Zimecki *et al*., 2005).Ward and Bruce(2003)** mentioned that whey proteins stimulate whole range of cytokines, particularly the proinflammatory cytokines tumor necrosis factor α (TNF-α) and INF γ and the anti-inflammatory cytokines interleukin-6 (IL-6) and interleukin-10(IL-10).

All of the cardiovascular effects of nandrolone have been demonstrated to be fully reversible within several months after cessation of the steroid use (**Wright, 1980; Haupt and Rovere ,1984).**

The PAS value recorded in this study was generally higher than the control during the whole periods of experimental observation in all the treated groups. The lowest rate of increment in PAS-positive material was observed in whey protein, while the highest rate was observed in the nandrolone treated group. In the group treated with whey protein + nandrolone the increment was intermediate between the two groups. Similar results were reported by other investigator such as **Silva *et al*. (2010)** who noticed that increased glycogen content of the cardiac muscle fibers in presence of AAS and suggested that this increase may reflect the effect of these steroids on changing the tissue responsiveness to other hormones, such as insulin-like growth factor on the glycogenic pathway.**Van *et al.* (1993)** reported that the use of AAS was associated with improvement in the physical performance by increasing the muscle energy reserves, such as the concentration of glycogen.

On the other hand **Byron (2008)** stated that leucine directly communicates to insulin, instructing it to work efficiently in muscle. This not only helps preserve your muscle mass, but it helps your muscles use glucose as fuel, in turn supporting healthy insulin function.

The results of this study also showed that the total protein content of cardiac muscle fibers for all the treated groups was significantly higher than the control value allover the experimental period, except whey protein group after month which recorded non significant decrease

The present findings are in accordance with those of **Giannoulis *et al.* (2008)** who reported that physiological testosterone administration in healthy older men resulted in a peak increase in muscle protein synthesis within the first month of treatment, but this anabolic effect wanes if treatment is continued over several months. **Dillon *et al.* (2010)** stated that androgen administration, either alone or in combination with other treatments, can be successful in improving muscle mass by increasing protein anabolism and reducing protein catabolism in men.

On the other hand, the high availability of amino acids in whey protein isolate, especially branched chain amino acids, is important for protein synthesis in the hours immediately after ingestion (**White *et al.*, 2008).**

Whey protein is effective in stimulating muscle protein synthesis for a number of reasons. Firstly, whey protein has been shown to exhibit the highest biological value of any known protein **(Renner, 1983)**. Biological value is the most accurate method of assessing the quality of a protein, as it is a measure of the protein's ability to retain nitrogen in the muscle (**Colgan, 1993).**Secondly, compared to other protein sources, whey protein contains a higher dose (45-55mgs/100gms) of essential amino acids (**Bucci and Unlu, 2000**). Essential amino acids are shown to be the most effective factor for stimulating protein synthesis and therefore promoting muscle growth in adult muscle (**Volpi *et al.*, 2003).**While non-essential amino acids contribute little to the overall response to protein synthesis, one might suggest that this compatibility would position whey as an effective anabolic agent (**Ha and Zemel, 2003**). Finally, there has been increased interest into the branched chain amino acid leucine found in high concentrations in whey protein isolate. Studies have shown that leucine may directly stimulate protein synthesis by activating an additional intracellular signalling pathway (**Karlsson *et al*.,2004).Burd *et al.* (2012)** concluded that ingestion of isolated whey protein supported greater rates of myofibrillar protein synthesis than micellar casein both at rest and after resistance exercise in healthy elderly men. This result was probably related to a greater hyperaminoacidaemia or leucinaemia with whey ingestion.

The present results showed a significant increase in total DNA content cardiac muscle fibers in all the treated groups all over the experimental periods. The value of this increase was the highest in nandrolone treated groups. This is in agreement with those of **Joubert *et al.* (1994)** who stated that muscle fibers hypertrophy induced by testosterone was characterizedby an increase in the number of myonuclei. Because the androgen receptors are located in the myonuclei, the increased nuclear number could potentiallygive rise to an elevation in the number of androgen binding sites (**Kadi *et al.*, 1999).**On the other hand whey protein was found to stimulate dose dependently total protein and DNA content **(Takada *et al.*, 1998**). In summary the present results suggested that the high dose of nandrolone not only elicit measurable increase in performance, but also is able to elicit adverse side effects on cardiac muscle fibers at the histological, histochemicals and ultrastructural levels. On the other hand whey protein supplementation elicited better maintenance of muscle strength and doesn't cause any damaging effects to the cardiac muscle fibers. The application of whey protein to the nandrolone-treated animals could provide a possible protective role from these adverse effects of nandrolone injection .So,nandrolone must be used under clinical supervision especially in the young athletes.

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