Early Prediction of the Acute Cardiotoxic Effects of Whey Protein and Nandrolone on Albino Rats

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Abstract

Background Whey protein (WP) is a dietary supplement. It helps bodybuilders and athletes recover faster, reduce excess weight, and enhance muscle mass. The excessive use of such supplements causes substantial stress on your body. Nandrolone decanoate (ND) is a synthetic testosterone derivative that is unconstitutionally abused by young athletes to enhance their physical endurance, despite its severe adverse effects and global restriction.

Aim Because we do not know how whey protein and nandrolone affect the cardiac system in supraphysiological doses taken by athletes, our objective is to investigate the cardiac effects of short-term use of whey protein and nandrolone.

Methods Thirty adult male albino rats were divided into four groups; control group: fifteen rats were divided into three equal subgroups; (control negative (CN): standard diet and free tap water, control distilled water (CDW): distilled water, and control peanut oil subgroup (CP): peanut oil). Whey protein group (WP): (five rats) WP was given by oral gavage daily except Friday at a dose of 5.4 g/kg. In addition, nandrolone decanoate group (ND): five rats got 36 mg/kg of ND intra-peritoneally once a week. Finally, five rats in the combined group (Comb) were given the exact doses of WP and ND. After four weeks, blood samples were taken from all the animals for further analysis. Moreover, the right and left ventricles were preserved for biochemical and histological study.

Results ND and Comb groups had severely deleterious effects on the ventricular muscle, while the WP rats showed considerably mild effects

Conclusions As a consequence of these findings, it is recommended that WP should be administered under medical supervision for restricted doses, while ND must be entirely prohibited.

Keywords: Whey protein, Nandrolone decanoate, Oxidative stress, Cardiotoxicity, Fibrosis, Apoptosis.

INTRODUCTION

Whey protein (WP) is a liquid protein separated from casein and purified to different concentrations during cheese production. It is a dietary supplement that comes in the form of easily digestible powdered milk protein (**1**). Due to the depletion of protein in the body during exercise, WP can be included in the process of restoring and constructing muscle. Furthermore, the glutamine content of WP helps to prevent muscle injury (**2)**. Also, it increases energy during exercise, reducing the recovery duration of severe training symptoms (**3)**. Because WP intake controls blood sugar levels in the body, WP is very

useful for dieters and those who want to lose some kilograms of their body (**4)**. Vasconcelos et al. (**5**) found that the widespread availability of WP in stores and on the internet has enabled their use without a prescription by amateur athletes, possibly disregarding the hazards connected with chronic and excessive protein ingestion.

Some researchers claim that excessive WP consumption can damage the kidneys and liver and lead to osteoporosis. Moreover, the short-term consuming WP can significantly impact renal functions, especially in predisposed people, as evidenced by an increased urinary plasma volume and calcium excretion while the pH of urinary citrate decreases, resulting in nephrocalcinosis **(6)**. Furthermore, it can cause unsupportable stress on the liver and may cause liver damage (**7**).

Aparicio et al. (**8)** stated that the higher intake of WP causes a mineral imbalance, especially calcium (Ca^{+2}), and a decrease in bone mineral density (which leads to osteoporosis).

Recent studies have reported that a high-protein diet increases cardiovascular risk. Melnik **(9**) showed that WP had a rule of increasing insulin and insulin-like growth factor-1 (IGF-1), which promotes atheromatous lesions. Also, high blood pressure may develop due to elevated IGF-1 levels in middle age. In addition, Melnik (**10**) stated that WP and milk products are associated with high insulin levels that may develop insulin resistance, resulting in glucose intolerance and cardiovascular diseases. Zhang and Reilly (1**1)** discovered that a high-protein diet increased levels of amino acids in the blood, reflecting a negative effect on mitochondrial function, macrophage apoptosis, and atherosclerotic plaques. The dominant cause of myocardial infarction is plaque rupture with superimposed thrombosis. Moreover, patients with ischemic heart disease who consumed a daily high-protein diet had an increase in mortality due to acute coronary syndrome (**12**). In addition, saturated fats and cholesterol contents found in dairy products, such as WPC contribute to hyperlipidemia and hypercholesterolemia and the risk of cardiovascular disease (**13**).

Anabolic androgenic steroids (AAS) are synthetic testosterone derivatives. It is gaining popularity among athletes to improve performance, power, and musculature. They are intended to be more anabolic than androgenic (**14)**, despite being prohibited in the majority of organized sports (**15)**.

According to Patanè et al. (**16**), nandrolone (a 19-nortestosterone derivative) is the AAS with the maximum anabolic potency. It has been used in medical contexts to treat osteoporosis in postmenopausal women, several types of anemia, and wasting syndrome associated with AIDS **(17**). Nevertheless, both athletes and non-athletes have dramatically increased their use of these substances in recent years **(18)**.

Erdal and Seyfullah (**19**) and Frankenfeld et al. (**20**) reported that ND has severe adverse effects on the cardiovascular, renal, reproductive, metabolic, neurological, and behavioral systems at supraphysiological doses.

Based on its effects on blood pressure, cholesterol levels, lipidemic profile, oxidative stress, and fluid retention, Ayubi et al. (**21**) found that ND abuse increases the risk of cardiovascular disorders. Depending on dose and duration, ND use may contribute to coronary artery disease. In addition, supraphysiological doses of ND would cause arrhythmias due to impaired systolic, diastolic, and autonomic nervous function in the ventricles. The increased cardiac muscle fibrosis caused by ND is well documented in animal and human studies. Furthermore, weight lifters who consume ND exhibit hypertrophy of the left ventricle **(15, 22**).

Because of the prevalence of protein supplements, including whey protein and anabolic steroids, among adolescents who use them to develop muscles and their accessibility and positive influence on muscle mass, their use remains one of the most significant health concerns in sports nowadays. This research aims to detect the effects of WP and ND on cardiac tissues and their associated parameters and toxicity markers.

Materials and Methods

Chemicals and Kits

- WP in the shape of a pale-yellow powder. The ingredients are protein blend (Whey Protein Isolate, Whey Protein Concentrate, Whey Peptides), natural and artificial flavor (vanilla ice cream), lecithin, cellulose gum, xanthan gum, salt, sucralose, acesulfame potassium, and lactase. It was purchased from Optimum Nutrition, INC, the USA.
- A 1 ml glass ampoule containing ND 25 mg/ml in the form of a yellowish oily solution was purchased from El-Nile Company for Pharmaceuticals and Chemical Industries.
- Peanut oil was ordered as a yellowish-green solution from the Biohayah® Company.
- SPINREACT's Total Cholesterol Assay Kit (colorimetric) BSIS11-E
- SPINREACT's Serum Triglyceride Quantification Kit (colorimetric) BSIS31-E
- SPINREACT's HDL- Cholesterol Assay Kit (precipitating reagent) BSIS12-E
- SPINREACT's LDL- Cholesterol Assay Kit (colorimetric) BSIS51-E
- Aviva Systems Biology's Rat Malondialdehyde (MDA) ELISA Kit (OKEH02548)
- Cusabio Biotech Co., Ltd.'s Rat Super Oxide Dismutase (SOD) ELISA kit (Cat. No. CSB-E08555r).
- Cusabio Biotech Co., Ltd.'s Rat Glutathione Peroxidase (GPx) ELISA kit (Cat. No. CSB-E12146r).
- SPINREACT's LDH Assay Kit (BEIS43)
- SPINREACT's CK-MB Assay Kit (BEIS04)

Experimental Animals

Thirty adult male albino rats (180-200 g) were acclimatized to their new environment over one week. They were housed in cages of polypropylene under lab-style temperature and lighting. Rats were received water supply and a standard laboratory meal during the experimental time. The research was conducted in compliance with the Zagazig University Ethics Committee for Animal Handling's rules at the Faculty of Medicine's Animal House at the university, Egypt, with authorization number **(*ZU-IACUC/3/F/87/2021*).** The sample size of the rats was calculated using Open Epi software by the Department of Community of the Faculty of Medicine, Zagazig University.

Experimental Groups

Rats were divided into four groups:

- Control Group: Fifteen rats were divided equally into three subgroups (control negative (CN): standard diet and free tap water, control distilled water (CDW): distilled water (the WP's solvent) (**23**), and control peanut oil (CP): peanut oil (the ND's vehicle) **(24).**

- Whey Protein group (WP): Five rats, each received 5.4 g/kg/day of the protein dissolved in distilled water orally for six days per week. The dose was chosen based on the typical dose of adults (70 kg) 1-2 scoops (30-60 g). The highest dose of 60 g extrapolated to rats by surface area, according to method described by Paget and Barnes **(25).**
- Nandrolone Decanoate group (ND): Five rats each treated IP once per week at a dose of 36 mg/kg/week of ND diluted in peanut oil (arachis oil). The dose was calculated based on the usual dose of athletes (70 kg) 200-600 mg/week. Based on the average dose of 400 mg extrapolated to rats by surface area, according to method described by Paget and Barnes **(25)**

- Combined group (Comb): Each rat in the Comb group got the before-clarified doses of WP for six days pers week and ND once per week.

Sampling and determination of heart weight/body weight ratio

Upon the experimental termination (after four weeks) (a 24-hour from the last dose), all rats were sacrificed after anesthesia. Blood samples were taken from the carotid artery **(26)**. Moreover, the hearts were removed and washed with cold saline and weighed. The index of cardiac hypertrophy was measured as a ratio of heart weight to the overall body weight of the rat (HW/BW in mg/g). Dissection, measuring, and homogenization of the right ventricle muscle were performed in 5 mL of ice-cold 0.1 M Tris-HCl buffer (pH 7.4). Before the biochemical analysis, the clear supernatant solution was then gathered and centrifuged. The left ventricle was preserved for histological examination.

Biochemical Investigations

To assess the blood lipid profile, including the concentrations of low-density lipoprotein (LDL) according to method described by Wieland and Seidel **(27**),and Friedewald et al. (**28**), total cholesterol (TC) according to method described by Meiattini et al. (**29**), and triglycerides (TG) according to method described by Bucolo and David (**30**) using enzymatic colorimetric methods. High-density lipoprotein (HDL) was evaluated using the phosphotungstate-magnesium ions precipitation method according to method described by Grove (**31**), and very low-density lipoprotein (VLDL) was determined from the TG level using Friedewald's formula: TG/5 according to method described by Friedewald et al. **(28**).

A rat MDA ELISA Kit (OKEH02548) obtained from Aviva Systems Biology was used to assess the serum MDA (nmol/ml) level to set oxidative stress parameters according to method described by Armstrong and Browne (**32**). Moreover, Cusabio Biotech Co., Ltd.'s rat SOD ELISA kit (Cat. No. CSB-E08555r) and its Rat GPx ELISA kit (Cat. No. CSB-E12146r) were used to quantify the total SOD (U/ml) level according to method described by Adachi et al. (**33**) and the GPx (U/mg) level in the heart tissue according to method described by McMurray et al. (**34**), respectively.

Serum lactate dehydrogenase (LDH) (U/L) was measured using the technique suggested by Baba and Sharma (**35**) to investigate the cardiac cytotoxic indicators, and serum creatine kinase-myocardial band (CK-MB) (U/L) was tested using the technique suggested by Lott and Abbott **(36**) and Gerhardt et al. (**37**)

Using a Cobas c702/8000 autoanalyzer (Roche Diagnostic, Mannheim, Germany) and its related reagents and the necessary chemicals were used, the tests were conducted in the Department of Biochemistry of the Faculty of Medicine, Zagazig University.

Histological Analyses

5 μm-thick slices of the left ventricle specimens were stained with H&E and Mallory trichrome after their fixation in 10% formalin (**38**). Moreover, paraffin-embedded slices were stained with immunohistochemistry using the marked streptavidin-biotin immune peroxidase method (**39**). A rabbit monoclonal antibody (EPR18297, ab184787, Waltham, MA, USA) diluted to 1:1000 was used to detect caspase-3. Alcohols of varying concentrations were rehydrated, xylene was deparaffinized, and the sections were washed in a phosphate buffer solution (PBS). Furthermore, the sections were processed with 3% hydrogen peroxide before being washed in PBS. The primary antibody was provided after application and washing in PBS. The biotinylated secondary antibody was added after rinsing with water, and the enzyme conjugate and 3, 3-diaminobenzidine tetrahydrochloride were incubated (DAB Substrate Kit, Thermo Fischer Scientific, Rockford, IL, USA). The sections were counterstained with Mayer's hematoxylin. PBS was used in place of antiserum to provide a negative control.

Morphometric analyses

The subsequent factors were measured:

- Cardiomyocyte nuclei count (considered the marker of cardiomyocyte hypertrophy).
- Percentage area of arrangement of tissue collagen stained with Mallory trichrome.
- Percentage area of Caspase-3 antibodies' immunoreactivity.

It was operated using a Leica Qwin 500 software image analyzer (Leica image system Ltd., Cambridge, UK). In each region, ten non-overlying fields were randomly selected at x-400 magnification. Measurements were conducted in Pathology Department, School of Dental Medicine, Cairo University.

Statistical analysis

SPSS 25.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis (**40**). Means and standard deviations (SD) were used to present the findings of the statistical study. To compare continuous data between the tested and control groups, a one-way analysis of variance (ANOVA) was also used. Values were considered statistically significant at $p < 0.05$.

Results

There were negligible differences ($p > 0.05$) between CN, CDW, and CP subgroups. In order to compare with the other experimental groups, a group of five rats was selected randomly.

Bio-Physiological Measures

Body Weights and Heart Weights (Table 1)

The obtained results from the control group's body and heart weights showed no significant changes. Moreover, the WP group gained more weight than the control group. Whereas the ND group had significantly lower body weights. The Comb group experienced a significant increase in body mass compared to the other groups.

There was no noticeable difference in heart weight between the WP and control groups. While the heart mass of the ND group was significantly higher. The hearts of the Comb group were substantially larger than those of the other groups.

Index of Cardiac Hypertrophy (heart weight / body weight ratio)

The ratio of heart weight to body weight was unaffected by the control group. The heart/body weight ratio showed a non-significant difference between the WP and control groups. At the same time, ND reflected an increased ratio than in the control and WP groups. The Comb group's heart hypertrophy indicator dramatically increased compared to the other groups **(Table 1) (Fig. 1)**

Table 1 Statistical comparison between mean values of body weights, heart weights, and heart/body weight ratio in control negative, nandrolone decanoate, whey protein, and combined groups after 4 weeks of the study using ANOVA test and LSD

Parameter	Group	N	Mean	S.D	F	P	P1	P2	P3
Body weight (g)	CN	5	230.6	0.894	164.9	<0.001 ***	-----	----	----
	WP	5	250.8	2.46			<0.001 ***	----	----
	ND	5	198.6	2.18			<0.001 ***	<0.001 ***	-----
	Comb	5	285.4	5.44			<0.001 ***	<0.001 ***	<0.001 ***
Heart weight (g)	CN	5	0.652	0.032	72.5	<0.001 ***	----	----	----
	WP	5	0.663	0.03			0.309 *	----	-----
	ND	5	0.748	0.07			0.03 **	0.03 **	-----
	Comb	5	1.29	0.137			<0.001 ***	<0.001 ***	<0.001 ***
Heart / Body weights ratio(mg/g)	CN	5	2.91	0.31	92.1	<0.001 ***	----	----	----
	WP	5	3.12	0.21			0.248 *	----	-----
	ND	5	4.29	0.37			<0.001 ***	<0.001 ***	-----
	Comb	5	5.48	0.17			<0.001 ***	<0.001 ***	<0.001 ***

*CN:control negative, Comb: Combined group, F: F value (ANOVA test), N: number of rats, , ND: nandrolone decanoate group ,P1: the difference between the control negative group and other groups, P2: the difference between the WP group and other groups, P3: the difference between the ND group and other groups, SD: standard deviation, WP: whey protein group, *: P >0.05 means are not significant, **: P <0.05 means are significant, ***: P <0.001 means are highly significant*

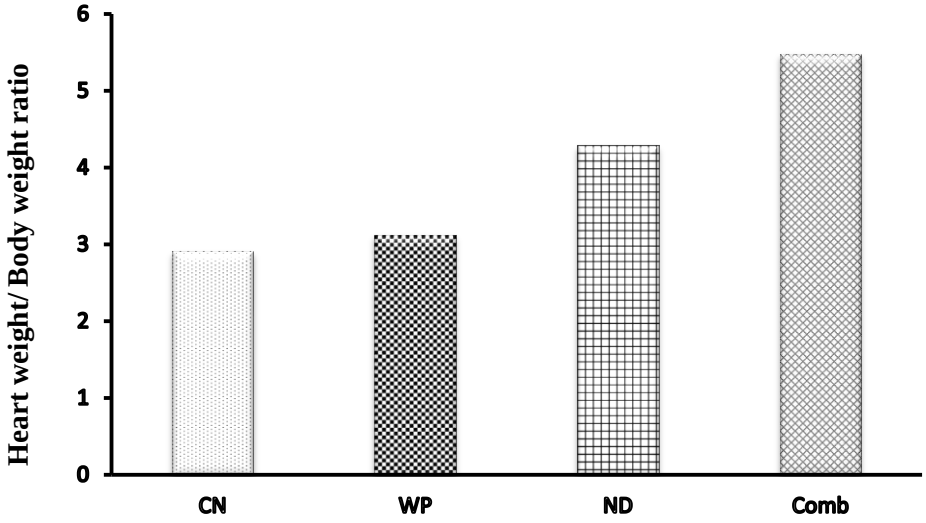

Fig. 1 Comparison between the heart/body weights ratio (mg/g) of the four groups (control negative (CN), whey protein (WP), nandrolone decanoate (ND), and combined (Comb) groups) after 4 weeks of the study using showing the mean values of heart/body weight ratio (number of rats = 30)

Biochemical Results

Serum Lipid Profile (Table 2)

TC level (mg/dl)

Compared to the WP and control groups and the ND group, the levels of TC were considerably higher ($p< 0.001$). The Comb group revealed a significant increase compared to the other groups. While TC levels in the WP and control groups were similar.

TG level (mg/dl)

The higher TG levels were found in the ND group compared to the WP and control groups ($p< 0.001$), while in the Comb group, its levels were considerably increased compared to other groups. In contrast, the TG levels in the WP and control groups were close.

HDL level (mg/dl)

Moreover, the levels of HDL were significantly lower in the ND group ($p< 0.001$) compared to the WP and control groups. The Comb group had a substantial drop compared to all other treated groups. Regarding HDL values, the WP group results did not show any differences from the control group.

LDL level (mg/dl)

Compared to WP and the control, ND exhibited higher levels of LDL ($p <0.001$). Similarly, LDL levels of the Comb group showed the highest, and similar LDL values were found in the WP and control groups.

VLDL level (mg/dl)

The ND treated group exhibited greater levels of VLDL ($p< 0.001$) when compared with WP and the control groups. In contrast, the Comb group showed the highest significant increase rather than other groups. While the VLDL levels in the WP and control groups were comparable.

Table 2 Statistical comparison between mean values of total lipid profile (TC, TG, HDL, LDL, and VLDL) in control negative, nandrolone decanoate, whey protein, and combined groups after 4 weeks of the study using ANOVA test and LSD

Parameter	Groups	N	Mean	S.D	F	P	P1	P2	P3
TC (mg\dl)	CN	5	94.6	1.71	107.3	<0.001 ***	------	-----	-----
	WP	5	96.5	2.14			0.159 *	-----	-----
	ND	5	121.8	10.93			<0.001***	0.001***	-----
	COMB	5	160.1	6.84			<0.001***	<0.001***	<0.001 ***
TG (mg\dl)	CN	5	73.6	2.75	138.5	<0.001 ***	-------	------	-----
	WP	5	75.4	2.98			0.323 *	-----	------
	ND	5	119.8	6.22			<0.001 ***	<0.001 ***	-----
	Comb	5	142.6	10.6			<0.001 ***	0.003 **	<0.001 ***
HDL (mg\dl)	CN	5	45.8	2.81	24.5	<0.001 ***	------	----	------
	WP	5	44.8	2.55			0.531 *	----	-------
	ND	5	36.9	2.04			0.001 **	0.002 **	-------
	COMB	5	33.2	1.48			<0.001 ***	0.01 **	<0.001 ***
LDL (mg\dl)	CN	5	34.3	0.599	34.7	<0.001 ***	-----	-----	----
	WP	5	35.4	0.671			0.221 *	-----	-----
	ND	5	42.21	1.12			<0.001 ***	<0.001 ***	-----
	Comb	5	46.99	4.31			<0.001 ***	0.04 **	0.001 **
VLDL (mg\dl)	CN	5	16.71	2.48	48.5	<0.001 ***	------	-----	-----
	WP	5	17.78	2.67			0.529 *	-----	-----
	ND	5	25.8	1.51			<0.001 ***	<0.001 ***	------
	Comb	5	33.5	3.13			<0.001 ***	0.007 **	<0.001 ***

*CN: control negative, Comb: Combined group, F : F value (ANOVA test),HDL: high density lipoprotein, LDL: low density lipoprotein, N: number of rats, ND: nandrolone decanoate group ,P1: the difference between the control negative group and other groups, P2: the difference between the WP group and other groups, P3: the difference between the ND group and other groups, SD: standard deviation, TC :total cholesterol, TG: triglycerides, VLDL: very low density lipoprotein, WP: whey protein group, *: P >0.05 means are not significant, **: P <0.05 means are significant, ***: P <0.001 means are highly significant*

Oxidative Stress Parameters (Table 3)

MDA level (nmol/ml)

Furthermore, the findings revealed that the MDA levels in the WP group were more elevated (p <0.001) than in the control group. Similarly, the ND group showed a remarkable increase compared to the WP and control groups. In addition, the MDA levels were considerably elevated in the Comb group compared to the other groups.

SOD level (U/ml)

On the other hand, the SOD levels had a depletion in the WP group than in the control group (p< 0.001). At the same time, the ND group had a significant reduction compared to the WP and control groups. The SOD level in the Comb group fell statistically significantly compared to the other groups.

GPx level (U/mg heart tissue)

A lower level of GPx was observed in the WP group than in the control group ($p<0.001$). The ND group experienced a significant decline compared with the WP and the control group. Similarly, GPx levels dropped significantly in the Comb group compared to the other groups.

Table 3 Statistical comparison between mean values of oxidative stress parameters (MDA, SOD, and GPx) in control negative, nandrolone decanoate, whey protein, and combined groups after 4 weeks of the study using ANOVA test and LSD

Parameter	Groups	N	Mean	S.D	F	P	P1	P2	P3
MDA (nmol/ml)	CN	5	5.96	0.016	181.7	<0.001 ***	----	-----	------
	WP	5	8.42	0.74			<0.001 ***	------	------
	ND	5	10.94	0.99			<0.001 ***	0.002 **	------
	Comb	5	15.4	0.49			<0.001 ***	<0.001 ***	<0.001 ***
SOD (U/ml)	CN	5	161.2	2.54	566.4	<0.001 ***	------	------	-----
	WP	5	130.8	2.37			<0.001 ***	-------	------
	ND	5	100.8	4.59			<0.001 ***	<0.001 ***	-------
	Comb	5	82.2	3.05			<0.001 ***	<0.001 ***	<0.001 ***
GPx (U/mg)	CN	5	215.5	3.55	144.6	<0.001 ***	-------		
	WP	5	186.4	2.65			<0.001 ***	-----	------
	ND	5	144.7	2.77			<0.001 ***	<0.001 ***	------
	Comb	5	120.8	1.98			<0.001 ***	<0.001 ***	<0.001 ***

*CN: control negative, Comb: Combined group, F : F value (ANOVA test),GPx: glutathione peroxidase, MDA: Malondialdehyde, N: number of rats, , ND: nandrolone decanoate group ,P1: the difference between the control negative group and other groups, P2: the difference between the WP group and other groups, P3: the difference between the ND group and other groups, SD: standard deviation ,SOD: superoxide dismutase, WP: whey protein group, *: P >0.05 means are not significant, **: P <0.05 means are significant, ***: P <0.001 means are highly significant*

Cardiac Cytotoxic Markers (Table 4)
LDH level (nmol/ml)

The results showed that the WP group had higher LDH levels than the control group ($p<0.001$). There was a significant rise in the ND group when compared to the WP and control groups. In the Comb group, the level of LDH was significantly higher.

CK-MB level (nmol/ml)

Furthermore, in the WP group, CK-MB levels were more elevated than in the control group ($p<0.001$). The ND group showed a significantly higher increase than the WP and control groups. Comparatively to the other groups, the Comb group had a significantly increased level of CK-MB

Table 4 Statistical comparison between mean values of cardiac cytotoxic markers (LDH and CK-MB) in control negative, nandrolone decanoate, whey protein, and combined groups after 4 weeks of the study using ANOVA test and LSD

Parameter	Groups	N	Mean	S.D	F	P	P1	P2	P3
LDH (nmol/ml)	CN	5	154.2	4.71	174.8	<0.001 ***	-----	-----	----
	WP	5	277.5	2.11			<0.001 ***	-----	------
	ND	5	1165.5	12.5			<0.001 ***	<0.001 ***	------
	Comb	5	1412.7	11.5			<0.001 ***	<0.001 ***	<0.001 ***
CK-MB (nmol/ml)	CN	5	55.4	3.84	185.9	<0.001 ***	-----	----	----
	WP	5	117.1	5.35			<0.001 ***	-----	-----
	ND	5	460.4	6.98			<0.001 ***	<0.001 ***	--------
	Comb	5	855.4	7.3			<0.001 ***	<0.001 ***	<0.001 ***

*CK-MB: creatine kinase-MB, CN: control negative, Comb: Combined group, F : F value (ANOVA test), LDH: lactate dehydrogenase , N: number of rats, ND: nandrolone decanoate group ,P1: the difference between the control negative group and other groups, P2: the difference between the WP group and other groups, P3: the difference between the ND group and other groups, SD: standard deviation , WP: whey protein group, *: P >0.05 means are not significant, **: P <0.05 means are significant, ***: P <0.001 means are highly significant*

Histological Results

The histopathological results of hematoxylin and eosin-stained left ventricle sections from the control group showed acidophilic, branching, and anastomosing cardiac muscle fibers with centrally located vesicular nuclei, narrow interstitial spaces, and little flattened nuclei of fibroblast **(Fig. 2a). Fig. 2b** represented areas of wavy muscle fibers with vacuolated myocytes in the WP group. Other sections revealed some areas of separated muscle fibers, widened endomysium plus areas of mild inflammatory cellular infiltrates **(Fig. 2c)**. In the ND group, disarrayed and wavy myofibers beside others with hyalinosis separated with abundant inflammatory cells and dark flat nuclei of fibroblasts (**Fig. 2d**). Moreover, dilated congested blood vessels were noticed in-between multiple wavy myofibers, besides some muscle fibers, were separated with many dark flat nuclei of fibroblasts in the field (**Fig. 2e)**. In addition, the left ventricular muscle of the Comb group revealed an obvious vacuolation separating multiple pale acidophilic degenerated myocytes with some areas of wavy myofibers (**Fig. 2f). Fig. 2g** reflecting that this section revealed a large dilated congested blood vessel within fields of wavy muscle fibers beside others with eosinophilic hyaline degeneration, some vacuolated myocytes, and infiltrates of inflammatory cells**.** Moreover, extensive wavy myofibers were investigated beside other disorganized fibers with widened endomysium in-between and pale acidophilic degenerated myocytes areas in comb group **(Fig. 2h).**

In the control group, Mallory trichrome-stained left ventricle sections displayed few small collagen fibers between cardiomyocytes **(Fig. 3a)**. Regarding the WP group, thin collagen fibers were noted in the interstitial between cardiomyocytes and around blood vessels **(Fig. 3b)**. Similarly, in the ND group there were increased collagen fibers between the cardiomyocytes and around blood vessels (**Fig. 3c).** Furthermore, the examination of Comb group sections showed a marked increase in collagen deposition between the cardiomyocytes and around blood vessels **(Fig. 3d).**

The immune localization of anti-caspase-3 antibodies in the left ventricle of the control group revealed a small grade of caspase-3 expression **(Fig. 4a).** Also, the WP group showed a minimal degree of caspase expression **(Fig. 4b).** While the ND group, a moderate degree of caspase expression was noticed **(Fig. 4c).** Conversely, in Comb group there was an extensive caspase- 3 expressions in left ventricular fields **(Fig. 4d).**

Morphometric Results

In the H&E-stained slices, the WP rats had a non-significant change in the number of cardiomyocyte nuclei compared to the control. Furthermore, a significant reduction was detected in the ND group compared to the control and the WP groups. Also, compared to the other experimental groups, in the Comb group, the mean values of the number of cardiomyocytes nuclei / high power field declined significantly **(Fig. 2i)**

In Mallory trichrome-stained sections, the WP rats reflected a non-significant difference in the area of collagen distribution compared to the control group. While the percentage of collagen dispersion in the ND left ventricle significantly augmented compared to the control and the WP groups. Compared to other groups, there was a significant elevation in the Comb group (**Fig. 3e).**

Regarding the area percentage of Caspase-3 immunoreactivity, the WP and the control groups were non-significant concerning each other. In the ND and the Comb groups, a significant increase was revealed in contrast to the control and the WP groups. Furthermore, a noticeable rise in the Comb rats was demonstrated in all other rats **(Fig, 4e).**

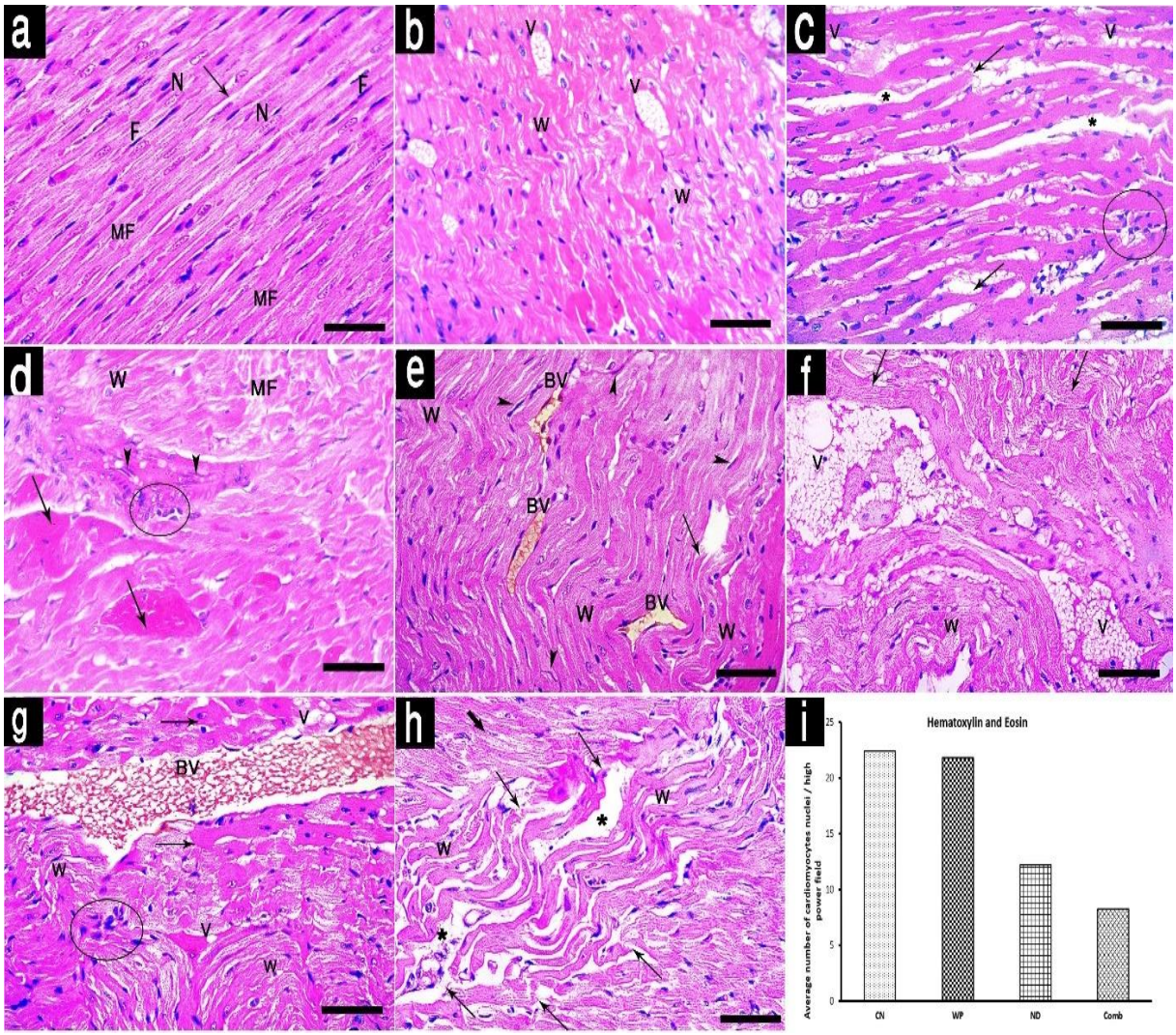

Fig. 2 ***Hematoxylin and eosin-stained section of the left ventricle: (a) control group; showing acidophilic, branching, and anastomosing cardiac muscle fibers (MF) with centrally located vesicular nuclei (N), narrow interstitial spaces (arrow), and flattened nuclei of fibroblast (F) are also seen. (b, c) WP group rats; wavy muscle fibers (W) with multiple vacuolated myocytes in-between (V) are obvious (b). Some fields show several areas of separated muscle fibers (arrow), vacuolated myocytes (V), widened endomysium (star), plus clumps of inflammatory cellular infiltrates (circle) are observed (c). (d, e) ND group; displays disarrayed muscle fibers (MF) with areas of hyalinization (arrow), wavy myofibers (W), multiple dark flat nuclei of fibroblasts (arrowhead) and areas of inflammatory cell (circle) (d). Other sections show extensive areas of wavy myofibers (W) and dilated congested blood vessels (BV), separated muscle fibers (arrow), plus multiple dark flat nuclei of fibroblasts (arrowhead) are notable (e). Comb group (f, g, h); showing focal pale acidophilic degenerated myocytes (arrow), wavy myofibers (W) with obvious vacuolation (V) (f). Other fields show areas of eosinophilic hyaline degeneration (arrow) in between the wavy myofibers (W), with vacuolated myocytes (V), dilated congested blood vessel (BV), and some inflammatory cellular infiltrates (circle) (g). Also, disorganized cardiac muscle fibers (light arrow) with pale acidophilic degenerated myocytes (broad arrow), wavy myofibers (W), and widened endomysium (star) are detected (h). [H&E, Scale bar= 50 µm]. (i) Number of cardiomyocytes nuclei/ high power field in H& E- stained sections in all studied groups. Data are presented as mean values (number of rats = 30)***

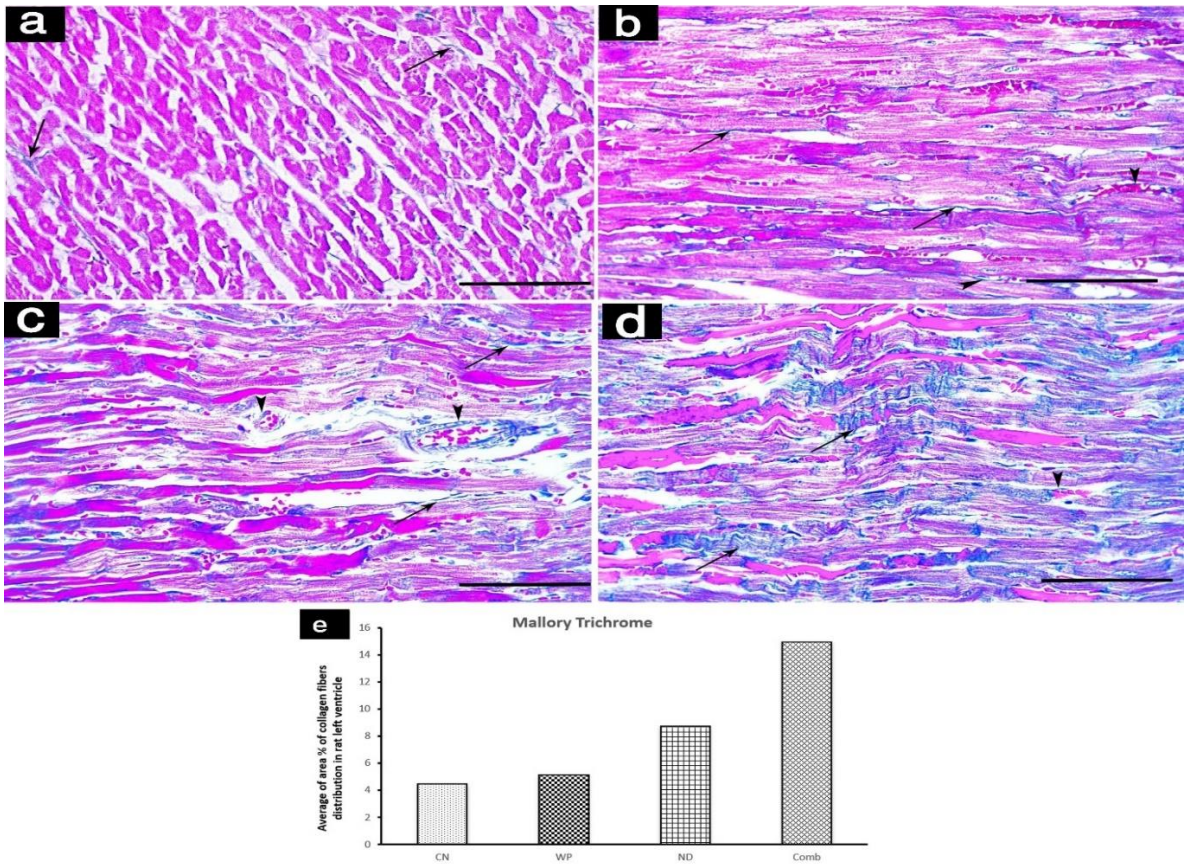

Fig. 3 ***Mallory trichrome stained section of the left ventricle: (a) In the control group, there are few small collagen fibers between cardiomyocytes (arrow) (b) Thin collagen fibers are notable in the interstitial between cardiomyocytes (arrow) and around blood vessels (arrowhead) regarding WP group (c) The ND group shows increased collagen fibers between the cardiomyocytes(arrow) and around blood vessels (arrowhead) (d) Marked increase in collagen deposition between the cardiomyocytes(arrow) and around blood vessels (arrowhead) are also seen concerning the Comb group. [Mallory trichrome, Scale bar= 50 µm]. (e) The area % of collagen distribution in Mallory trichrome-stained sections for all groups investigated. Results are provided as mean values (number of rats = 30)***

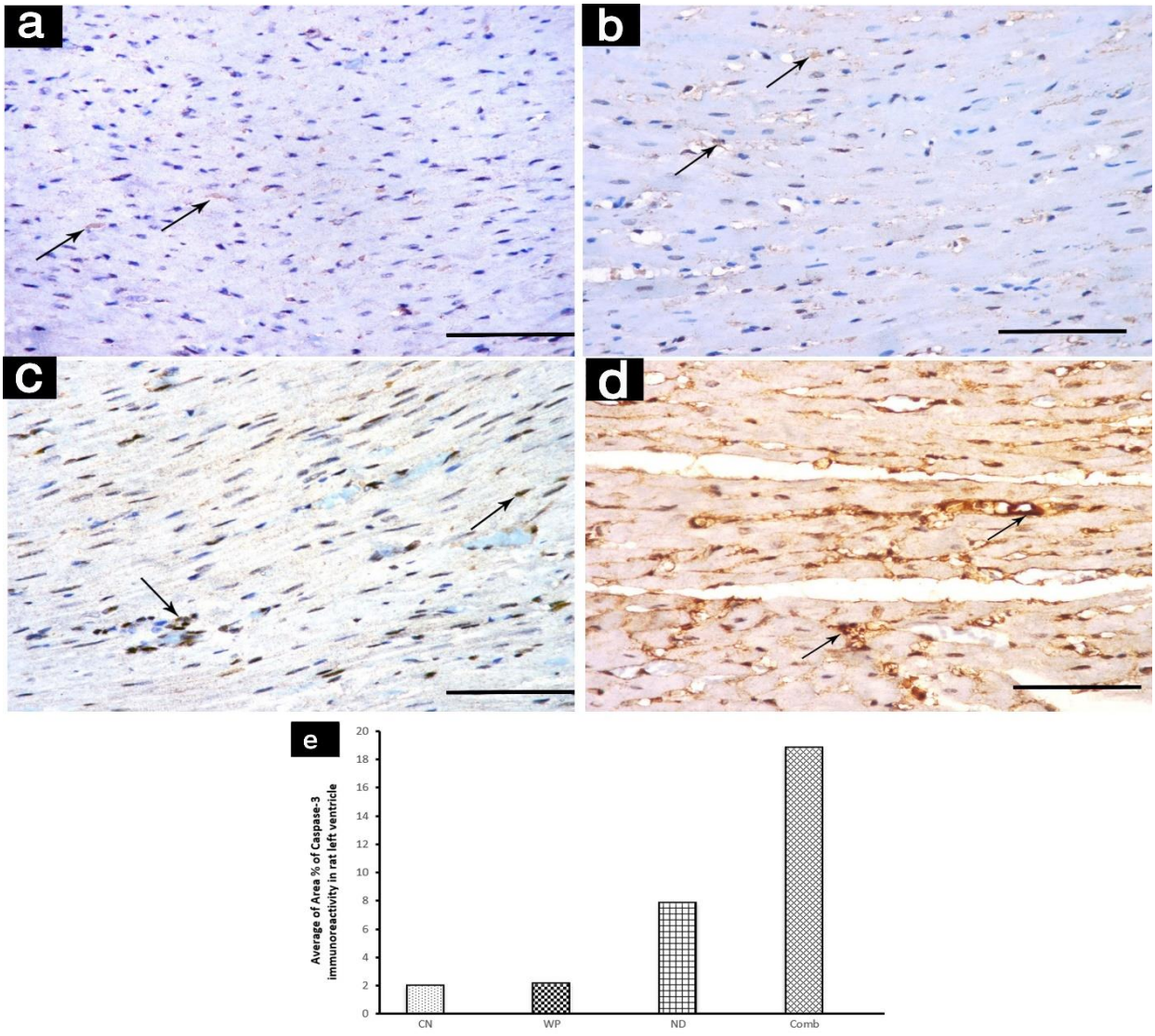

Fig. 4 Caspase-3-stained section of the left ventricle: (a) control group: showing a small grade of caspase-3 expression (arrow). (b) The WP group shows minimal caspase expression(arrow). (c) the ND group reflects a moderate degree of caspase expression (arrow). While in the Comb group, there are extensive caspase- 3 expressions in the left ventricular fields(arrow) (d). [Immune peroxidase, Scale bar = 50µm]. (e): Area % of caspase- 3 immunoreactivities in all the study groups. Data are presented as mean values (number of rats = 30)

Discussion

The nandrolone decanoate (ND) is one of the most used AAS by bodybuilders worldwide as a doping agent to improve muscle performance or physical appearance. Typically, Individuals take doses 10 to 100 times higher than the therapeutic dose, up to 700 mg/week for an average 80 kg man, for extended periods to enhance muscle mass; this abuse can have many adverse consequences**(15, 20)**

The whey protein (WP) has become the most prominent protein supplement amongst athletes and anyone who engages in physical activity, because it provides high percentages of energy during exercise and significantly improving, athletic performance**(5)**. Witard et al. (**41**), showed that a dosage exceeding 40 g/day may be associated with harmful consequences in humans. Athletes typically consume 1-2 scoops of WP daily, equivalent to 30-60 g protein/day (**42**).

The results indicated that the ND group gained less weight compared to the WP and the control

groups. A study by Pereira-Junior et al.(**43**) found that after 4 weeks of nandrolone decanoate there was an evident weight gain reduction than control group, and this could be attributed to decrease in food intake or fat and lean body masses loss (**44**).

Regarding heart weight and heart/body weight ratio, rats of the ND and the Comb groups showed a significant increase in comparison to the control and the WP rats, which was supported by our morpho histometric results revealing the considerable decrease in cardiomyocytes nuclei/ high power fields, indicating cardiac muscle hypertrophy at both physiological and histological levels.

Firstly, among the mechanisms responsible for cardiac hypertrophy following the ND treatment is an increase in myocardial collagen content (**45**). Secondly, Ganesan et al. (**46**) found that ND promotes nitrogen retention and amino acid reserve in the muscles, thereby encouraging an anabolic state. Thirdly, under the influence of ND, sarcomeres were added, leading to an increase in the volume of cardiomyocytes (**47, 48)**.

Other underlying mechanism of AAS was described in the literature that reinforces cardiac hypertrophy is a shift in the expression of the alpha and beta-myosin heavy chain (α-MHC/β-MHC) isoforms caused by protracted nandrolone treatment, with or without intensive exercise. This is demonstrated by an increase in α-MHC mRNA and the ratio of α-MHC mRNA/β-MHC mRNA expression, as well as an elevation in monoamine oxidase (MAO) and calcium/calmodulin-dependent protein kinase II-activities (CaMKII-) in cardiac tissue (**49**).

Franquni et al. (**22**) showed that myocyte hypertrophy and cardiac remodeling (related to ND) are related to the augmentation of Angiotensin Converting Enzyme (ACE) activity and the development of a pro-inflammatory state in animals treated with ND for 4 weeks.

In the current research, the final body weight of WP rats was higher than the control group. Similarly, several researchers found that when female chickens were fed on dried WP for 42 days, their body weight increased and their water consumption increased due to the high protein content **(50, 51**). Moreover, HIV-infected patients acquired weight more rapidly after WP administration (**52**).

Heart weight and heart-to-body weight ratio did not differ significantly between the WP and control groups. The present results are in constant with Lollo et al. and Helal et al. (**53, 54)** as they reported that absolute and relative heart weights did not change significantly in rats fed WPs + 6% leucine and 10% WP, respectively. Moreover, Lollo et al. (**53**) explained why the heart mass remains constant although increased anabolic pathway activation in WP intake, as long-term activation of protein synthesis pathways in healthy animals might result in activation of catabolic pathways like the ubiquitin-proteasome as a form of negative feedback.

In contrast, authors discovered that individuals who consumed 35% isolated WP of the total caloric content for 6 weeks had a greater increase in cardiac mass than those who consumed 14% WP (**55**). These varied results may be attributed to different diet styles. Also, they hypothesized that a high protein diet increased muscle protein synthesis and promoted muscle hypertrophy, which was supported by their findings.

In the WP group, the number of cardiomyocytes nuclei showed a non-significant difference when compared to the control group. The current results agree with Chen et al. (**56**), who found that neither skeletal nor cardiac myocyte hypertrophy in response to WP. Moreover, the WP could cause muscle fiber enlargement but combine it with tension, and exercise training, which differs in promoting muscle remodeling.

In comparison to the WP and control groups, all serum lipids parameters in the ND and Comb-treated groups deteriorated significantly. Tofighi et al. (**57**) reported outcomes similar to the present findings, but TG levels were non-significantly changed. This may be due to dosage, administration method (such as IM), AAS type (metabolizable or not), or species differences. To explain current observations, Balgoma et al. (**58**) also discovered the formation of sphingolipids and glycerolipids as a result of the AAS-induced decrease of several intracellular proteins expression. Furthermore, androgen interactions may stimulate production of de novo fatty acids and cholesterol by enhancing the expression of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase and the LDL receptor (**58**). Apo-lipoprotein B (ApoB), the precursor of LDL and VLDL, appeared to rise after a single dose of ND, and this effect persisted two weeks later (**16**).

Regarding lipid profile investigation, the WP group had a non-significant difference in all lipid profile mean values compared to the control group. These results are in harmony with Pal et al. (**59**), who stated that a single oral dose of 45 g of WP isolates had no effect on TC, LDL-C, and HDL-C in obese women. However, they noticed that TG levels were significantly decreased, which may be attributed to some factors, such as training and caloric restriction.

The nandrolone decanoate (ND) group exhibited increased oxidative stress in the form of elevated MDA and decreased SOD and GPx. The outcomes were consistent with those of Riezzo et al. (**60**), who described that intramuscular injection of ND at concentrations of 1.875 and 5 mg /kg for 42 days, twice a week, exhibited elevated MDA levels and reduced antioxidant defense. It can be clarified that ND alters the physiological redox state's homeostasis and encourages ROS **(61**).

In the present work, the WP-treated rats induced oxidative stress after a short period. Moreover, many inflammatory cytokines, including Interleukin 1,6 (IL-1,6) and TNF-α, were elevated in the liver after four weeks of the WP administration at a dose of 252 g/kg without practice (**7**). Inflammation also contributes to oxidative stress by impairing antioxidant defenses and increasing TNF- and TGF- levels (**62**). Under the effect of ROS, chemokine up-regulation, leukocyte integrin stimulation, and surface adhesion molecules attract migrating inflammatory cytokines and fibroblast precursors to endothelial cells (**63**). On the contrary, Brown et al. **(64**) indicated that when males took 33 g/day of WP equivalent to one scoop, WP had no effect on radical scavenging capacity. This difference may be attributed to variations in species (human), smaller doses, and training activities.

Our research aligned with those of Camiletti-Moirón (**65**), who emphasized that anabolic androgenic steroids combined with a high-protein diet damage renal function and the central nervous system by oxidizing protein and lipids. However, oxidative deoxyribonucleic acid (DNA) or chromosomal aberrations were unaffected by the use of AAS or protein powders; these conditions may be responsible for compensatory mechanisms in highly active people, such as enhanced expression of DNA repair processes and improvement of antioxidant potential, as suggested by Shafi et al. (**66**).

The treated groups had higher LDH and CK-MB levels compared to the control group. Numerous cardiac enzymes, including CK-MB and LDH, are found in the myocardium. In the WP, lipid peroxidation caused an increase in these cardiac cardiotoxicity enzymes (**67**), so their elevation can be used to diagnose myocardial injury. Similar findings were reported by Tousson et al. (**68**), who stated that AAS increased NADPH oxidases, thereby increasing oxidative stress and cardiac damage. In addition, Aydın et al. **(69**) demonstrated that co-treatment with acrolein (hazardous substance has the same specific toxicological mechanism as ND) and WP had a negative influence on myocardial injury markers, such as CK-MB and LDH.

In current experiment, microscopic examination of the left ventricular specimens from rats of the ND

group revealed the following histopathological changes: disarrayed muscle fibers with areas of hyalinization, wavy myofibers, multiple dark flat nuclei of fibroblasts, areas of moderate inflammatory cells in addition to separated muscle fibers, and dilated congested blood vessels. Germanakis et al. (**70**) connected these histopathological effects in the heart of rabbits after AAS (1g/kg/day) intake to the ND-induced changes in local myocardial oxidative stress response. Some researchers emphasized that these cardiac myopathy alterations are associated with dyslipidemia, corresponding with enhanced NF-κB and hypoxic-ischemic factor (HIF-1) production **(71, 72**).

Regarding the left ventricle of the WP group, it displayed undulating muscle fibers and vacuolated myocytes. Other sections revealed some areas of separated muscle fibers, enlarged endomysium, and mild inflammatory cell infiltration. Gürgen et al. **(7**) observed inflammatory markers and hepatotoxicity in rats that consumed 252 g/kg of WP for 4 weeks. Additionally, Mustafa et al. **(73**) reported an expansion of renal tubules and a widening space around glomeruli with glomerular segmentation after 30 days of 1 and 2 g/kg WP consumption. These renal changes mimic our findings in the cardiac muscle, which include a widened endomysium and separation of muscle fibers.

In the left ventricle sections of ND treated rats, deposition of collagen fibers increased significantly, accompanied by moderate caspase expression. In investigations of athletes who abused ND, areas of cardiac fibrosis were identified as the most characteristic pathological change **(74**). Caspase induces apoptosis via aspartate-directed cysteine-dependent proteases. Proteases break the cytoplasmic and nuclear structural components of cardiac cells. Apoptosis of myocytes has been demonstrated as an incomplete process (**75**). In the absence of nuclear fragmentation, there is a possibility of continuous cytoplasmic protein loss allowing cardiomyocytes to survive for prolonged periods. Actin I and II are proteins that are vulnerable to early loss **(76**).

Concerning the Comb group, microscopic examination of the left ventricle specimens revealed the presence of acidophilic, degenerated myocytes with prominent vacuoles. Some sections showed areas of eosinophilic hyaline degeneration between the undulating myofibers, along with dilated congested blood vessels, extensive inflammatory cellular infiltrates, disorganized cardiac muscle fibers, and widened endomysium in addition to an extensive heavy expression of caspase-3 in cardiac muscle fibers.

Some bodybuilders who consumed WP supplements with ND, suffered from an acute renal injury. Their kidney biopsies revealed interstitial fibrosis, inflammatory lymphocytic infiltrations, and acute tubular necrosis (**77**).

Conclusions and Recommendations

To conclude, as displayed by histopathology, albino male rats exposed to short-term supraphysiological doses of ND and WP resulted in structural changes in their cardiac muscle, including inflammation, fibrosis, and apoptosis. Besides dyslipidemia, an unbalanced redox system and increased oxidative stress were also present. Under these conditions, the oxidant chemical MDA was elevated, and the antioxidant defenses SOD and GPx were depleted. Furthermore, the Comb group had the highest levels of histological structural modifications and biochemical disturbances of all other experimental rats; therefore, we believe athletes and bodybuilders need to make better choices about their cardiac health:

1. Athletes should be cautious when taking AAS along with a high-protein diet like WP, particularly if not under the supervision of a physician.
2. It is advised to take other substitutes that are available as plant-based foods with fewer calories than animal sources to meet daily protein needs, like soybeans and quinoa.
3. Instead of synthetic anabolic androgenic steroids, athletes can depend on natural plant sources or

vitamins with anabolic properties for building muscles and recovering fast after training, like Ashwagandha, Ginseng, Zinc, Magnesium, and Vitamin D.

Declarations

I. Funding: No funding was received for this work
"Not applicable"

II. Conflicts of Interest: There are no conflicts of interest for all authors.
"Not applicable"

III. Availability of data and material: This published article (and its additional information files) contains all data produced or analyzed during this investigation. The corresponding author will provide the datasets used and/or analyzed during the current work upon reasonable request.

IV. Ethics approval: The research was conducted in compliance with the Zagazig University Ethics Committee for Animal Handling's rules at the Faculty of Medicine's Animal House at the university, Egypt, with authorization number (ZU-IACUC/3/F/87/2021).

V. Consent to participate:
"Not applicable"

VI. Consent for publication:
I attest that all authors have agreed to submit the work.

VII. Code availability:
"Not applicable"

VIII. Author contributions:
Ghada E. Elmesallamy contributed to the design of the work. Nadia El-Akabawy contributed to the analysis and interpretation of histopathological data for the work. Hadir Adel A. Albaz drafted the first version of the manuscript. Nadia El-Akabawy and Marwa T. Abaza revised and provided feedback on the initial draught for important intellectual content. Hadir Adel A. Albaz worked on the version, following feedback, until a final version was developed. Ghada E. Elmesallamy approved the final version for publishing. Hadir Adel A. Albaz, Nadia El-Akabawy, Marwa T. Abaza, and Ghada E. Elmesallamy agreed to be accountable for all aspects of the work, ensuring that the accuracy or integrity of any part of the work is appropriate. The final draught was reviewed by all authors who were involved until the final manuscript was submitted.

References

1. Deeth HC, Bansal N. Whey proteins: from milk to medicine: Academic Press; 2018.
2. Torre-Villalvazo I, Alemán-Escondrillas G, Valle-Ríos R, Noriega LG. (2019). Protein intake and amino acid supplementation regulate exercise recovery and performance through the modulation of mTOR, AMPK, FGF21, and immunity. Nutr Res.72:1-17.
3. Camera DM. (2022).Evaluating the Effects of Increased Protein Intake on Muscle Strength, Hypertrophy and Power Adaptations with Concurrent Training: A Narrative Review. Sports Med.52(3):441-61.
4. Gao R, Rapin N, Elnajmi AM, Gordon J, Zello GA, Chilibeck PD. (2020). Skim milk as a recovery beverage after exercise is superior to a sports drink for reducing next-day postprandial blood glucose and increasing postprandial fat oxidation. Nutr Res.82:58-66.
5. Vasconcelos QDJS, Bachur TPR, Aragão GF. (2021). Whey protein supplementation and its potentially adverse effects on health: a systematic review. Appl Physiol Nutr Metab. 46(1):27-33.
6. Martin WF, Armstrong LE, Rodriguez NR. Dietary protein intake and renal function. In: Coles L, editor. Clin Nutr Interface Between Metab Diet, Dis. NEW JERSY: Apple Academic Press; 2013.

7. Gurgen SG, Yucel AT, Karakus AC, Cecen D, Ozen G, Kocturk S. (2015). Usage of whey protein may cause liver damage via inflammatory and apoptotic responses. Hum Exp Toxicol.;34(7):769-79.
8. Aparicio V, Nebot E, Porres J, Ortega F, Heredia J, Lopez-Jurado M, et al. (2011). Effects of high-whey-protein intake and resistance training on renal, bone and metabolic parameters in rats. Br J Nutr.105:836-45.
9. Melnik BC. (2009). Milk–the promoter of chronic Western diseases. Medical Hypotheses.72(6):631-9.
10. Melnik BC. Evidence for Acne-Promoting Effects of Milk and Other Insulinotropic Dairy Products. In: Clemens RA HO, Michaelsen KF (eds), editor. Milk and Milk Products in Human Nutrition Nestlé Nutr Inst Workshop Ser Pediatr Program. 67: Nestec Ltd., Vevey/S. Karger AG, Basel,; 2011. p. pp 131–45.
11. Zhang H, Reilly MP. (2020). Novel mechanistic links between high-protein diets and atherosclerosis. Nature Metab.2(1):7-8.
12. Moore KJ, Koplev S, Fisher EA, Tabas I, Bjorkegren JLM, Doran AC, et al. (2018). Macrophage Trafficking, Inflammatory Resolution, and Genomics in Atherosclerosis: JACC Macrophage in CVD Series (Part 2). J Am Coll Cardiol.72(18):2181-97.
13. Zhubi-Bakija F, Bajraktari G, Bytyci I, Mikhailidis DP, Henein MY, Latkovskis G, et al. (2021). The impact of type of dietary protein, animal versus vegetable, in modifying cardiometabolic risk factors: A position paper from the International Lipid Expert Panel (ILEP). Clin Nutr. 40(1):255-76.
14. Sretenovic J, Zivkovic V, Srejovic I, Milosavljevic Z. (2016).The effects of high doses of nandrolone decanoate on cardiac muscle tissue. Life.
15. Vasilaki F, Tsitsimpikou C, Tsarouhas K, Germanakis I, Tzardi M, Kavvalakis M, et al. (2016). Cardiotoxicity in rabbits after long-term nandrolone decanoate administration. Toxicol Lett.241:143-51.
16. Patane FG, Liberto A, Maria Maglitto AN, Malandrino P, Esposito M, Amico F, et al. (2020). Nandrolone Decanoate: Use, Abuse and Side Effects. Medicina (Kaunas).56(11):606.
17. Geusens P. (1995). Nandrolone decanoate: pharmacological properties and therapeutic use in osteoporosis. Clin Rheumatol.14 Suppl 3(3):32-9.
18. Vieira RP, Franca RF, Damaceno-Rodrigues NR, Dolhnikoff M, Caldini EG, Carvalho CR, et al. (2008). Dose-dependent hepatic response to subchronic administration of nandrolone decanoate. Med Sci Sports Exerc.40(5):842-7.
19. Erdal T, Seyfullah H. (2013). The administration of nandrolone decanoate may cause multiple organ failure. Ovidius University Annals, Series Physical Education & Sport/Science, Movement & Health.13.
20. Frankenfeld SP, de Oliveira LP, Ignacio DL, Coelho RG, Mattos MN, Ferreira AC, et al. (2014). Nandrolone decanoate inhibits gluconeogenesis and decreases fasting glucose in Wistar male rats. J Endocrinol.220(2):143-53.
21. Ayubi N, Kusnanik NW, Herawati L, Komaini A, Cholik T, Syafawi A. (2023). Abuse of Anabolic-Androgenic Steroids and Adverse Effects on Human Organ Health: A Review. Biointerface Res Appl Chem.;13(3):1-11.

22. Franquni JV, do Nascimento AM, de Lima EM, Brasil GA, Heringer OA, Cassaro KO, et al. (2013). Nandrolone decanoate determines cardiac remodelling and injury by an imbalance in cardiac inflammatory cytokines and ACE activity, blunting of the Bezold-Jarisch reflex, resulting in the development of hypertension. Steroids.;78(3):379-85.
23. Amah AK, Makena W, Ezekwe AS, Ejiofor DC, Nwanegwo C, Ewa O. (2019). Hematological evaluation of aqueous and methanolic leaf extracts of Thaumatococcus daniellii and Alchornea cordifolia in Wistar rats. GSC Biological and Pharmaceutical Sciences.;8(1):123-7.
24. Ibrahim B, Mehmet O, Kadir P, Ozgur O, Alparslan C. (2011). Morphometric evaluation of the effect of methenolone enanthate on humeral development in adolescent rat. Sci Res Essays.;6(13):2676-81.
25. Paget G, Barnes J. Toxicity tests. In: D R Laurence ALB, editor. Evaluation of drug activities: pharmacometrics. 1: New York; 1964. p. 135-65.
26. Parasuraman S, Raveendran R, Kesavan R. (2010). Blood sample collection in small laboratory animals. J pharmacol pharmacother.;1(2):87-93.
27. Wieland H, Seidel D. (1983). A simple specific method for precipitation of low density lipoproteins. J Lipid Res.;24(7):904-9.
28. Friedewald WT, Levy RI, Fredrickson DS. (1972). Estimation of the Concentration of Low-Density Lipoprotein Cholesterol in Plasma, Without Use of the Preparative Ultracentrifuge. Clin Chem.;18(6):499-502.
29. Meiattini F, Prencipe L, Bardelli F, Giannini G, Tarli P. (1978). The 4-hydroxybenzoate/4-aminophenazone chromogenic system used in the enzymic determination of serum cholesterol. Clin Chem.;24(12):2161-5.
30. Bucolo G, David H. (1973). Quantitative determination of serum triglycerides by the use of enzymes. Clin Chem.;19(5):476-82.
31. Grove TH. (1979). Effect of reagent pH on determination of high-density lipoprotein cholesterol by precipitation with sodium phosphotungstate-magnesium. Clin Chem.;25(4):560-4.
32. Armstrong D, Browne R. (1994). The Analysis of Free Radicals, Lipid Peroxides, Antioxidant Enzymes and Compounds Related to Oxidative Stress as Applied to the Clinical Chemistry Laboratory. In: Armstrong D, editor. Free Radicals in Diagnostic Medicine: A Systems Approach to Laboratory Technology, Clinical Correlations, and Antioxidant Therapy. Boston, MA: Springer US;. p. 43-58.
33. Adachi T, Ohta H, Yamada H, Futenma A, Kato K, Hirano K. (1992). Quantitative analysis of extracellular-superoxide dismutase in serum and urine by ELISA with monoclonal antibody. Clin Chim Acta.;212(3):89-102.
34. McMurray CH, Davidson WB, Ball HJ, Todd D. (1986). Characterisation of an antiserum and development of an ELISA for glutathione peroxidase. Vet Res Commun.;10(4):269-81.
35. Baba N, Sharma HM. (1971). Histochemistry of lactic dehydrogenase in heart and pectoralis muscles of rat. J cell biol.;51(3):621-35.
36. Lott JA, Abbott LB. (1986). Creatine kinase isoenzymes. Clin Lab Med.;6(3):547-76.

37. Gerhardt W, Ljungdahl L, Herbert A-K. (1992). Troponin-T and CK MB (mass) in early diagnosis of ischemic myocardial injury. The Helsingborg Study,. Clin Biochem. 1993;26(4):231-40.
38. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. 5th ed. ed. Philadelphia: Churchill Livingstone., ; 2002.
39. Janardhan KS, Jensen H, Clayton NP, Herbert RA. (2018). Immunohistochemistry in investigative and toxicologic pathology. Toxicol pathol.;46(5):488-510.
40. Morgan GA, Barrett KC, Leech NL, Gloeckner GW. IBM SPSS for introductory statistics: Use and interpretation: Routledge; 2019.
41. Witard OC, Jackman SR, Breen L, Smith K, Selby A, Tipton KD. (2014). Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. Am J clin nutr.;99(1):86-95.
42. Alhakbany MA, Alzamil HA, Alnazzawi E, Alhenaki G, Alzahrani R, Almughaiseeb A, et al. (2022). Knowledge, Attitudes, and Use of Protein Supplements among Saudi Adults: Gender Differences. Healthcare.;10(2):394.
43. Pereira-Junior PP, Chaves EA, Costa ESRH, Masuda MO, de Carvalho AC, Nascimento JH. (2006). Cardiac autonomic dysfunction in rats chronically treated with anabolic steroid. Eur J Appl Physiol.;96(5):487-94.
44. Binayi F, Joukar S, Najafipour H, Karimi A, Abdollahi F, Masumi Y. (2016), The Effects of Nandrolone Decanoate Along with Prolonged Low-Intensity Exercise on Susceptibility to Ventricular Arrhythmias. Cardiovasc Toxicol.;16(1):23-33.
45. Tanno AP, das Neves VJ, Rosa KT, Cunha TS, Giordano FC, Calil CM, et al. (2011). Nandrolone and resistance training induce heart remodeling: role of fetal genes and implications for cardiac pathophysiology. Life Sci.;89(17-18):631-7.
46. Ganesan K, Rahman S, Zito PM. Anabolic Steroids: StatPearls Publishing, Treasure Island (FL); 2022 2022.
47. Mata EFD, Nascimento AMd, Lima EMd, Kalil IC, Endringer DC, Lenz D, et al. (2020). Finasteride promotes worsening of the cardiac deleterious effects of nandrolone decanoate and protects against genotoxic and cytotoxic damage. Braz J Pharmaceut Sci.;56.
48. Sretenovic J, Zivkovic V, Srejovic I, Pantovic S, Jovic JJ, Nikolic M, et al. (2022). Nandrolone Decanoate and Swimming Affects Cardiodynamic and Morphometric Parameters in the Isolated Rat Heart. Life (Basel).;12(8):1242.
49. Shirpoor A, Heshmatian B, Tofighi A, Eliasabad SN, Kheradmand F, Zerehpoosh M. (2019). Nandrolone administration with or without strenuous exercise increases cardiac fatal genes overexpression, calcium/calmodulin-dependent protein kinaseiidelta, and monoamine oxidase activities and enhances blood pressure in adult wistar rats. Gene.;697:131-7.
50. Shariatmadari F, Forbes JM. (2005). Performance of broiler chickens given whey in the food and/or drinking water. Br Poult Sci.;46(4):498-505.

51. Kheiri F, Rahimian Y, Nasr J. (2015). Application of sumac and dried whey in female broiler feed. Arch Animal Breeding.;58(1):205-10.
52. Kumar R, Chauhan SK, Shinde G, Subramanian V, Nadanasabapathi S. (2018). Whey Proteins: A potential ingredient for food industry-A review. Asian J Dairy Food Res.;37(4):283-90.
53. Lollo PCB, Batista TM, Moura CS, Morato PN, Cruz AG, Faria JAF, et al. (2013). l-Leucine supplemented whey protein. Dose–response effect on heart mTOR activation of sedentary and trained rats. Food Res Int.;53(1):543-50.
54. Helal HM, Samy WM, El-Fakharany EM, Kamoun EA, Mortada SM, Sallam MA. (2020). Maltodextrin-α-tocopherol conjugates of vitamin E: Influence of degree of derivatization on physicochemical properties and biological evaluation. J Drug Deliv Sci Technol.;60:102097.
55. PC DEA, T DARL, Avila ETP, Tolazzi GJ, Cavalcante LP, Navalta JW, et al. (2020). High-Protein Diet Associated with Resistance Training Improves Performance and Decreases Adipose Index in Rats. Int J Exerc Sci.;13(2):1366-81.
56. Chen WC, Huang WC, Chiu CC, Chang YK, Huang CC. (2014). Whey protein improves exercise performance and biochemical profiles in trained mice. Med Sci Sports Exerc.;46(8):1517-24.
57. Tofighi A, Shirpoor M, Ansari MHK, Shirpoor A, Zerehpoosh M. (2017). The effect of nandrolone treatment with and without enforced swimming on histological and biochemical changes in the heart and coronary artery of male rats. Anatol J Cardiol.;17(3):176-83.
58. Balgoma D, Zelleroth S, Grönbladh A, Hallberg M, Pettersson C, Hedeland M. (2020). Anabolic androgenic steroids exert a selective remodeling of the plasma lipidome that mirrors the decrease of the de novo lipogenesis in the liver. Metabolomics.;16(1):1-13.
59. Pal S, Ellis V, Ho S. (2010). Acute effects of whey protein isolate on cardiovascular risk factors in overweight, post-menopausal women. Atherosclerosis.;212(1):339-44.
60. Riezzo I, Turillazzi E, Bello S, Cantatore S, Cerretani D, Di Paolo M, et al. (2014). Chronic nandrolone administration promotes oxidative stress, induction of pro-inflammatory cytokine and TNF-alpha mediated apoptosis in the kidneys of CD1 treated mice. Toxicol Appl Pharmacol.;280(1):97-106.
61. Sretenovic J, Joksimovic Jovic J, Srejovic I, Zivkovic V, Mihajlovic K, Labudovic-Borovic M, et al. (2021). Morphometric analysis and redox state of the testicles in nandrolone decanoate and swimming treated adult male rats. Basic Clin Androl.;31(1):17.
62. Aimo A, Castiglione V, Borrelli C, Saccaro LF, Franzini M, Masi S, et al. (2020). Oxidative stress and inflammation in the evolution of heart failure: From pathophysiology to therapeutic strategies. Eur J Prev Cardiol.;27(5):494-510.
63. Frangogiannis NG. (2014). The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol.;11(5):255-65.
64. Brown EC, DiSilvestro RA, Babaknia A, Devor ST. (2004). Soy versus whey protein bars: effects on exercise training impact on lean body mass and antioxidant status. Nutr J.;3(1):22.

65. Camiletti-Moirón D. (2016). Consequences of high-protein diets consumption, anabolic androgenic steroids administration and high-intesity exercise on brain and kidney oxidative stress markers in wistar rats..
66. Shafi FAA, Jabbar EAK, Yousif RM, Lafta FM. (2020); Effect of exercise, synthetic anabolic steroids and protein intake on DNA damage in trained and untrained men. Meta Gene.;24:100685.
67. Saka W, Kehinde B, Akintola A. (2022). Cardiotoxic effects of car paint fumes exposure on cardiac tissues of male wistar rats. J Bio Med Open Access.;3(2):126.
68. Tousson E, Elgharabawy RM, Elmasry TA. (2018). Grape Seed Proanthocyanidin Ameliorates Cardiac Toxicity Induced by Boldenone Undecylenate through Inhibition of NADPH Oxidase and Reduction in the Expression of NOX2 and NOX4. Oxid Med Cell Longev.;2018:9434385.
69. Aydin B, Atli Sekeroglu Z, Sekeroglu V. (2018). Effects of whey protein and conjugated linoleic acid on acrolein-induced cardiac oxidative stress, mitochondrial dysfunction and dyslipidemia in rats. Biomed Pharmacother.;107:901-7.
70. Germanakis I, Tsarouhas K, Fragkiadaki P, Tsitsimpikou C, Goutzourelas N, Champsas MC, et al. (2013). Oxidative stress and myocardial dysfunction in young rabbits after short term anabolic steroids administration. Food Chem Toxicol.;61:101-5.
71. Udomkasemsab A, Prangthip P. (2019). High fat diet for induced dyslipidemia and cardiac pathological alterations in Wistar rats compared to Sprague Dawley rats. Clínica e Investigación en Arteriosclerosis.;31(2):56-62.
72. Soliman GF, Abdel-Maksoud OM, Khalifa MM, Rashed LA, Ibrahim W, Morsi H, et al. (2022). Effect of nebivolol on altered skeletal and cardiac muscles induced by dyslipidemia in rats: impact on oxidative and inflammatory machineries. Arch Physiol Biochem.;128(2):463-73.
73. Mustafa MN, Shaban RK, abdalkader Mahdy S. (2020). EFFECT OF WHEY PROTEIN IN THE DEVELOPMENT OF HISTOLOGICAL CHANGES IN THE LIVER AND KIDNEYS OF MALE WHITE RATS. Kidney.;3:4.
74. Torrisi M, Pennisi G, Russo I, Amico F, Esposito M, Liberto A, et al. (2020). Sudden Cardiac Death in Anabolic-Androgenic Steroid Users: A Literature Review. Medicina (Kaunas).;56(11):587.
75. Bouche L, Kamel R, Tamareille S, Garcia G, Villedieu C, Pillot B, et al. (2021). DRP1 haploinsufficiency attenuates cardiac ischemia/reperfusion injuries. PloS One.;16(3):e0248554.
76. Bouitbir J, Alshaikhali A, Panajatovic MV, Abegg VF, Paech F, Krähenbühl S. (2019). Mitochondrial oxidative stress plays a critical role in the cardiotoxicity of sunitinib: Running title: Sunitinib and oxidative stress in hearts. Toxicology.;426:152281.
77. Davani-Davari D, Karimzadeh I, Khalili H. (2019). The potential effects of anabolic-androgenic steroids and growth hormone as commonly used sport supplements on the kidney: a systematic review. BMC Nephrol.;20(1):198.