ISSN NO. 2320-5407

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

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Effect of nandrolone and/or whey protein on the soleus muscle and testis of adult male albino rats

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Manuscript Info

Manuscript History:

Received: 12 September 2014
Final Accepted: 21 October 2014
Published Online: November 2014

Key words:
Nandrolone, whey protein, soleus muscle,testis, histopathology, ultrastructure.

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Abstract

Nandrolone is one of the most anabolic androgenic steroids which are largely used by amateur and professional athletes to improve the athletic performance. The misuse of that anabolic drug by athletes is a very common problem. Therefore, the present study was carried out to investigate the possible harmful effect of nandrolone on the soleus muscle and testis of male albino rats, in comparison with whey protein as a natural anabolic product, also to investigate whether whey protein can ameliorate the deleterious responses induced by nandrolone. The experimental rats were sacrificed at days 1 and 30 post –treatment. Blood samples were obtained for biochemical and immunological investigations. Soleus muscles and testes were removed for light and electron microscopic studies. In the present results, serum total protein level was significantly decreased after one day of nandrolone treatment (10 mg/kg B.wt/week for 3 months), while elevation of this level was observed after whey protein treatment (5 g/kg B.wt/day for three months). The ameliorative effect was observed post-treatment with both whey protein and nandrolne as manifested by the non significant change of total protein level as compared to the control group. A decrease in collagen ΠI in nandrolone group was recorded, while whey protein group showed a significant increase of this level. LDH was increased after nandrolone and /or whey protein treatment. Testosterone level in groups treated with nandrolone or with whey protein + nandrolone was significantly decreased all over the experimental periods conversely, a significant increase was observed after whey protein supplementation. Lowered levels of TNF-α and IL-1β were recorded after nandrolone treatment, while a significant increase was recognized as a result of whey protein administration. A significant decrease of growth hormone and transforming growth factor-β was noticed under the effect of nandrolone while, whey protein administration was associated with a significant increase of both hormones. Microscopically, in soleus muscle, nandrolone treatment showed hypertrophied muscle fibers with a significant increase in the cross sectional area as compared to the control value. Some muscle fibers were distorted and atrophied with increased cellular infiltration in between and others were totally replaced by dense collagen fibers. On the other hand, whey protein showed increased muscle size accompanied with normal collagen distribution. The double treatment revealed remarkable protection of myofibers against the harmful effects of nandrolone. In testis, nandrolone treatment showed many disorganized seminiferous tubules with loss of sperms and spermatids. Others showed malformed sperms, hyaline material and distorted Sertoli cells with numerous vacuoles, lipid droplets and ruptured mitochondria. Widened interstitial spaces with degenerated Leydig cells were also observed. Whey protein did not cause any adverse effect on testis tissue during the whole experiment periods. Administration of

whey protein before nandrolone treatment induced an obvious recovery from the testicular damage, some tubules still exhibited hypocellularity and germ cell separation. **Conclusion**: Whey protein supplementation elicited better maintenance of muscle strength. Since the amino acid composition of whey protein is very similar to that of the skeletal muscle it may provide amino acids essential for muscle remodeling after injury. Thus whey protein could be used as a safe muscle building; even it could counteract some of the adverse effects of nandrolone.

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Introduction

Anabolic androgenic steroids (AAS) are compounds formed from testosterone or one of its derivatives (**Cunha *et al.*, 2006)**.They are proved to have more anabolic than androgenic activity (**Reynold, 1983)**. They increase protein synthesis within cells, which results in the buildup of cellular tissue (anabolism), especially in muscles and have positive anabolic actions on the musculoskeletal system, influencing lean body mass, muscle size, strength, protein metabolism, bone metabolism and collagen synthesis**(Evans, 2004).** Structurally nandrolone is very similar to testosterone, although it lacks a carbon atom at the 19^{th} position (hence its other name 19-nortestosterone) (**Llewellyn, 2007)**. After administration, nandrolone is metabolized rapidly in the body to a number of metabolites, which are then excreted in the urine. Serious health risks can be produced by long-term use or excessive doses of anabolic steroids**, t**hese effects include harmful changes in the skeletal muscles which are the primary target for the anabolic effects of anabolic androgenic steroids **(Carson *et al.*, 2002),** psychological effects such as aggression and depression, reproductive abnormalities such as infertility, virtualization and feminization as well as impaired liver, heart and kidney functions (**Graham and Kennedy, 1990). Shokri *et al.* (2014)** reported that high doses of anabolic-androgenic steroids associated with male infertility so the combination of exercise and high doses of nandrolone decanoate negatively influences the DNA integrity and protamine content resulting in lower sperm quality.

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, a by-product of cheese and curd manufacturing, was once considered a waste product. The two primary sources of protein in milk are the casein and whey. After processing occurs, the casein is the protein responsible for making curd, while whey remains in an aqueous environment. Whey protein contains an optimal balance of amino acids for muscle growth, especially glutamine or glutamic acid and taurine**(Douglas *et al*,. 1981)**. Whey proteins contains up to 26-percent branched chain amino acids**(Walzem*et al*., 2002)** which are efficient substrates for synthesizing new proteins, the branched chain amino acid leucine was found in high concentrations in whey protein isolate. Studies have shown that leucine may directly stimulate protein synthesis (**Kimball and Jefferson, 2002**). The components of whey include beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulins, lactoperoxidase enzymes, glycomacropeptides, lactose and minerals **(Marshal, 2008).**
Whey proteins are also rich in the sulfur containing amino acids cysteine and methionine, the high concentration of these amino acids enhanced immune function through intracellular conversion to glutathione. Therefore, whey has potent antioxidant activity. In addition, the amino acids found in whey are efficiently absorbed and utilized relative to free amino acid solution (**Daenzer *et al.*, 2001).**Whey protein is used amongst bodybuilders and athletes for its ability to promote muscle growth **(Antonio *et al.*, 2002).**

Because of the wide and irregular use of anabolic androgenic steroids in high doses by professional athletes and amateurs, with the objective of increasing muscle mass, improve performance and physical aesthetics body, so the current study was designed to investigate the harmful effect of the most widely used anabolic androgenic steroids (nandrolone decanoate) on the soleus muscle and testis of male albino rats, in comparison with whey protein as a natural anabolic product, also to investigate whether whey protein can ameliorate the deleterious changes induced by nandrolone injection.

Material and Methods

Experimental animals

A total of 140 male albino rats weighing about 120 g were used. The animals were housed in especially designed cages, 7 rats per cage, with controlled air, temperature and relative humidity. Food and water were made available *ad-libitum* throughout the whole experimental period.

Chemical and drugs:

-Nandrolone

Nandrolone decanoate (Deca-Durabolin) oily solution is manufactured by the Nile Co. Pharmaceuticals-Cairo, under license of N.V. Organon-OSS-Holland.

-Whey protein

100% gold standard whey protein isolate powder was used. Whey protein is manufactured in the U.S.A. by ON Company and dissolved in distilled water.

Experimental design:

The experimental animals were divided into 4 groups:

Group 1: Untreated control rats.

Group 2: Rats treated with nandrolone, 10 mg/kg B.wt /week were intramuscularly injected for 3 months according to **Joumaa and Leoty (2001).**

Group 3: Rats administered whey protein extract orally by gastric tube at a dose of 5 g/kg B.wt /day for 3 months according to **Press (2003).**

Group 4: Rats treated with whey protein at a dose of 5 g/kg B.wt daily for 6 weeks and then they were injected with 10 mg/kg B.wt /week of nandrolone for 6 week.

The experimental rats were sacrificed at days 1 and 30 post –treatment.

Samples collection:

Directly, after the animal was anesthetized by ether, blood was collected from the heart puncture by plastic syringes and left to coagulate and the serum was separated by centrifugation at 3000rpm for 15 min. for biochemical and immunological analysis. Also, samples of soleus muscle and testis were rapidly removed for light and electron microscopic investigations.

Biochemical and immunological analysis:

Quantitative colorimetric determination of total protein in serum was performed by the method of **Tietz (1994)**. LDH was determined according to the method of **Van *et al.* (1994**). Testosterone was determined according to the method of **Burtis *et al. (*1994).** Collagen type III was determined using enzyme immunoassay (ELISA) test kit,Uscn life science Inc.Wuhan. Tumor necrosis factor (TNF-α) was determined using Fluorokine MAP rat base kit.Serum IL- 1β was determined using enzyme immunosorbent assay (ELISA) method, Immuno Biological Laboratories. Serum growth hormone was estimated by enzyme linked immunosorbent assay (ELISA) method, Biovendor, Research and Diagnostic Products. Transforming growth factor-beta 1(TGFβ1) was determined by using enzyme linked immunosorbent assay (ELISA) method, Genscript, (catalog number: L00367).

Histological technique

Following rats sacrifice, they were rapidly dissected and suitable pieces of right soleus muscles and testes were quickly removed and stained by haematoxylin and eosin (**Drury and Wallington 1980)** and Mallory′ trichrome **(Humason, 1972).**Meanwhile, the left soleus muscles and testes were processed for electron microscopic examination.

Quantitative morphometric analysis:

The cross sectional areas of the soleus muscles and seminiferous tubules were measured using Image Pro Plus image analysis software. The mean cross sectional area of the soleus muscles and seminiferous tubules were calculated for each group. The percent value of the mean of treated groups relative to the control value was calculated and graphically presented using Microsoft excel.

Results

Biochemical and immunological studies

The rats injected with nandrolone exhibited a significant decrease in the level of total protein as compared to the control group while, non significant decrease was observed on 30th day post-treatment. On the other hand, the rats treated with whey protein showed non significant increase in such level all over the experimental periods. Treatment with whey protein before injection with nandrolone exhibited non significant decrease in total protein all over the experimental periods (Table 1).Injection of rats with nandrolone exhibited a significant decrease in the level of collagen111after one day and a **n**on significant decrease was recorded after 30days. In contrast, treatment of rats with whey protein resulted in a significant increase in this level on the1st day post-treatment, while non significant decrease was recorded after 30days. Treatment with whey protein and nandrolone showed a significant decrease in the level of collagen111on day one post-treatment, while non significant increase was recorded on day30 post-treatment (Table 1).

In Table (2) the rats injected with nandrolone showed a significant increase in LDH all over the experimental periods. The treatment with whey protein or with whey protein+ nandrolone exhibited a significant increase in this level. Such increase was lower than that in nandrolone alone. The rats injected with nandrolone exhibited a significant decrease in serum testosterone on one and 30 days post-treatment, conversely drenching whey protein to the rats induced a significant increase at the same intervals. Treatment of rats with whey protein and nandrolone intensified the decrease in serum testosterone for the 1st and 30th days post-treatment.

The rats injected with nandrolone exhibited a significant decrease in the level of TNF-α as compared to the control group after one day of treatment, while a significant increase was observed on day30 post-treatment(Table 3). The treatment with whey protein showed a significant increase in TNF-α one day post-treatment, while non significant increase was observed after 30 days. Treatment of rats with whey protein and nandrolone exhibited a significant decrease in this level after one day of treatment, while non significant increase was observed on day 30 post-treatment.

Nandrolone treatment exhibited a significant decrease in the level of IL-1β on day one post-treatment, while non significant decrease was recorded after 30days. Treatment with whey protein resulted in a significant increase in such level on the 1st day post-treatment, while non significant decrease was recorded after 30days. Treatment with whey protein and nandrolone showed a significant decrease in the same level on day one post- treatment, while non significant increase was recorded on day 30 (Table 3).

The treatment with nandrolone exhibited a significant decrease in the level of growth hormone as compared with the control group after one day of treatment, while non significant decrease was observed after 30 days (Table 4).The treatment with whey protein showed a significant increase in serum growth hormone on day one post- treatment, while non significant decrease was observed after 30 days. The treatment with whey protein and nandrolone exhibited a significant decrease in such level after one day of treatment, while non significant decrease was observed after 30days.

Injection of rats with nandrolone exhibited a significant decrease in level of TGFβ1 on day one post-treatment, such decrease turned to be significant increase after 30days (Table 4). Treatment of rats with whey protein resulted in a significant increase in this level on the1stand 30thdays post-treatment, while the treatment with whey protein and nandrolone showed a significant decrease on day one post -treatment, while non-significant increase was observed on day 30 of treatment.

Table (1): Effect of nandrolone and/ or whey protein on serum total protein and collagen111 in adult male albino rats.

Parameter	Total Protein((g/L)				Collagen111 (ng/mL)			
Time	One day		One month		One day		One month	
Groups	Mean± S.E	% change	Mean ± S.E	% change	Mean± S.E	% change	Mean± S.E	% change
Control	7.57 ±0.28	0.0 %	7.57 ±0.28	0.0 %	139.35 ±2.43	0.0 %	139.35 ±2.43	0.0 %
Nandrolone	5.76 ±0.14[acd]	-23.92%	7.29 ±0.36	-3.7%	101.63 ±2.33[acd]	-27.07%	137.84 ±9.25	-1.08%
Whey protein	7.77 ±0.43[b]	2.64%	7.65 ±0.38	1.06%	155.30 ±1.54[abd]	11.45%	135.79 ±6.05	-2.55%
Whey protein+ nandrolone	6.88 ±0.34[b]	-9.11%	6.81 ±0.34	-10.0%	119.81 ±1.21[abc]	-14.02	143.92 ±4.31	3.28%

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

Table (2): Effect of nandrolone and/ or whey protein on serum lactate dehydrogenase (LDH) and testosterone in adult male albino rats.

Parameter	LDH (U/L)				Testosterone (ng/mL))			
Time	One day		One month		One day		One month	
Groups	Mean± S.E	% change	Mean± S.E	% change	Mean± S.E	% change	Mean± S.E	% change
Control	631.33 ±42.83	0.0 %	631.33 ±42.83	0.0 %	3.09 ±0.35	0.0 %	3.09 ±0.35	0.0 %
Nandrolone	2139.83 ±102.62[ac]	238.94 %	1224.00 ±48.29[acd]	93.88 %	1.63 ±0.10[acd]	-47.25%	1.74 ±0.05[acd]	-43.69%
Whey protein	981.00 ±43.20[abd]	55.39%	893.17 ±46.50[abd]	41.47 %	4.12 ±0.22[abd]	33.33%	8.24 ±0.46[abd]	166.67 %
Whey protein+ nandrolone	2001.33 ±96.03[ac]	271.0%	1047.67 ±14.06[abc]	65.95 %	1.33 ±0.09[abc]	-56.96%	1.20 ±0.02[abc]	-61.17%

legand as table (1)

Table (3): Effect of nandrolone and/ or whey protein on serum tumor necrosis factor (TNF-α) and interleukin-1 (IL-1β) in adult male albino rats.

Parameter	TNF-α (pg/mL)				IL-1β (pg/mL)			
Time	One day		One month		One day		One month	
Groups	Mean± S.E	% change	Mean± S.E	% change	Mean± S.E	% change	Mean± S.E	% change
Control	*285.47 ±2.10*	0.0 %	*285.47 ±2.10*	0.0 %	*63.02 ±1.15*	0.0 %	*63.02 ±1.15*	0.0 %
Nandrolone	*197.22 ±4.09*^{acd}	-30.91%	*335.02 ±16.32*^{a}	17.36%	*47.28 ±1.00* ^{acd}	-24.98%	*57.01 ±3.03*	-9.54%
Whey protein	*317.12 ±9.52* ^{abd}	11.09%	*305.54 ±14.02*	7.03%	*71.73 ±0.95* ^{abd}	13.82%	*58.60 ±2.82*	-7.01%
Whey protein +nandrolone	*240.75 ±3.55* ^{abc}	-15.67%	*325.34 ±21.23*	13.97%	*55.97 ±1.22* ^{abc}	-11.19%	*63.14 ±2.70*	0.19%

legand as table (1)

Table (4): Effect of nandrolone and/ or whey protein on serum growth hormone (GH) and transforming growth hormone-beta (TGFβ1) of adult male albino rats.

Parameter	GH(pg/mL)				TGFβ1(pg/mL)			
Time	One day		One month		One day		One month	
Groups	Mean± S.E	% change	Mean± S.E	% change	Mean± S.E	% change	Mean± S.E	% chang
Control	*3804.56 ±108.97*	**0.0 %**	*3804.56 ±108.97*	0.0 %	*27.28 ±0.58*	**0.0 %**	*27.28 ±0.58*	0.0 %
Nandrolone	*2184.63 ±38.21*^{acd}	**-42.57%**	*3386.45 ±252.06*	-10.99%	*20.62 ±0.49* ^{acd}	**-24.41%**	*34.17 ±1.10*^{a}	25.26 %
Whey protein	*4182.86 ±81.86* ^{abd}	**9.96%**	*3766.15 ±202.32*	-1.01%	*31.21 ±0.40* ^{abd}	**14.41%**	*32.35 ±0.90* ^{a}	18.59 %
Whey protein +nandrolone	*2550.23 ±27.77*^{abc}	**-32.97%**	*3798.90 ±201.38*	-0.15%	*24.03 ±0.29* ^{abc}	**-11.91%**	*29.99 ±1.77*	9.93%

legand as table (1)

The histological studies

1-Soleus muscle-Light microscopeic results

Examination of longitudinal section ofcontrol rat soleus muscle is illustrated in Fig. (1). The rat soleus muscle one day post –treatment with nandrolone showed hypertrophied muscle fibers with increased myonuclei (Fig.2a).Highly degenerated muscle fibers and increased cellular infiltration in the widened endomysium were seen (Fig.2 b). Also, many muscle fibers were partially or totally replaced by dense collagen fibers (Fig. 2c).Following thirty days of nandrolone treatment, some muscle fibers were distorted, the soleus muscle exhibited prominent nuclei with abnormal arrangement (Fig.3).

Soleus muscles of rats administered whey protein and examined one or thirty days post- administration showed increased muscle size as well as myonuclei appeared numerous than in the control group (Fig.4). Examination of rat soleus muscle one day post-treatment with whey protein and then treated with nandrolone revealed a noticeable protection of myofibers against deleterious changes induced by nandrolone, however increased nuclei and wide spacing of myofibers were still present (Fig.5). Following thirty days, the majority of soleus muscles appeared normal, while partial increase in the collagen fibers was observed inside the hypertrophied muscle fibers (Fig. 6).

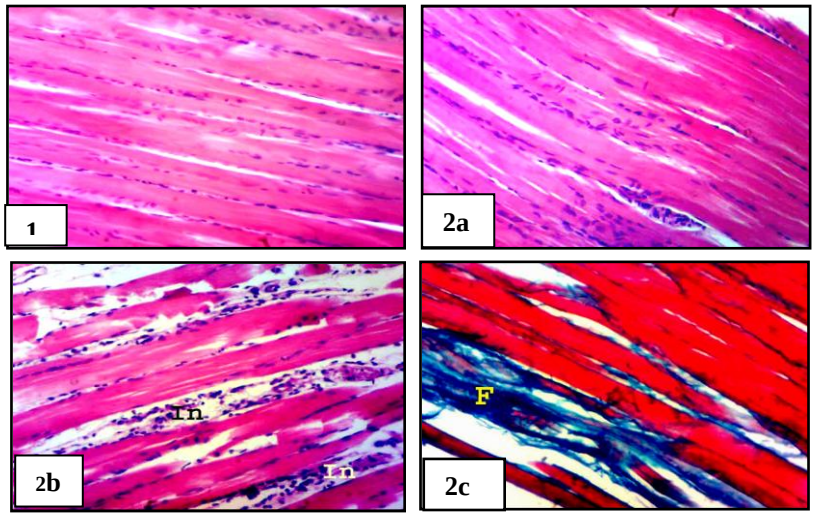

Figure (1): Photomicrograph of **c**ontrol soleus muscle of a rat showing elongated and arranged parallel striated muscle fibers, the spacing between them being occupied by small amounts of endomysial supporting tissue (L.S., Hx&E X100). **Figure (2):** One day post-treatment with nandrolone showing a: hypertrophy of the muscle fibers accompanied by increased myonuclei (L.S., Hx&E X100),b: highly degenerated muscle fibers with increased cellular infiltration (In) in the widened endomysium (L.S., Hx&E X100)**,** c: many muscle fibers were replaced by dense collagen fibers **(F)** (L.S., Mallory's trichrome stain X250).

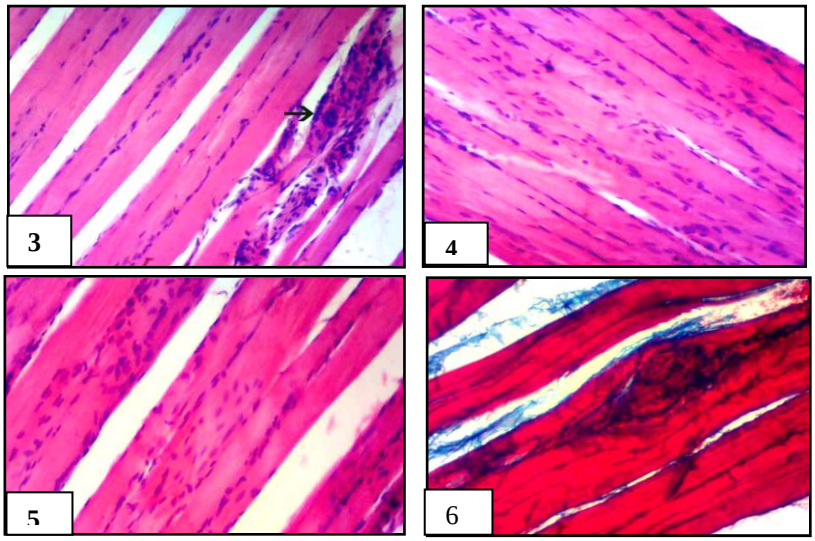

Figure (3: Photomicrograph of rat soleus muscle thirty days post-treatment with nandrolone showing intact and hypertrophied degenerated muscle fibers (→) with increased swollen nuclei (L.S., Hx &E X100). **Figure (4):** Thirty days post-treatment with whey protein showing hypertrophied muscle fibers (L.S.,Hx &E X100). **Figure (5):** One day post-treatment with whey protein and nandrolone showing increased myonuclei and widened endomysium (L.S.,Hx &E X100). **Figure (6):** Thirty days post-treatment with whey protein and nandrolone showing increased collagen fibers in the endomysium and inside the hypertrophied muscle fibers (L.S., Mallory's trichrome stain X100).

The electron microscopic results

The ultrastructure of control rat soleus muscle is illustrated in Fig. (7a&b). The rat soleus muscle one day post – treatment with nandrolone showed dividing satellite cells and the endomysium is occupied by numerous collagen fibers (Fig. 8). Following thirty days of treatment, the soleus muscle exhibited well developed myonuclei, mitochondria, myofilaments, RER, free ribosomes and dividing satellite cells (Fig.9).One and thirty days following whey protein administration, the soleus muscle showed well developed mitochondria, myonuclei and dividing

satellite cells (Fig10). Both one and thirty days post-treatment with whey protein and nandrolone revealed numerous satellite cells and collagen fibers were observed in the narrow endomysium with numerous mitochondria and myonuclei inside the sarcolemma (Fig. 11).

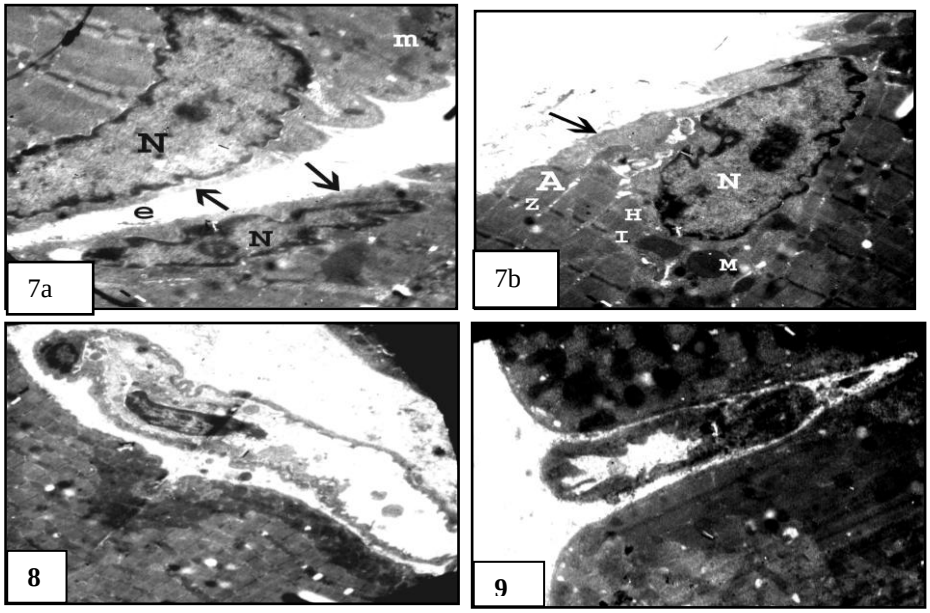

Figure (7): Electron micrographs of control rat soleus muscle showing a&b: elongated myonuclei **(N)** located inside the sarcoplasm **(→),** endomysium **(e)**, mitochondria **(m),** dark segment (**A)** (anisotropic) contains a pale zone (**H)** or Hensen's disk and the pale segment (**I)** (isotropic) bisected by a dark line (Z)(a-X 2000 , b-X 3000). **Figure (8)**: One day post-treatment with nandrolone showing two satellite cells near the sarcolemma with numerous mitochondria in their cytoplasm and inside the muscle fibers (X 1000). **Figure (9)**: Thirty days post-treatment with nandrolone showing satellite cell penetrating the sarcolemma beside the myonucleus(X2000).

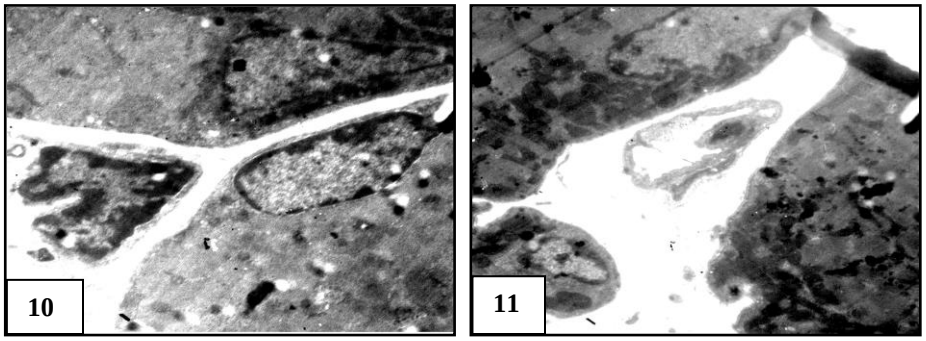

Figure (10): Electron micrograph of rat soleus muscle **t**hirty days post-treatment with whey protein showing myonucleus inside the sarcoplasm, numerous mitochondria and outside the satellite cell piercing the sarcolemma (X 3000). **Figure (11)**: Thirty days post-treatment with whey protein and nandrolone showing numerous mitochondria and myonucleus inside the sarcoplasm with satellite cells embedded in endomysium (X 2000).

2-Testis

The light microscopic results

The normal histological structure of rat testis is illustrated in Fig. (12).Examination of testes of rats one day post – treatment with nandrolone revealed marked evidence of disorganized seminiferous tubules. As shown in Figs. (13a,bc), some tubules exhibited partial separation of the germ cells, others showed loss of sperms and spermatids. Such tubules showed partial or complete hyalinization as well as irregular and disrupted outer surfaces. Exfoliated spermatogenic cells in the tubular lumina and wide separation between the deeper tubules were also noticed. Vacuolations of various sizes, deeply stained Sertoli cells nuclei and necrotic spermatogenic cells were observed

besides interstitial exudation and highly degenerated Leydig cells. With Mallory's trichrome stain, thickened tunica albuginea and increased collagen fibers in and around the tubules were seen. By the end of thirty day, marked deleterious consequences were frequently perceived in the majority of the seminiferous tubules. Some tubules showed germ cell depletion, giant cells and hyalinization (Fig.14).

Testes sections examined one or thirty days following whey protein administration did not show any detectable structural abnormalities when compared with the control group (Fig.15). Testes of rats administered whey protein pre- nandrolone treatment and examined after one and thirty days showed a striking recovery from the testicular damage. In Figs. (16&17), most seminiferous tubules were apparently resuming their normal architecture. However, spermatogenic arrest at various stages of spermatogenesis was observed in some tubules. Such tubules showed partial separation of germ cells, exudates and reduced Leydig cells.

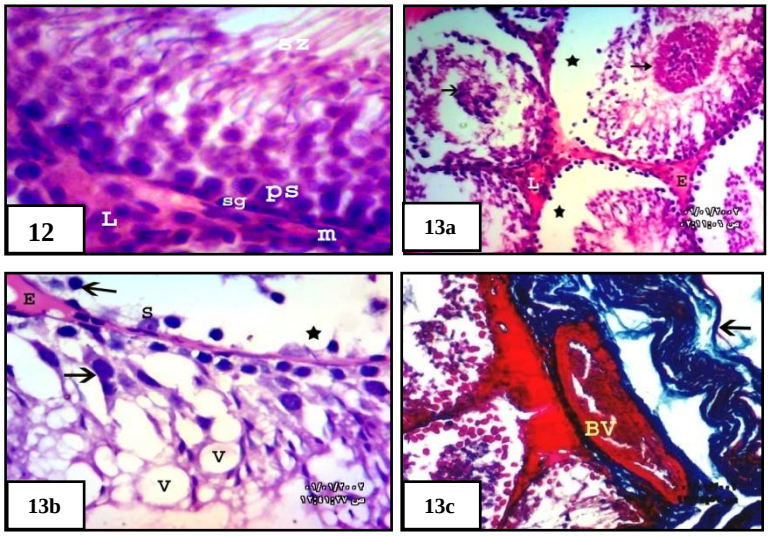

Figure (12): Photomicrograph of control rat testis showing seminiferous tubules lined with germinal epithelium, spermatogonia (**sg),** primary spermatocytes (**ps)**, spermatozoa **(sz)** and myoid cells **(m)**. Interstitial spaces between the seminifrous tubules containing Leydig cells **(L)** were seen (Hx&E X250). F**igure (13)**: **O**ne day post- treatment with nandrolone showing **a:** partial separation of germ cells (✦), hyalinization, exfoliated spermatogenic cells, hypocellularity, aggregations of some late spermatids (→), exudation**(E**) and diffusely stained patches of Leydig cells **(L)** (Hx&E X100). **b:** partial separation (✦) of germ cells, vacuolations (**V**), germ cells depletion, deeply stained Sertoli cells nuclei **(S)** and nuclei of some spermatogenic cells→) besides interstitial exudates **(E)(**Hx&E X250). **c:**highly thickened tunica albuginea (→) with increased collagen fibers in and around walls of the engorged subcapsular blood vessels (Mallory's trichrome stain X100).

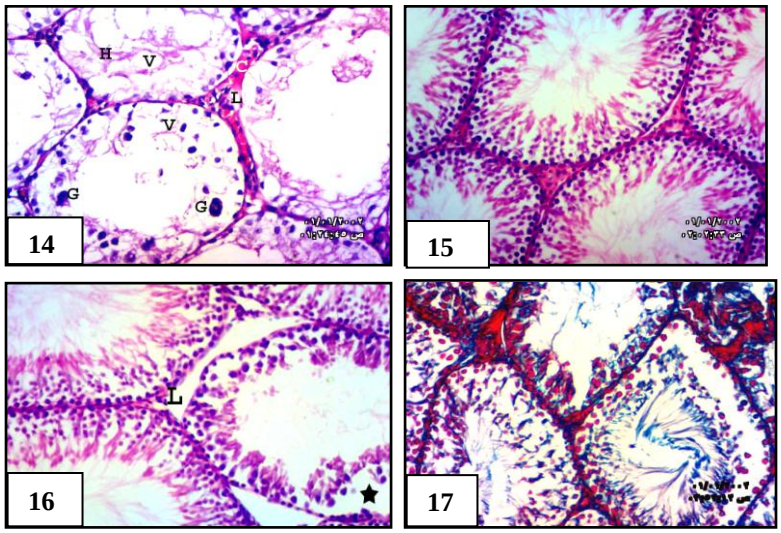

Figure (14): Photomicrograph of rat testis thirty days post-treatment with nandrolone showing seminiferous tubules with germ cells depletion, giant cells **(G)**, vacuolation **(V),** hyaline materials **(H)**,interstitial congestion (**C**), bleeding and degenerated Leydig cells **(L**) (Hx&E X100). **Figure (15)**: One day post-treatment with whey protein showing almost normal seminiferous tubules with normal interstitial Leydig cells (Hx&E X100). **Figure (16)**: One day post-treatment with whey protein and nandrolone showing gradual reappearance of normal spermatogenic cells while reduced spermatids and spermatozoa, partial separation (✦ of germ cells and reduced Leydig cells **(L)** are still detected(Hx&E X100). **Figure (17)**: Thirty days post-treatment with whey protein and nandrolone showing some exudates and collagen fibers in the interstitial spaces (Mallory's trichrome stain X100).

The electron microscopic results

The ultrastructur of control rat testis is shown in Fig. (18). Testes taken from rats one day post–treatment with nandrolone showed destructed spermatogenic cells, distorted myoid cells, vacuoles, lipid droplets ,thick and irregular basement membrane, spermatogonia type B lost their nuclei and contained degenerated mitochondria, primary spermatocytes with organoids debris, besides disintegrated chromatin with corrugated , elongated and pyknotic nuclei(Fig.19a).Malformed sperms with atrophied heads, absence of centrioles, degenerated or absent mitochondria, ruptured outer sheath of the middle piece with increased deposition of collagen fibers around them were noticed in Fig. (19b). Thirty days following the treatment with nandrolone showed Sertoli cells with hardly detected cellular membranes, apoptotic and undifferentiated spermatogenic cells, primary spermatocytes with vacuolated and ruptured cellular organoids, ill defined nuclear and cellular membranes and myoid cells with disintegrated chromatin,(Fig.20a**)**.The spermatids in Golgi phase contained hypertrophied mitochondria with ruptured cristae (Fig. 20b).

On the other hand, normal ultrastructural appearance was observed in the testes of rats one and thirty days following whey protein administration. A noticeable recovery was observed in the testicular tissue of rats administered whey protein and nandrolone after one or thirty days, some seminiferous tubules revealed normal Sertoli cells, myoid cells, spermatogonia type (B)and spermatids. Others showed degenerative changes in the spermatogenic cells and spermatids in the acrosomal phase, numerous vacuoles in the primary spermatocytes and dilated cisternae of RER (Fig. 21).

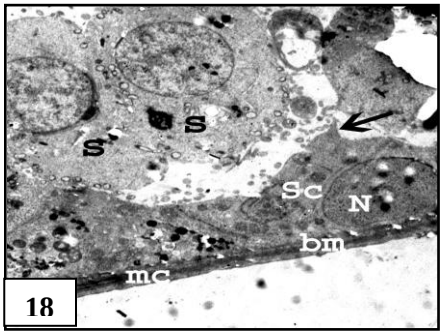

Figure (18): Electron micrograph of control rat testis showing basement membrane **(bm)**, myoid cell with flattened nucleus **(mc),** two rounded spermatid **(s)** with their characteristic ring form mitochondria, Sertoli cells **(Sc)** with slightly indented nucleus **(N),** voluminous cytoplasm with plenty of mitochondria and long process joint (⟶)(X1000). **Figure (19)**: One day post –treatment with nandrolone showing a: scattered collagen fibers, lipid droplets, degenerated myoid cell **(mc),** degenerated spermatogonia type B **(B**), primary spermatocytes **(ps)** contained debris of degenerated organoids, disintegrated chromatin, with corrugated, elongated and pyknotic nucleus(X 2000). b**:** distorted head **(a**), middle piece **(b)** and tail**(c)** of the sperms, most of spermatogenic cells with pyknotic nuclei (**pn**), elongated cells and numerous vacuoles **(v)** (X 1000).

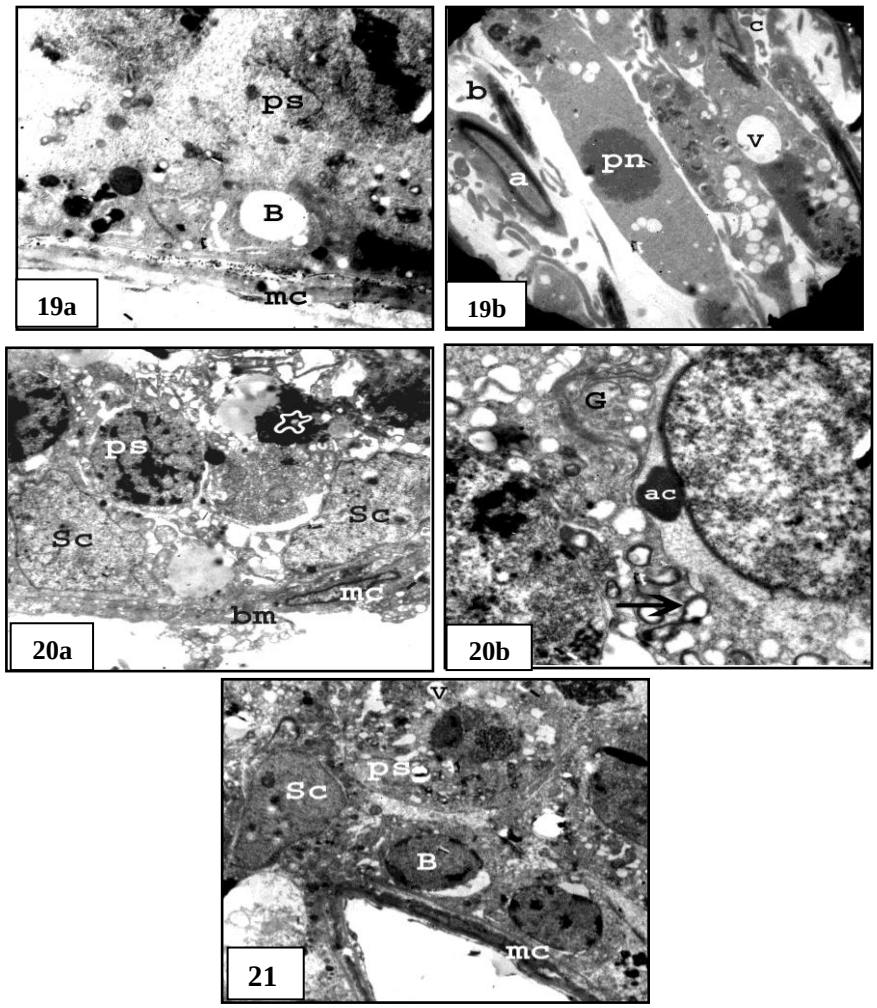

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Figure (20): Electron micrograph of rat testis thirty days post-treatment with nandrolone showing a**:** ruptured basement membrane **(bm)**, myoid cells **(mc)** with disintegrated chromatin, and irregular Sertoli cells **(Sc**), with ill defined nucleolus., two primary spermatocytes**(ps**) with vacuolated and ruptured cellular organoids and ill defined nuclear and cellular membranes, debris of degenerated cells or electron dense masses are detected between the spermatogenic cells (★) (X 1000).b**:** spermatid in the acrosomal phase, normal appearance of Golgi apparatus **(G)**, irregular acrosome **(ac)** and numerous hypertrophied mitochondria with ruptured cristae **(→)**(X 3000). **Figure (21)**: Thirty days post-treatment with whey protein and nandrolone showing normal Sertoli cell **(Sc),** dividing spermatogonia type B **(B**),myoid cell **(mc),**basement membrane, primary spermatocytes **(ps)** contained numerous vacuoles **(v)** and dilated cisternae of RER
(X 1000).

Morphometric measurement

Cross-sectional area:

1-the soleus muscle

Rats injected with nandrolone exhibited a significant increase in the value of cross-sectional area of the soleus muscle all over the experimental periods. While rats treated with whey protein or with whey protein+ nandrolone showed a significant increase after one day only, but non significant increase was observed for these groups after thirty days of treatment (Table 6).

2-The seminiferous tubules

Rats treated with nandrolone exhibited a significant decrease in the value of cross sectional area of the seminiferous tubules after one and thirty days of treatment, while non significant change was observed for the other groups (Table6).

Table (6): Effect of nandrolone and / or whey protein on cross sectional area of soleus muscles and seminiferous tubules in adult male albino rats.

Parameter	Area of soleus muscle				Area of seminiferous tubules			
Time	One day		One month		One day		One month	
Groups	Mean± S.E	% change	Mean± S.E	% change	Mean± S.E	% change	Mean± S.E	% change
Control	*11.90*±1.7	0.0	*11.90*±1.7	0.0	*170.57*±19.11	0.0	*170.57*±19.11	0.0
Nandrolone	*18.01±0.84**	51.34 %	*13.45*±0.47*	13.03 %	*55.06±5.64**	-67.72%	*119.29*±4.66*	-30.06 %
Whey protein	*15.92±0.43**	33.78 %	*12.61±0.30*^{NS}	5.97%	*182.54±7.56*^{NS}	7.02%	*200.43±12.12*^{NS}	17.5%
Whey protein +nandrolone	*17.62±0.80**	48.07 %	*12.50±0.27*^{NS}	5.04%	145.67±*6.5*^{NS}	-14.60%	*146.42±4.8*^{NS}	-14.16 %

*** Significant difference from control at P ≤ 0.05**
NS, Non significant change from control value

Discussion

Androgenic anabolic steroids have been used by athletes for decades to increase lean body mass, strength and overall athletic performance (**Yesalis, 2000**). The anabolic steroid nandrolone decanoate has long biological half-life and previous studies demonstrated that skeletal muscle is a biological target of AAS (**Carson *et al.,* 2002). Ferrari *et al.*(2013)** reported that the treatment with nandrolone decanoate in both sedentary and trained rats
Showed many morphological and functional changes in male reproductive system that resulted in reduced efficiency of spermatogenesis.
Today, whey is a popular dietary protein supplement purported to provide antimicrobial activity, immune modulation, improved muscle strength and prevents cardiovascular disease and osteoporosis. Also, whey has the ability to act as an antioxidant, antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial and chelating agent **(Kanwar *et al.,* 2007).**Injury elucidated in the soleus muscle and testis by nandrolone decanoate and the possible protective role of whey protein against this injury have been demonstrated in this study by using biochemical, immunological and histological parameters.

As shown in the present study, total protein level was significantly decreased after one day of nandrolone treatment. Confirming with this result ,**Lok *et al. (*2010)** stated that long term testosterone implication during puberty may lead to heart and liver defects at early ages and cause a decrease in the total protein, albumin and calcium levels. The current study presented elevation of total protein after whey protein treatment; similar findings were reported by **Davenport *et al*. (2000)** who suggested that much of whey protein was absorbed into the circulation. Moreover, it was found that whey protein contains up to 26-percent branched chain amino acids (BCAA) which are efficient substrates for synthesizing new proteins. For example, the BCAA leucine acts as a signaling molecule for initiation of protein synthesis **(Walzem *et al.,* 2002)**. The ameliorative effect was observed in the group of rats treated with whey protein + nandrolne as manifested by the non significant change of total protein level as compared to the control group.

Enzymatic skeletal muscle proteins such as creatine kinase(CK),lactate dehydrogenase (LDH), myoglobin and myosin heavy chain (MHC) may spill from muscle cells to the serum and be used as quantitative markers of cellular damage and recovery **(Claudia *et al.,* 2011).**In the present study LDH was increased all over the experimental periods after nandrolone and / or whey protein treatment. The increase of LDH was very pronounced in nandrolone

group in comparison with whey protein group. **Maravelias *et al.* (2005)** showed that the elevations of the liver enzymes levels (aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase) were common in the athletes who use steroids.In myocardial cell cultures; testosterone cypionate caused a significant release of lactate dehydrogenase indicating cellular injury (**Brilla *et al.*, 1993)**. Similar elevation in LDH activity was observed by **Matthew *et al.* (2010)** after supplementation with whey protein and a trend towards recovery was recorded during a month. The beneficial effect of the whey protein supplement is 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).**

The male hormone testosterone, derived mainly from the testis, is an anabolic and androgenic steroid responsible for the production of male physical feature **(Corrigan, 1996).** The present study showed that testosterone level in treated groups with nandrolone or with whey protein +nandrolone was significantly decreased all over the experimental periods. Also **Hartgens and Kulpers (2004)** proved that AAS administration suppressed the hypothalamic-pituitary-gonadal axis, producing reversible suppression and disturbance of the regular endogenous production of testosterone and gonadotrophins that persist for months after drug withdrawal. The main changes in reproductive system of males using AAS are a decrease in testis size, concomitant to a decrease in testosterone levels (Torres-**Calleja *et al.*, 2001).**The present study showed a significant increase in serum testosterone followed whey protein supplementation. This finding agrees with **Kalman *et al.* (2007)** who mentioned that the testosterone/estradiol ratio was increased in groups receiving whey protein, while estradiol was decreased. Group analysis showed significant increase in the testosterone/estradiol ratio in soy isolate + whey blend group.

Cytokines are non-antigen proteins secreted by both immune and non-immune cells and have autocrine/paracrine action **(De la Fuenta *et al*., 2004).**Some cytokines such as TNF-α, (IL)-1β and IL-6 have received more attention than others because they have traditionally been classified as proinflammatory cytokines**(McKay *et al*., 2000).** Lowered levels of TNF-α and IL-1β recorded in this study are in line with the observations of **Langen *et al*. (2002)** who demonstrated that both TNF-α and IL-6 can act as skeletal muscle growth factors at low concentrations, but can also induce muscle wasting at higher concentrations or with chronic exposure. On the contrary, **Thomas *et al*. (1995)** demonstrated that nandrolone decanoate directly induced the production of the inflammatory cytokines IL-1β and TNF-α from human peripheral blood lymphocytes. They mentioned that anabolic steroids can have significant effects on immune responses. **Hsu *et al*. (2009)**attributed the elevation of such inflammatory cytokines to the dosage abuse of nandrolone which hastens mortality due to septic shock and increases serum malondialdehyde, liver TNF-α and spleen IFN-γ levels.

Whey proteins enhance immune function (**Bounous, 2000),** and the ability of whey protein extract to enhance the generation of superoxide anionand 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 *et al.* (1989**) indicated that whey protein contains cysteine is considered to be a rate limiting substrate for the synthesis of glutathione which is necessary for lymphocyte proliferation. 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).Ward and Bruce(2003)** mentioned that whey proteins stimulate whole range of cytokines, particularly the proinflammatory cytokines TNF-α and INF γ. These data go in hand with this study in which a significant increase of TNF-α and IL-1β was recognized under the effect of whey proteins.

Karila *et al*. (1998) provided evidence that AAS could lower the levels of certain hormone-binding proteins in circulation including the growth hormones. Transforming growth factor-β (TGF-β) signaling is important for fibroblast cell activation and regulates gene transcription through signaling cascades utilizing intracellular proteins **(Flanders *et al*., 2003).** Androgen receptor activation can repressTGF-β signaling through protein-protein interaction with Smad3in prostate cells **(Chipuk *et al*., 2002).** These findings illustrate the results of this work that showed a significant decrease of growth hormone (GH) and transforming growth factor-β (TGF-β) under the effect of nandrolone. On the other hand, whey protein administration was associated with a significant increase of GH and TGF-β when compared to nandrolone. This could be explained by **Alba-Roth *et al.* (1988)** who mentioned that arginine which is one of the ingredients of whey protein is a precursor of nitric oxide and creatine, and its administration promotes the secretion of growth hormone (GH) which may lead to an increase of muscle mass and strength. As reported by **Murray *et al.* (1995),**L-arginine, seems to increase GH by decreasing somatostatin(SS) release from the hypothalamus. It is well known that whey protein is potentially a rich source of low abundance proteins, containing higher amounts of immunoglobulins, growth factors, cytokines and nucleosides than are found in milk (**Kelly, 2003)**.

Concerning the histological examination, androgens, in addition to having effects on the male reproductive system, have anabolic effects on skeletal muscles (**Antonio *et al.*, 1999**). The present light and electron microscopic investigations recorded in the soleus muscles for nandrolone treated rats after one and thirty days of treatment

showed hypertrophied muscle fibers accompanied by increased number and abnormal arrangement of myonuclei. Also, increased cellular infiltration and widened endomysium were observed. The most striking change was that some muscle fibers were replaced by dense collagen fibers. These findings were supported by **Sinha-Hikim *et al.* (2002)** who found that testosterone supplementation; even without strength training has been reported to induce hypertrophy in human skeletal muscles. **Bhasin (2003)** proved that androgen-induced increase in muscle mass appeared to arise from muscle fiber hypertrophy rather than hyperplasia.

In this study, soleus muscle of rats examined one and thirty days after whey protein administration exhibited increased muscle size with prominent myonuclei compared with the control group, this increase come in parallel with increased GH level. These results were in agreement with **Burke *et al*. (2001)** who declared that the ingestion of protein is advantageous when muscle fiber hypertrophy is desirable; again suggesting that whey protein is an effective method of increasing muscle size, possibly due to enhanced protein and contractile synthesis, as evident by increased muscle fiber area. However, enhanced protein levels within the muscle can enhance muscle regeneration from injury.

On the other hand, examination of rat soleus muscle one or thirty days post-treatment with whey protein and nandrolone revealed an obvious protection of myofibers against the deleterious changes of nandrolone, however increased collagen fibers in the endomysium and inside the hypertrophied muscle fibers were still present. **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. 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)**.. **Ha and Zemel (2003)**stated that whey protein supplementation may be increasing muscle cross sectional area and/or muscle mass of the injured muscle fibers possibly due to its ability to influence net muscle protein balance within the muscle**.**

In the testis, nandrolone treatment after one and thirty days induced marked evidence of disorganized seminiferous tubules, some tubules exhibited partial separation of the germ cells and others showed loss of sperms and spermatids,. Such tubules revealed deeply stained Sertoli cells nuclei and obvious degeneration of spermatogenic cells which in turn showed necrosis to the extent that cellular debris only persisted in the lumens. Confirming with these results, **Richburg (2000)** deduced that the observation of detached germ cells, amorphous head sperm and missed location of spermatid and spermatozoa that are closely related to the basement membrane may be due to the rapid disruption of the Sertoli and germ cells interaction. This physical interaction ultimately leads to the sloughing of the germ cells from seminiferous epithelium.**Takahashi *et al*. (2004)**proved that prolonged treatment of nandrolone in male led to germ and Sertoli cells' sloughing with reduction of testicular volume and seminiferous tubule length. As reported by **Mesbah *et al*. (2007)** administration of nandrolone decanoate induced marked degenerative changes of germ cells, Sertoli cells and Leydig cells. These are accompanied by changes in semen parameters and testis atrophy.

In the present study, vacuolations of the seminiferous tubules were seen within the germinal cells as a results of nandrolone treatment. Similarly, **Chakravarty and Singh (1998)** found that nandrolone administration induced shrinkage of the seminiferous tubules, such tubules showed vacuoles in their epithelial lining. The authors explained this vacuolation by the decreased population of spermatogenic cells leaving empty spaces in between the lining epithelial cells.

The present nandrolone treatment showed thickening of the tunica albuginea with increased collagen fibers in and around the seminiferous tubules. In this respect, **Karila (2003)** suggested that anabolic androgenic steroids abuse at supraphysiological doses decreased the degradation of type I collagen and significantly increases the overall metabolism of type III collagen in all the soft tissues and this opinion may discuss the increase of collagen fibers observed in the present study.

The results of this study also showed widened interstitial spaces between seminiferous tubules with highly degenerated Leydig cells as well as irregular nuclear and cellular membranes. **Salem *et al. (*2007)** reported that nandrolone (1mg/rat weekly for 10 weeks) treatment led to shrunken seminiferous tubules with variable degeneration of the germinal epithelium. Also, **Naraghi *et al.* (2010**) found a remarkable decrease in the number and size of Leydig cells and a depletion of intact cells in nandrolone-treated rats. The close relationship between Leydig cells and blood vessels suggested that these cells are at high risk of exogenous toxicants and multivacuolated Leydig cells are probably a form of cell involution **(Feinberg *et al.*, 1997)**.Decreased tubular area in the nandrolone-treated group was markedly seen in the present study .This decrement was significantly different from the control. It can thus be noted that the extent of necrosis was evident from the statistically significant decrease in cross sectional areas of the seminiferous tubules. As reported by **Creasy and Foster (1991)** the loss of the germ cells from the seminiferous

tubules generally resulted in a decrease in the tubular diameter and this could be explained the observed widening of the interstitial spaces.

In the present nandrolone study, Sertoli cells appeared distorted and contained highly atrophied nuclei, numerous vacuoles, and lipid droplets with ruptured mitochondria. There are also malformed sperms with atrophied heads, absence of centrioles, degenerated or absent mitochondria, and ruptured outer sheath of the middle piece. Altered basement membrane structure recorded in this study has been associated with severe functional impairment of the pyknotic nuclei of Sertoli cells .These results are in agreement with the reports showing that exogenous stimulants may cause progressive apoptosis of the Sertoli cells, which affect spermatogenesis and sperm parameters (**Gopalkrishnan *et al.*, 1998).**High doses of anabolic-androgenic steroids may influence the hypothalamic-pituitary-gonadal axis, which can in turn affect testicular apoptosis **(Shokri *et al*., 2009**). Increased lipid droplets in Sertoli cells post-treatment with nandrolone were discussed by **Bensoussman *et al. (*1998)** who attributed the appearance of numerous lipid droplets in the cytoplasm of Sertoli cells to the accumulation of the breakdown products of the spermatogenic cells.

On the other hand, the present light and electron studies revealed that whey protein administration did not cause any adverse effect on testis tissue during the whole experimental period. These findings were supported by **Mercola (2011)** who reported that whey protein prevented the decrease in cell viability and also inhibited markers associated with DNA oxidative damage. It appeared that the amino acids found in whey protein activate certain cellular mechanisms including mTORC-1 (Mammalian Target of Rapamycin), which in turn promote muscle protein synthesis and also protect against declining testosterone levels after exercise.

Therefore in this study administration of whey protein before nandrolone treatment induced an obvious recovery from the testicular damage. Most seminiferous tubules were resuming their normal architecture. However, some tubules exhibited hypocellularity, exudation and germ cell separation. In this group, there was a significant increase in the mean tubular areas versus nandrolone- treated group, while a non significant increase versus the control group was observed. These findings were supported by **Rieu *et al.* (2007)** who stated that whey is an abundant source of branched-chain amino acids, which are used to fuel working muscles and stimulate protein synthesis. Kimball and Jefferson (2006) reported that leucine, found in whey protein isolate, plays a key role in initiating the transcription pathway that fires up protein synthesis. When leucine is ingested in high amounts, 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 are cysteine rich. 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**).The possible protective role of whey protein as suggested by **Bounous (2000)** may be attributed to the increases in blood and tissue GSH concentrations, which in turn increase the scavenger of the free radicals produced by harmful agents.

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