WHEY PROTEIN AND ANABOLIC STEROIDS: CURRENT TREND AND FUTURE POTENTIAL EFFECT ON THE HEART: BIOCHEMICAL, HISTOLOGICAL, AND IMMUNOHISTOCHEMICAL STUDY

BY

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ABSTRACT

Background: Whey protein (WP) is considered an ergogenic aid for athletes to improve muscle performance and recovery after sports. Because of its anabolic properties and potential to improve exercise tolerance, Nandrolone Decanoate (ND) is one of the most often abused anabolic steroids among bodybuilders worldwide. **Objective**: This study aimed to evaluate the possible toxic effects of WP and ND on the heart of adult male albino rats. **Methods**: Thirty mature male albino rats were separated into four main groups; Control Group: (15 rats) were classified into three equal subgroups: control negative (CN): fed a regular diet and tap water. Control distilled water (CDW): received 2ml/kg/day of distilled water orally. Control peanut oil (CP): received intra-peritoneal peanut oil 0.5 ml/rat onceweekly. WP group: (5 rats) received WP orally at a dose of 5.4g/kg/day. Nandrolone decanoate group (ND): (5 rats) received ND intra-peritoneal at a dose of 36mg/kg once weekly. Combined group (Comb): (5 rats) received WP and ND at the same doses. All animals were euthanized after eight weeks, and blood samples were obtained for study. The heart was preserved for biochemical and histological examination. **Results**: WP and ND proved to have significant toxic effects on blood parameters and histopathological changes in the heart. **Conclusion**: These findings support that the heart is highly sensitive to WP and ND administration, and it is necessitated careful administration of dose and for such period under medical supervision for better health.

Keywords: *Whey protein supplement, Nandrolone decanoate, oxidative stress, cardiotoxicity.*

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INTRODUCTION

Whey protein (WP) supplement is a high-quality protein powder derived from cow's milk. Milk has two proteins: whey, which makes up about 20% of the overall protein composition, and the rest 80% casein ***(Sharma et al., 2022).*** WPs are a collection of different main proteins; β-lactoglobulin (β-Lg), α-lactalbumin (α-La), and several minor fractions extracted from milk during cheese production ***(Gangurde et al., 2011).***

Whey Protein Concentrate (WPC), Whey Protein Hydrolysate (WPH), and Whey Protein Isolate (WPI) are the most prevalent forms of WP (***Sakkas et al., 2022***). ***Rani and Kumar (2022)*** said that WPC is considered the lowest-quality option due to its restricted refinement process. It has a 70–80% protein concentration, with fat, cholesterol, and lactose. This contributes to a higher caloric content, which can be detrimental to bodybuilders searching for a well nutritional plan. On the other hand, a WPI is the purest form. It is treated to eliminate lactose and fat and contains >90% protein. This modified process makes WPI the de-facto protein powder source for most athletes due to a lower caloric content than most other options. In addition, a decreased amount of carbohydrates makes WPI relatively low in lactose, which can be a life-preserver for people suffering from allergic responses ***(Sharma et al., 2022).*** Finally, WPH is quickly absorbed and easily tolerated by most athletes, although its cost is often higher ***(Gangurde et al., 2011).***

WP has several health benefits, especially for athletes. WP is a complete protein since it includes all of the essential branched-chain amino acids (BCAAs), especially leucine

necessary for a healthy body ***(Gorissen et al., 2018).*** Leucine enhances the body's muscle protein production &growth by generating anabolic hormones, such as insulin. So, the high leucine content participates in WP's muscle-building efficacy and strength ***(Volpi et al., 2013)***. For these benefits, WP is commonly utilized by athletes in the form of protein bars, drinks and dietary supplements ***(Agin et al., 2000)***.

Whey protien also has other benefits rather than a sport, like chronic fatigue syndrome ***(Krissansen, 2007),*** weight loss ***(Frestedt et al., 2008),*** an increase in immune power ***(Arnal et al., 1999)***, and HIV-associated muscle wasting; due to its anabolic action ***(Agin et al., 2000)***.

It is also used in cancer treatment and hepatitis; due to its cysteine content which encourages glutathione levels, a cellular antioxidant ***(Bounous, 2000)***.

Vasconcelos et al. (2021) 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.

The safety of WP is controversial; some claim that too much can damage the kidneys and liver and even cause osteoporosis even during a short period.

Knight et al. (2003) found that it has proven to incur the following effects: elevated serum plasma volume and calcium excretion while decreasing the PH of the citrate in the urine. Essentially, it increases the taxing of the kidneys, which is the first step in kidney disease ***(Martin et al., 2013)***.

Furthermore, it can place an unsupportable strain on the liver and may cause liver damage ***(Gürgen et al., 2015)***.

Recent studies have been reported to raise cardiovascular risk with high-protein consumption. ***Zhang and Reilly (2020)***, discovered that a high-protein diet raises blood levels of amino acids, macrophage apoptosis and insulin like growth factor-1(IGF-1) that has been incorporated in the promotion of atherosclerotic plaques. Plaque rupture with superimposed thrombosis is the leading cause of myocardial infarction, heart attack and sudden death ***(Moore et al., 2018)***.

Moreover ***Zhubi-Bakija et al. (2021)*** found the dairy products such as WP contains saturated fat and cholesterol that increase the risk of hyperlipidemia and hyper cholestrolemia as well as cardiovascular disease.

Anabolic Androgenic Steroids (AAS) are derivatives of synthetic testosterone were developed to create a testosterone-like steroid with more anabolic actions and less androgenic effects ***(Sretenovic et al., 2016),*** however being prohibited in many organized sports, AAS became widely popular among weightlifters, bodybuilders, swimmers, and bikers to increase performance, and strength, as well as for enhancing physical appeal and body image ***(Vasilaki et al., 2016***).

Nandrolone is a 19-nortestosterone derivate and is thought to have the highest anabolic-to-androgenic ratio of any AAS ***(Patanè et al., 2020).*** It has been applicable in medical practice, like osteoporosis in postmenopausal women, various forms of anemia, and acquired immunodeficiency syndrome (AIDS)-associated wasting syndrome ***(Geusens, 1995).*** Nevertheless, the misuse of these agents has escalated dramatically recently by athletes and non-athletes ***(Vieira et al., 2008).***

Erdal and Seyfullah (2013) and Frankenfeld et al. (2014) found that supraphysiological doses of Nandrolone decanoate (ND) have severely deleterious effects on multiorgan systems such as cardiovascular, endocrine, reproductive, metabolic, neurological and behavioral functions.

Ayubi et al., (2023) found that ND misuse raises the risk of cardiovascular disorders, due to its effects on blood pressure, cholesterol levels, lipidemic profile, oxidative stress, and fluid retention. Depending on doses and duration of use, chronic ND use contributes to coronary atherosclerosis. Also, supra physiological doses of ND would induce the impairment of left ventricular systolic, diastolic, and autonomic nervous function of heart leading to arrhythmia. According to both human and animal studies, ND-induced myopathy of the cardiac muscle is caused by increased myocardial fibrosis. Additionally,

hypertrophy of left ventricle has been observed in weight lifters consuming ND ***(Franquni et al., 2013; Vasilaki et al., 2016)***.To our knowledge, until now there are few studies concerning the toxicity of WP on cardiac muscle especially when combined with ND.

THE AIM OF THE WORK

The aim of the current study was to evaluate Whey protein and Nandrolone decanoate effect on lipid profile, oxidative stress parameters, and cardiotoxicity markers. Also, our research was conducted to investigate cardiac muscle histopathology and area percentage of fibrosis and apoptosis in heart tissue in sedentary rats for 8 weeks.

MATERIAL AND METHODS

I. Chemicals and kits

- WP in the shape of a pale-yellow powder was acquired from Optimum Nutrition, INC in the USA.
- ND 25 mg/ml in the form of a yellowish oily solution contained in a 1 ml glass ampoule was acquired from El-Nile Company for Pharmaceuticals and Chemical Industries.
- Peanut oil in the form of a yellowish-green solution was purchased from Biohayah Egypt Company.
- Rat Malondialdehyde (MDA) ELISA Kit (OKEH02548) from Aviva Systems Biology
- Rat Super Oxide Dismutase (SOD) ELISA kit (Cat. No.CSB-E08555r) from Cusabio Biotech Co., Ltd.
- Rat Glutathione Peroxidase (GPx) ELISA kit (Cat. No. CSB-E12146r) from Cusabio Biotech Co., Ltd.

II.Experimental Animals:

One week was spent acclimating 30 mature male albino rats (190±100 g) to their environment. They lived in polypropylene cages under laboratory lighting and temperature. Rats were given a conventional laboratory meal and had access to water for the duration of the experiment. The research was done at the Animal House of the Faculty of Medicine at Zagazig University, Egypt, in accordance with the guidelines of the Zagazig University Ethical Committee for Animal Handling, permission number (***ZU-IACUC/3/F/87/2021***).

3. Experimental groups

Four groups of rats were randomly selected:

- **Control Group:** (15 rats) were classified into three subgroups equally: **Control negative subgroup (CN):** each rat was left freely to eat a regular diet and drink tap water for measuring the fundamental values of performed tests. **Control distilled water subgroup (CDW):** each rat received 2ml/kg of distilled water (solvent of WP) orally by gavage 6 days per week ***(Amah et al., 2019)*****. Control peanut oil subgroup (CP):** each rat received 0.5 ml peanut oil, once weekly, intraperitoneal (IP) ***(Ibrahim et al., 2011).***
- **Whey Protein group (WP)**: (5 rats): each rat received WP at a dose of 5.4g/kg/day dissolved in distilled water by oral gavage for 6 days per week.

  The dose was chosen based on the usual dose of adults (70 kg) 1-2 scoops (30-60 g), so depending on the large dose of 60g, extrapolated to rats by surface area, the effect might be expected at a dose of 60000mg × 0.018=1080mg for 200g rat, then converted to g/kg (÷1000 and ×5) =5.4g/kg rat ***(Paget and Barnes, 1964).***
- **Nandrolone Decanoate group (ND)** (5 rats): each rat received IP once weekly ND diluted in peanut oil at a dose of 36mg/kg/week. The dose was calculated based on the usual dose of athletes (70 kg) 200-600mg/week, so depending on the average dose of 400mg, extrapolated to rats by surface area, the effect might be expected at a dose of 400mg × 0.018=7.2 mg for 200g rat, then convert to kg (×5)=36mg/kg rat ***(Paget and Barnes, 1964).***
- **Combined group (Comb):** (5 rats)**:** each rat received WP for 6 days per week and NDonce weekly with the same previously mentioned doses.

I.Sampling and determination of heart weight/body weight ratio

All rats were weighed every two weeks. According to ***Van Herck et al. (2001)***, animals were given mild ether anesthesia and retro-orbital plexus samples taken using a capillary tube after 8 weeks (after a 24-hour fasting period after the conclusion of the trial). The hearts were retrieved, cleaned with cold saline, and weighed. The animal's total body mass (HW/BW in mg/g) adjusted the heart weight. This ratio indicated heart

enlargement. The right half of the heart was dissected, weighed, and homogenized in 5 mL of ice-cold 0.1 MTris–HCl buffer (pH 7.4). The transparent supernatant solution was then collected and centrifuged before biochemical examination. Furthermore, the left half of the heart is left for histological analysis.

II. Biochemical investigation:

- To evaluate the serum lipid profile, we determined the low-density lipoprotein (LDL), total cholesterol (TC), and levels of triglycerides (TG) by routine enzymatic colorimetric method using Cobas c702/8000 autoanalyzer (Roche diagnostic, Mannheim, Germany) and its corresponding chemicals. Using the phosphotungstate-magnesium ions precipitation method, high-density lipoprotein (HDL) was measured, and very low-density lipoprotein (VLDL) was derived from TG level using Friedewald's formula: TG/5.
- To evaluate the oxidative stress parameters, serum MDA (nmol/ml) level was measured using a rat MDA ELISA Kit (OKEH02548) from Aviva Systems Biology. In addition, the total SOD (U/ml) level was determined using a rat SOD ELISA kit (Cat. No.CSB-E08555r) from Cusabio biotech co., Ltd, and heart tissue GPx (U/mg) level was measured using Rat GPx ELISA kit (Cat. No. CSB-E12146r) from Cusabio biotech co., Ltd.
- To evaluate the cardiac cytotoxic markers, Lactate dehydrogenase (LDH) in heart tissue (U/L) was assayed according to the method proposed by ***Baba and Sharma (1971)***, and Creatine Kinase –myocardial band (CK-MB) in heart tissue (U/L) was assayed according to the method proposed by ***Lott and Abbott (1986) and Gerhardt et al. (1993).***
- The analyses were performed in the Biochemistry Department, Faculty of Medicine, Zagazig University, utilizing a Cobas c702/8000 auto-analyzer (Roche diagnostic, Mannheim, Germany) and the required chemicals.

Histological analysis:

Heart specimens were placed in 10% formalin and processed into 5µm slices stained with H&E and Mallory trichrome ***(Bancroft and Gamble, 2002)***.

Furthermore, immunohistochemistry staining was performed on paraffin slices utilizing the marked streptavidin-biotin immune peroxidase approach ***(Janardhan et al., 2018)***. For Caspase- 3, a rabbit monoclonal antibody was used (1:1000) (EPR18297, ab184787, Waltham, MA, USA). Xylene deparaffinized, alcohols of decreasing concentration rehydrated, and phosphate buffer solution washed the segments (PBS). The segments were also treated with hydrogen peroxide at a concentration of 3% and then rinsed with PBS. After applying and washing with PBS, the main antibody was administered. Following washing with Water and incubation with the enzyme conjugate and 3, 3-diaminobenzidine tetrahydrochloride, add the biotinylated secondary antibody (DAB Substrate Kit, Thermo Fischer Scientific, Rockford, IL, USA). Mayer's Hematoxylin was used to counterstain segments. Antiserum was replaced with PBS to provide negative controls.

III. Morphometric analysis:

The subsequentfactors were measured:

- The number of nuclei in cardiomyocytes. Marker of hypertrophy of the cardiomyocytes.
- An area percentage of the following:

a. Mallory trichrome-stained tissue collagen arrangement.

b. The immunoreactivity of anti-Caspase-3 antibodies.

Leica Qwin 500 software image analyzer computer system (Leica image system Ltd., Cambridge, UK) was utilized. At X400 magnification, ten non-overlying fields were randomized for each region. The measurements were performed in the Pathology Department of the School of Dental Medicine at the University of Cairo.

STATISTICAL ANALYSIS:

SPSS 25.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis ***(Voelkl and Gerber, 1999)***. The results of the statistical analysis were given as means and standard deviation (SD). One-way analysis of variance (ANOVA) was also employed to compare continuous data between the test and control groups. At $p<0.05$, values were deemed statistically significant.

RESULTS

A. Bio physiological measures:

Subgroups CN, CDW, and CP did not vary significantly ($p > 0.05$). Consequently, one group (5 rats) was chosenrandomly as a reference to contrast with the other research groups.

1. Body weights and Heart weights (Table 1):

The control group exhibited non-significant changes in body and heart weights at the conclusion of the trial. WP lost more weight than the control group. The ND group had considerably lower body weights than the WP and control groups. In comparison to the other groups, the combined group had a considerable gain in body mass.

2. In terms of heart weight, non-significant change was found between the WP and control groups. The ND group had a considerable increase in heart content in comparison to the WP and control groups.

In comparison to the other groups, the combined group had much heavier hearts.

3. Index of cardiac hypertrophy (heart/ Body weight) ratio:

The control group did not affect heart-to-bodyweight ratio. The WP group had non-significant heart/body weight ratio differences from the control group. ND outperformed WP and control groups.

Relative to all other groups, the index of heart hypertrophy increased significantly in the combined group **(Fig. 1)**.

B-Biochemical results:

Serum Lipid profile (Table 2):

TC level (mg/dl)

The levels of TC were significantly elevated in the ND group in comparison to the WP and control groups (p-value < 0.001). Compared to all other groups, the combined group had a considerable rise. TC levels were similar in the WP and control groups.

TG level (mg/dl)

The ND group had greater TG levels than the WP and control groups ($p < 0.001$). Relative to other categories, the combined group rose significantly. TG levels were similar in the WP and control groups.

HDL level (mg/dl)

The levels of HDL were considerably lower in the ND group in comparison to the WP and control groups ($p < 0.001$). The combined group had a considerable decline relative to all other groups. Regarding HDL levels, the WP group did not differ from the control group.

LDL level (mg/dl) ND had greater LDL levels than WP and control ($p < 0.001$). The combined group rose more than all others. WP and control groups had similar LDL values.

Concerning VLDL level (mg/dl)

ND had higher VLDL levels than WP and control groups ($p < 0.001$). The combined group rose more than all others. WP and control groups had similar VLDL levels.

Table (1): Comparative statistical analysis of the four major groupings as regard rats' body weights and heart weights at the end of the study by ANOVA test and least significant difference test (LSD).

parameter	group	N	Mean	S. D	F test	P	P1	P2	P3
Body Weight (g)	Control	5	293.4	2.19	18.9	<0.001 HS	------	------	------
	ND	5	220.2	3.96			<0.001 HS	<0.001 HS	-----
	WP	5	277	7.18			0.001 S	------	------
	Comb	5	325	20			<0.001 HS	<0.001 HS	<0.001 HS
Heart Weight (g)	Control	5	0.922	0.11	10.5	<0.001 HS	----	------	------
	ND	5	1.19	0.07			<0.001 HS	<0.001 HS	-----
	WP	5	0.819	0.21			0.348 NS	------	------
	Comb	5	1.48	0.179			<0.001 HS	<0.001 HS	<0.001 HS

P1: difference between control group and other groups, P2: difference between WP group and other groups, P3: difference between ND group and other groups, S: P<0.05 means significant, HS: P<0.001 is highly significant, NS: P>0.05 means not significant, SD: standard deviation, N: number of rats, ND: nandrolone decanoate group, WP: whey protein group, and Comb: combined group.

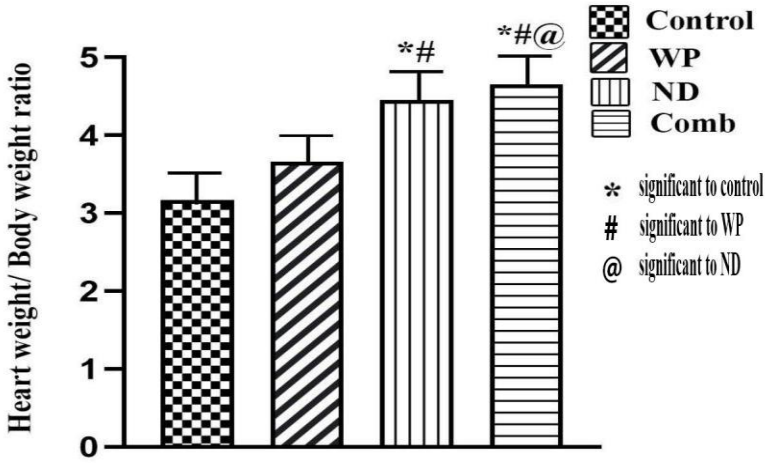

Fig. (1): ***Comparison between the heart/body weight ratio (mg/g) of the four main groups (Control, WP, ND, and Comb groups) (sample size of 30 albino rats) showing the mean values, SD of heart/body weight ratio.***

Oxidative stress parameters (Table 3):

MDA level (nmol/ml):

Compared to the control group, the WP group had higher MDA levels ($p<0.001$). Compared to the WP and control groups, the ND group exhibited a significantly significant rise. A significantly significant rise in MDA level was reported in the combined group relative to the other groups.

SOD level (U/ml):

The WP group had lower SOD levels than the control group ($p<0.001$). Compared to WP and the control group, the ND group had a considerable decline. In comparison to the other groups, the SOD level in the combined group decreased in a manner that was statistically significant.

GPx level (U/mg)

The WP group had lower GPx levels than the control group ($p<0.001$). Compared to WP and the control group, the ND group had a considerable decline. A significantly significant drop in GPx level was seen in the combined group compared to the other groups.

Cardiac cytotoxic markers (Table 4):

LDH level (nmol/ml)

The WP group had greater LDH levels than the control group ($p<0.001$). Compared to the WP and control groups, the ND group exhibited a significantly significant rise. LDH levels rose considerably in the combined group.

CK-MB level (nmol/ml)

The WP group had higher CK-MB levels than the control group ($p<0.001$). Compared to the WP and control groups, the ND group exhibited a significantly significant rise. A significantly significant rise in CK-MB level was detected in the combined group compared to the other groups.

Table (2): Comparative statistical analysis of the four major groupings as regards lipid profile (TC, TG, HDL, LDL, and VLDL) by ANOVA test and LSD along the period of the study.

parameter	Group	N	Mean	S. D	F	P	P1	P2	P3
TC mg\dl	Control	5	100.6	2.85	14.5	<0.001 HS	------	------	------
	ND	5	164.5	5.53			<0.001 HS	<0.001 HS	-----
	WP	5	105 .9	5.43			0.09 NS	------	------
	Comb	5	180.7	2.27			<0.001 HS	<0.001 HS	<0.001 HS
TG mg\dl	Control	5	76.63	0.88	18.3	<0.001 HS	------	-----	------
	ND	5	115.7	2.85			<0.001 HS	<0.001 HS	-----
	WP	5	77.5	2.51			0.611 NS	------	------
	Comb	5	148.8	3.63			<0.001 HS	<0.001 HS	<0.001 HS
HDL mg\dl	Control	5	47.6	1.21	11.4	<0.001 HS	-----	------	------
	ND	5	25.73	2.22			<0.001 HS	<0.001 HS	------
	WP	5	46.7	2.89			0.441 NS	-------	------
	Comb	5	20.81	2.86			<0.001 HS	<0.001 HS	<0.001 HS
LDL mg\dl	Control	5	36.6	1.22	22.4	<0.001 HS	-------	------	------
	ND	5	49.1	1.77			0.202 NS	------	------
	WP	5	38.5	2.58			<0.001 HS	<0.001 HS	------
	Comb	5	55.8	5.39			<0.001 HS	<0.001 HS	<0.001 HS
VLDL mg\dl	Control	5	19.9	1.61	12.3	<0.001 HS	------	------	------
	ND	5	30.5	1.14			0.302 NS	------	------
	WP	5	19.5	1.89			<0.001 HS	<0.001 HS	------
	Comb	5	40.9	1.48			<0.001 HS	<0.001 HS	<0.001 HS

P1: difference between control group and other groups, P2: difference between WP group and other groups, P3: difference between ND group and other groups, S: P <0.05 means significant, HS: P <0.001 is highly significant, NS: P >0.05 means not significant, SD: standard deviation, N: number of rats, ND: nandrolone decanoate group, WP: whey protein group, and Comb: combined group

Table (3): Comparative statistical analysis of the four major groupings as regards oxidative stress parameters (MDA, SOD, and GPx) by ANOVA test and LSD along the period of the study.

Parameter	Group	N	Mean	SD	F test	P	P1	P2	P3
MDA nmol/ml	Control	5	6.25	0.21	22.6	<0.001 HS	-------	-------	-------
	ND	5	18.4	0.85			<0.001 HS	<0.001 HS	-----
	WP	5	15.3	0.87			<0.001 HS	-------	-------
	Comb	5	22.7	0.79			<0.001 HS	<0.001 HS	<0.001 HS
SOD (U/ml)	Control	5	165.6	3.46	43.2	<0.001 HS	-------	-------	-------
	ND	5	80.6	2.26			<0.001 HS	<0.001 HS	-----
	WP	5	92.7	10.46			<0.001 HS	-------	-------
	Comb	5	76.2	9.01			<0.001 HS	<0.001 HS	<0.001 HS
GPx (U/mg)	Control	5	218.9	8.621	120.5	<0.001 HS	-------	-------	-------
	ND	5	68.6	17.73			<0.001 HS	<0.001 HS	------
	WP	5	100.7	16.92			<0.001 HS	-------	-------
	Comb	5	52.5	12.1			<0.001 HS	<0.001 HS	<0.001 HS

P1: difference between control group and other groups, P2: difference between WP group and other groups, P3: difference between ND group and other groups, S: P <0.05 means significant, HS: P <0.001 is highly significant, NS: P >0.05 means not significant, SD: standard deviation, N: number of rats, ND: nandrolone decanoate group, WP: whey protein group, and Comb: combined group.

Table (4): Comparative statistical analysis of the four major groupings as regards cardiac cytotoxic markers (LDH and CK-MB) by ANOVA test and least LSD along the period of the study.

parameter	Group	N	Mean	SD	F test	P	P1	P2	P3
LDH nmol/ml	Control	5	157.2	4.92	243.1	<0.001 HS	-------	-----	-----
	ND	5	1483.1	14.6			<0.001 HS	<0.001 HS	-----
	WP	5	485.8	10.1			<0.001 HS	-----	------
	Comb	5	1595.6	21.8			<0.001 HS	<0.001 HS	<0.001 HS
CK-MB nmol/ml	Control	5	56.4	1.95	322.2	<0.001	------	-----	-----
	ND	5	847.2	30.99			<0.001 HS	<0.001 HS	-----
	WP	5	184.1	7.4			<0.001 HS	-----	-----
	Comb	5	1437.9	30.7			<0.001 HS	<0.001 HS	<0.0001 HS

P1: difference between control group and other groups, P2: difference between WP group and other groups, P3: difference between ND group and other groups, S: P <0.05 means significant, HS: P <0.001 is highly significant, NS: P >0.05 means not significant, SD: standard deviation, N: number of rats, ND: nandrolone decanoate group, WP: whey protein group, and Comb: combined group

Histological results:

Hematoxylin and eosin-stained myocardial slices from the control group showed cardiomyocytes with acidophilic sarcoplasm and central vesicular nuclei. Cardiac muscle fibers were branching and anastomosing with narrow interstitial spaces and few flattened nuclei of fibroblasts **(fig. 2a)**. In WP group, areas of muscle fibers separation were notable, with vacuolated myocytes, multiple dark flat nuclei of fibroblasts, interstitial edema, and widened endomysium **(fig. 2b)**. Some fields revealed disarrayed muscle fibers with inflammatory cellular infiltrates in between, and focal areas of pale acidophilic degenerated myocytes were noticed **(fig. 2c)**. In ND group, multiple muscle fibers were separated with extensive inflammatory cellular infiltrates, interstitial edema and widened endomysium (**fig. 2d**). Moreover, multiple dilated congested blood vessels were seen beside focal areas of pale acidophilic degenerated myocytes with dark deeply stained pyknotic nuclei (**fig. 2e)**. In addition, dilated, clogged blood capillaries were found between the undulating muscle fibers (**fig. 2f)**. The cardiac muscle of the combined group; revealed multiple areas of pale acidophilic degenerated myocytes**,** wavy myofibers with vacuolated myocytes, abundant dark flat nuclei of fibroblasts, dilated congested blood vessels and multiple areas of blood extravasation (**fig. 2g).** Some muscle fibers were disarrayed within broad fields of pale acidophilic degenerated myocytes beside others with hyalinosis, interstitial edema, and inflammatory cellular infiltrates **(fig. 2h)**.

In the control group, Mallory trichrome-stained heart slices exhibited few tiny collagen fibers between cardiomyocytes and blood vessels **(fig. 3a)**.

In the WP group, cardiomyocyte interstitium had thin collagen fibers **(fig. 3b)**. The ND group had more collagen fibers between cardiomyocytes and blood arteries **(fig. 3c).** Collagen deposition increased significantly between cardiomyocytes and surrounding blood arteries in the combined group **(fig. 3d)**.

Immune localization of anti-caspase-3 antibodies in control group cardiac muscle; revealed a minimal degree of caspase expression **(fig. 4a)**. WP group revealed a mild degree of caspase immune expression **(fig. 4b)**. In the ND group, a heavy degree of caspase expression was noted **(fig. 4c)**. Combined group showed extensive heavy caspase- 3 immune expression **(fig. 4d)**.

Morphometric results:

In H&E-stained slices, WP patients had fewer cardiomyocyte nuclei than controls. Comparing the ND group to the control and WP groups, a significantly significant decrease was seen. Also, the combined group also decreased significantly compared to the other groups **(fig. 2i)**.

In Mallory trichrome-stained sections, the WP group showed non-significantly more collagen dispersion area than the control group. ND and combined groups increased significantly compared to control and WP groups. With comparison to the ND group, the combined group exhibited a significant rise in % **(fig. 3e)**.

WP and control groups had similar Caspase-3 immunoreactivity area %. Yet, a considerable rise was seen in the ND and combination groups in comparison to the control group. In addition, the combined group had a very significant rise relative to all other groups **(fig, 4e)**.

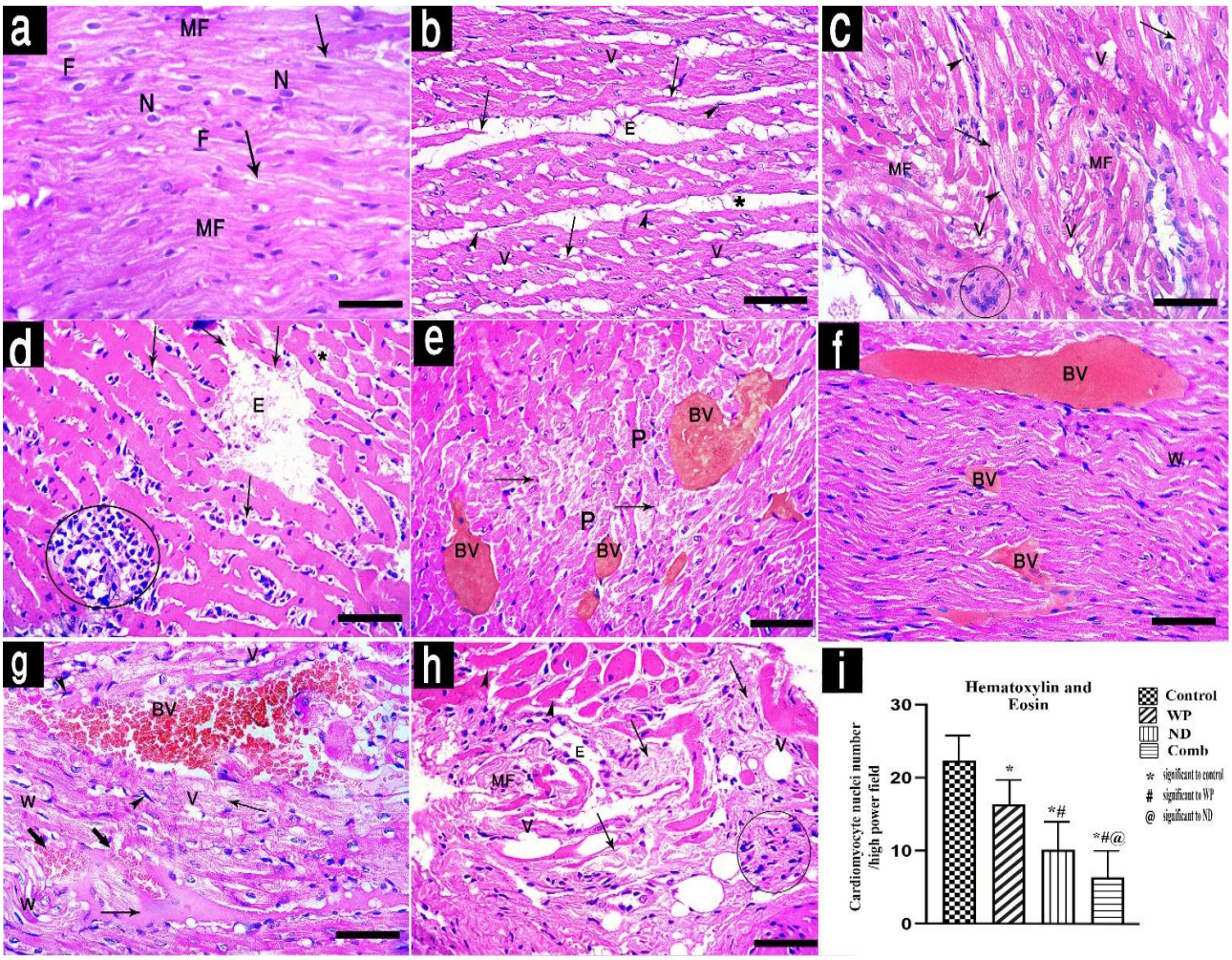

Fig. (2): *Hematoxylin and eosin-stained section of the cardiac muscle:* ***(a)*** *control group; showing acidophilic, branching, and anastomosing cardiac muscle fibers (MF) with centrally located vesicular nuclei (N) and narrow interstitial spaces (arrow). Flattened nuclei of fibroblasts (F) are also seen.* **(b, c)** *WP group rats; multiple areas of muscle fibers separation are notable (arrow), with vacuolated myocytes (V), multiple dark flat nuclei of fibroblasts (arrowhead), interstitial edema (E), and widened endomysium (star) are observed* ***(b)****. Some fields showed is arrayed muscle fibers (MF), appearing with focal areas of pale acidophilic degenerated myocytes (arrow), some vacuolated myocytes (V), multiple darkflat nuclei of fibroblasts (arrowhead), and inflammatory cellular infiltrate (circle) are observed****(c)****.* ***(d, e, f)*** *ND group; shows multiple areas of separated muscle fibers (arrow), with extensive inflammatory cellular infiltrates (circle), interstitial edema (E), and widened endomysium (star)****(d)****. Also, multiple dilated congested blood vessels (BV) are seen beside focal areas of pale acidophilic degenerated myocytes (arrow) with dark, deeply stained pyknotic nuclei (P)****(e)****. The dilated congested blood vessel and capillaries (BV) are observed in between the wavy muscle fibers (W)****(f)****. (****g, h****) Combined group; shows multiple areas of pale acidophilic degenerated myocytes (thin arrow), wavy myofibers (W) with vacuolated myocytes (V), abundant dark flat nuclei of fibroblasts (arrowhead), dilated congested blood vessels (BV), and multiple areas of blood extravasation (thick arrow) are also observed****(g).*** *Many disarrayed muscle fibers (MF) are seen with some fields of pale acidophilic degenerated myocytes (arrow) besides others with hyalinosis (arrowhead), large vacuolated myocytes (V), interstitial edema (E), and inflammatory cellular infiltrates (circle)****(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 ± SD (n = 30).*

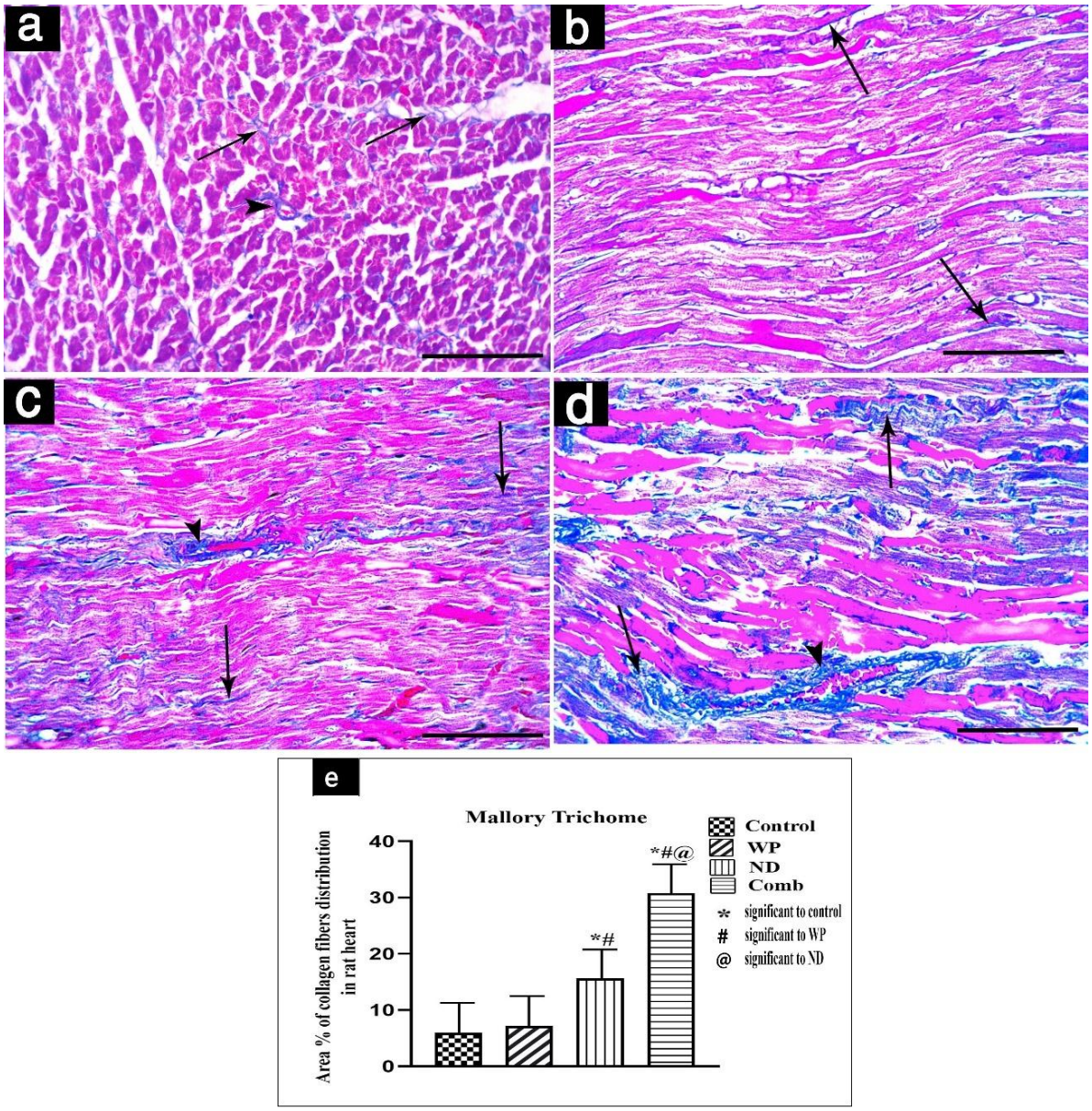

Fig (3): *Mallory trichrome stained section of the cardiac muscle:* ***(a)*** *In the control group, there are few fine collagen fibers (arrow) between both the blood vessels and cardiomyocytes (arrowhead).* ***(b)*** *In the interstitium between the cardiomyocytes of the WP group, thin collagen fibers (arrow) are seen.* ***(c)****Increased collagen fibers are seen between the cardiomyocytes (arrow) and surrounding the blood vessels (arrowhead) in the ND group.* ***(d)****The combined Whey and ND groups exhibit a significant increase in collagen deposition between cardiomyocytes (arrow) and blood arteries (arrowhead). [Mallory trichrome, Scale bar= 50 µm].* ***(e)****The area % of collagen distribution in Mallory trichrome-stained sections for all groups investigated. (N = 30) Results are provided as mean ±SD.*

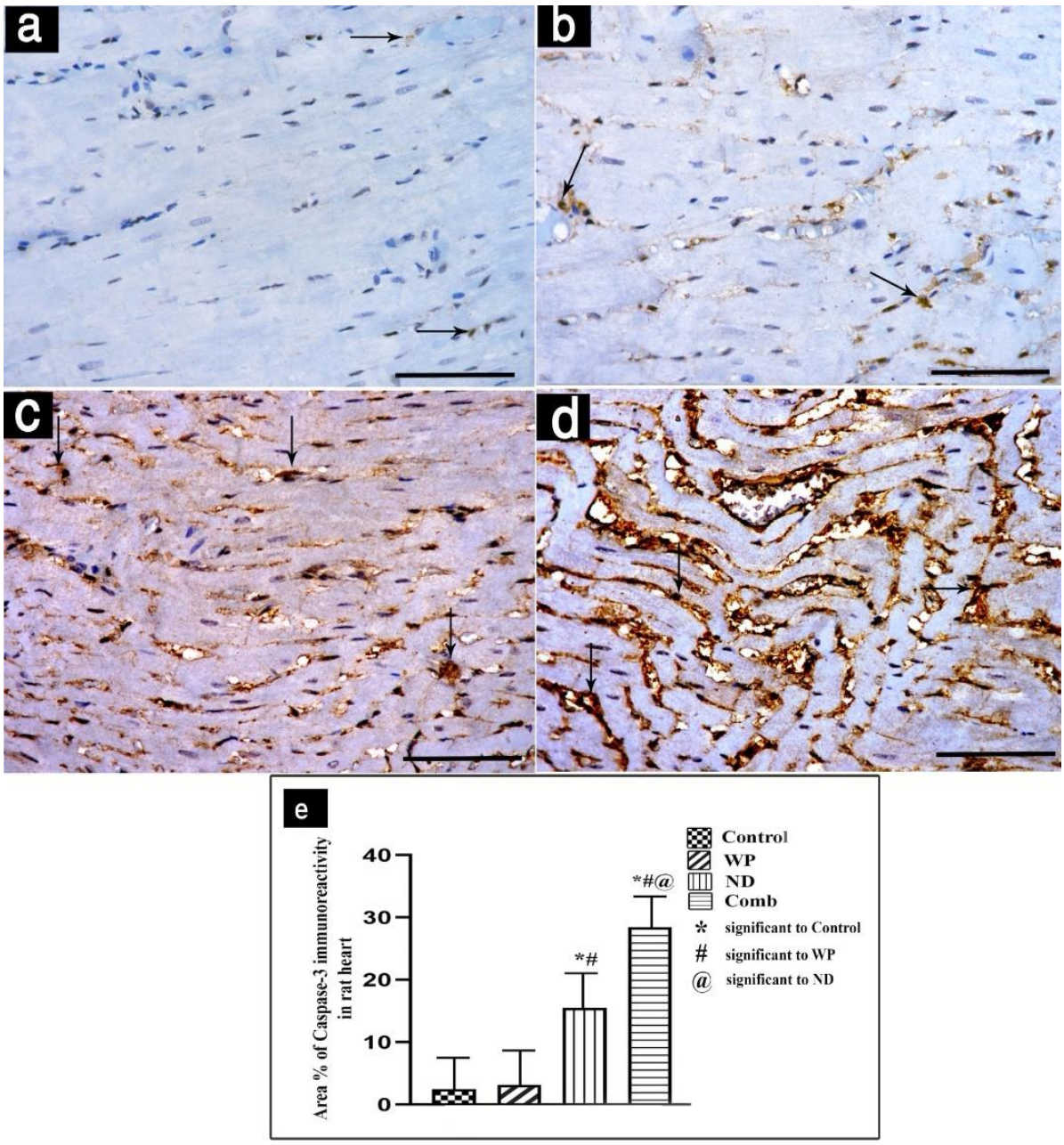

Fig (4): *Caspase-3-stained section of the cardiac muscle: (**a**) control group: showing immune localization of caspase - 3 in the cardiac muscle of the control group revealed minimal degree of caspase expression (arrow). **(b)** WP group, showing the mild degree of caspase expression (arrow). **(c)** ND-treated group shows a heavy degree of caspase- 3 expressions (arrow). **(d)** Combined group; showing extensive heavy caspase- 3 expressions (arrow). [Immune peroxidase, Scale bar = 50µm]. **(e):** Area percentage of caspase- 3 immunoreactivity in all the study groups. Data are presented as mean ± SD (n = 30).*

DISCUSSION

WP has become the most prominent protein supplement amongst athletes and anyone who engage in physical activity because it provides energy during exercise while significantly improving athletic performance (***Vasconcelos et al., 2021)***. Several researches, such as ***Witard et al. (2014)*** showed that a dosage exceeding 40 g/day may be associated with deleterious consequences in people. Besides that, the usual daily consumed dose by athletes is 1-2 scoops of WP, equivalent to 30-60 g protein/day ***(Alhakbany et al., 2022)***, so in our study, we chose 60 g in humans, equivalent to 5.4 g/day in rats to detect potential side effects on the heart.

Thetoxicity of WP in the heart contributes to various mechanisms.The WP has an insulinotropic effect.

Butkowski and Jelinek (2017) showed that elevated insulin levels are associated with increased inflammatory cytokines like Interleukin-6 (IL-6). ***Willerson and Ridker (2004)*** proved that IL-6 as well as the inflammatory response was major factors in cardiovascular diseases. Moreover, BCAA levels exceeding 10 mmol/L (especially with a catabolic defect) increase reactive oxygen species production, resulting in mitochondrial damage and oxidative stress. Several cardiovascular diseases have been linked to dysfunctional mitochondria. Furthermore, the redox-sensitive nuclear transcription factor kappa-light-chain-enhancer of activated B cells (NF-κB) was stimulated by BCAA, resulting in the release of inflammatory mediators. The NF-κB is an important key in heart diseases related to the inflammatory process ***(Zhenyukh et al., 2017; Liu et al., 2021)***.

ND is one of the most used AAS by bodybuilders worldwide as a doping agent to improve muscle performance or physical appearance. Individuals typically take doses 10 to 100 times higher than the therapeutic dose, up to 700 mg per week for an average 80 kg man for long duration ***(Frankenfeld et al., 2014; Vasilaki et al. 2016).*** These finding support the cardiotoxic effect of ND & underlying mechanisms, including activation of the renin–angiotensin system (RAS), increasing blood pressure overload, direct effect on cardiac myocytes and oxidative reactions***(Cowan and Young, 2009; Andrade et al., 2011).***Moreover, ***Carteri et al. (2021)*** suggested that altering the potential of the mitochondrial membrane and its Ca+2 metabolism leaded to cardiotoxicity.

Marques-Neto et al. (2014), found the ND use is associated with antioxidant enzyme impairment and an increase in tumor necrosis factor (TNF-α). TNF-α and angiotensin II play roles in redox system imbalance and oxidative stress. ***Aimoet al. (2020)*** added that oxidative stress activates transforming growth factor- β (TGF-β), which promotes fibroblasts differentiation into myofibroblasts that induce fibrosis in cardiac muscle.

In the present research, the WP group's mean body weight values were lower than those of the control group. ***Wirunsawanya et al. (2018)*** coincide with our results. ***Faipouxet al. (2008)***, demonstrated that chronic consumption of high-protein meals like WP boosted the noradrenergic/adrenergic neurons responsible for the cholecystokinin-induced satiety effect. Additionally, ***Leidy et al. (2015) and Englund et al. (2017)*** proposed that the thermal effect of high-WP meals is the cause of the increased fat burning and reduction of intramuscular fat.

The WP group did not vary significantly from the control group in terms of heart weight and heart/body weight ratio.

Helal et al. (2020) study was constant with this conclusion. ***Lollo et al. (2013)*** explained the long-term activation of protein synthesis pathways might result in negative feedback in the form of activation of catabolic pathways like the ubiquitin-proteasome.

In contrast, ***de Almeida et al. (2020)*** found a greater growth in cardiac mass in 35% isolated WP group than the 14% WP group. These diverse results may be attributed to different diet regimens as our WP-fed rats consumed a balanced diet plus 5.4g WP, also they believed that a high protein diet augmented muscle protein synthesis and promote muscle hypertrophy supported by their results. At cellular level, we noticed significant decrease in cardiomyocytes nuclei/ high power fields in WP group, which is an indicator of increased cardiomyocytes size. Our results were in harmony with ***Tang et al. (2007)***, ***Hayes and Cribb (2008) and Lollo et al. (2013)***, they reported that WP induced skeletal myofibrils and myocardial cells hypertrophy respectively via boosting muscle protein synthesis.

On the contrary, ***Chen et al. (2014)*** found neither skeletal nor cardiac myocytes hypertrophy with WP, this may be explained by their usage of lower doses, about one third of the dose we used in the current experiment; muscle hypertrophy is therefore dose-dependent.

Compared to the WP and control groups, the ND group lost more weight. ***Marocolo et al. (2019)*** was in line with our results. Slow body weight gain in the ND group was due to high doses induced food intake reduction, total fat mass lowering and fasting plasma insulin and glucose levels reduction in a dose-dependent manner ***(Alsiö et al., 2009).*** On the hand, the combined group had a significantly elevated body weights compared to all other groups**.** In line with our experiment, ***Jabbar et al. (2018)***, observed overweight bodybuilders consuming AAS and dietary protein supplements. Regarding heart weight and heart/body weight ratio, rats of ND and combined groups showed significant increase in comparison to control and WP rats, which was supported by our morpho-histometric results revealing cardiac muscle hypertrophy at both physiological and histological levels. According to research done by ***Camargo Filho et al. (2006) and Jain and Goel (2020),*** a hyperproteinic diet as WP supplements following anabolic steroid administration for long periods caused hypertrophy in immobilized muscles, and cardiomegaly with left ventricular hypertrophy respectively. ***Khan et al. (2022)*** confirmed our work, as muscular biopsies in weightlifters revealed that AAS users had a greater number of muscular fibers and bigger average fiber size.

Mechanisms responsible for cardiac hypertrophy after ND treatment described respectively by ***Tanno et al. (2011)***, ***Abdollahi et al. (2016) and Ganesan et al. (2020)***, a rise in myocardial collagen content, also a decrease in phosphatase activity and increase in activity of the enzyme that converts angiotensin into angiotensin II, moreover ND promotes nitrogen retention, and amino acid reserve in the muscles encouraging an anabolic state.

On the other hand, ***Chaves et al. (2013) and Santos Neto (2022)***, found no significant change in body mass, heart weight, and cardiac hypertrophy index. We tried to understand the idea behind this conflict; we could attribute it to the different doses, duration of treatment, species of rats, lastly the exercise effect.

Regarding lipid profile investigation, Our WP group had non-significant differences

in all lipid profile mean values compared to the control group. In harmony with ***Zhang et al. (2016) and Bell et al. (2017)***, who had similar results, however, the authors of the first mentioned study noticed TG was significantly decreased, which may be attributed to other factors such as training and calorie restriction. As in the present study, we did not reduce the total energetic value plus the sedentary style of our rats, which could explain the non-significant TG in our experiment.

The ND and combined-treated groups presented a significant deterioration in all lipid profile parameters compared to the WP and control groups. In line with ***Taher et al. (2008) and Tofighi et al. (2017)***, they showed the same results as our work, but TC levels, and TG levels respectively were not significantly affected may be due to unsimilar dosage, technique of administration (like IM), AAS type (metabolizable or not), or species. Our study showed a harmony with ***Flachi et al. (2018)***, ***Jain and Goel (2020), and Tashiro et al. (2021)*** as they found that bodybuilders who consumed very high doses of anabolic steroids and highprotein (WP), their lipid profiles revealed dyslipidemia. On the contrary, ***Aparicio et al. (2013)*** found that high protein with AAS had favorable effects on lipid profile; this could be explained by the combination of exercise with treatment.

In terms of oxidative stress measures, the WP, ND, and combined groups exhibited statistically substantial increases in the mean levels of MDA, but the mean levels of SOD and GPx fell considerably in comparison to the control group. In agreement with our findings, ***Lacroix et al. (2004)***, ***Namikoshi et al. (2007) and Camiletti-Moirón et al. (2015)*** reported that rats fed high-protein diet exhibited a mitochondrial oxidation rise and oxidative stress in the liver cells, in the kidney and aorta of obese rats and brain respectively. The relationship between oxidative stress and inflammation is tight. Through several processes, including ROS attract circulating inflammatory cells and fibroblast progenitors ***(Frangogiannis, 2014)***. Also, inflammation causes oxidative stress by impeding antioxidant defenses, boosting TNF-α and TGF-β ***(Aimo et al., 2020)***. Using WP for long periods without training increased many inflammatory markers like Interleukin 1 (IL-1), IL-6 and TNF- α in the liver ***(Gürgen et al., 2015)***. This could explain why WP caused oxidative stress.

On the contrary, ***Brown et al. (2004), Rankin et al. (2006) and Behboudi et al. (2019)***, found that high dietary protein like WP didn't affect radical scavenging capacity. Other studies noticed that WP had a favorable effect on redox status; this difference may be attributed to different species, dose, duration, and playing exercises.

Regarding ND group, our results matched ***Mohamed and Mohamed (2015) and Riezzo et al. (2014)***. We can explain this finding as ND could induce lipid peroxidation by disturbing the cellular redox system balance and promoting the oxidant agents like superoxide anion radicals ***(Abdollahi et al., 2016; Sretenovic et al., 2021)***. Our work concerning combined group matched with ***Camiletti-Moirón (2016)***, who emphasized that a high-protein diet with anabolic androgenic steroids caused brain and kidney damage by inducing lipid and protein oxidation.

WP, ND, and combined groups had higher LDH and CK-MB levels than the control group. ***Handelsman (2006) and Tousson et al. (2018)***, reported similar findings to our study. The myocardium contains many cardiac enzymes, such as CK-MB and LDH, the elevation of these enzymes can

diagnose myocardial injury, as they are released in the blood stream under effect of increased lipid peroxidation ***(Saka et al., 2022)***.

This is in contrast to the current findings by ***Pergolizzi et al. (2017)***, who observed that ND treatment significantly reduced LDH and CK-MB levels.

In the current work, WP-fed rats had disorganized cardiac muscle fibers beside focal regions of pale acidophilic vacuolated and degenerated myocytes. Our results agreed with ***Whitt et al. (2008) and Gürgen et al. (2015)***, who noticed similar findings in hepatocytes of both human and animals respectively after WP exposure.

Furthermore, ***Patra et al. (2019) and Mustafa et al. (2020)***, suggested that the main incriminated inflammatory marker for these cytological changes was TNF-α released from liver Kupffer cells, and active natural killer (NK) cells.

On the other hand, ***Chen et al. (2014)*** showed no pathological effects of WP-fed animals on heart tissue. This opposite finding may be due to different species (mice). However, rats are more physiologically, morphologically, and genetically closer to humans than mice; a lower dose (4.1 g/kg) is equivalent to 20 g in humans, a safe dose of WP, so it was not high enough to cause cardiac pathology.

In the present study, we followed a dosage regime following various other animal studies to enable exporting information about the pathogenesis of chronic administration on cardiac muscle ***(Shokri et al., 2014).*** In the current work, ND induced various myocardial histological alterations, such as multiple areas of separated muscle fibers with dilated congested blood vessels.

Moreover, some fields showed extensive inflammatory cellular infiltrates, interstitial edema, and widened endomysium. Our results were in harmony with ***Vasilaki et al. (2016),*** who revealed a picture of chronic myocardial inflammation detected at high doses of ND.

Germanakis et al. (2013), connected these histopathological effects to the ND-induced changes in local myocardial oxidative stress response. Some researchers added that these cardiac myopathy changes are linked to dyslipidemia, which is related with enhanced NF-κB and hypoxic-ischemic factor (HIF-1) production ***(Udomkasemsab and Prangthip, 2019; Soliman et al., 2022)***.

Recent studies have suggested that oxidative stress was regulated via CD40-CD40L interaction, thus controlling various cellular pathways in both the immunological and cardiovascular systems, explaining our finding of extensive inflammatory cellular infiltrates within the myocardium ***(Al-Oanzi et al., 2022)***.

A previous study by ***Ahmed (2015)*** on testicular tissue showed that ND administration disorganized the histological structure of the seminiferous tubules with various apoptotic changes in spermatogonia and Sertoli cells, plus remarkable atrophied interstitial cells of Leydig. Similar to these findings, cardiac muscle expressed a significant increase in collagen fibers deposition and caspase-3 in the present work. Foci of myocardial fibrosis were the most representative histological alterations in the literature concerning AAS-abusing athletes ***(Torrisi et al., 2020)***. Caspases induce apoptosis via aspartate-directed cysteine-dependent proteases. Proteases break the structural cytoplasmic and nuclear components of cardiac cells. Apoptosis of myocytes has been demonstrated as an incomplete process ***(Bouche et al., 2021).***

In case of lack of nuclear fragmentation, there is a chance for continuous cytoplasmic protein loss allowing cardiomyocytes to survive for prolonged periods.

The vulnerable proteins to be early lost are actin I and II ***(Bouitbir et al., 2019)***. In addition, the oxidative damage with a diminished antioxidant defense system resulted in a mitochondrial-activated apoptotic signaling pathway, ending in caspase-3-mediated cardiomyocyte apoptosis, as demonstrated in our ND group ***(Sandamali et al., 2022)***.

Regarding the combined group, the myocardial histological alterations were extensive for signs of inflammation, fibrosis, or apoptotic changes. Our findings were in the same line with ***Flachi et al. (2018)***, who described a case report of a young athlete that used to abuse different types of AAS for 12 years; he suffered from an end-stage renal disease, with chronic interstitial inflammation, generalized cytoplasmic vacuolization, sclerosis, hyperplastic podocytes, and interstitial fibrosis. Moreover, when bodybuilders consumed WP supplements with ND, they experienced an acute renal injury that was manifested as interstitial fibrosis, inflammatory lymphocytic infiltrations, and acute tubular necrosis in kidney biopsies ***(Davani-Davari et al., 2019)***.

CONCLUSION

In conclusion, chronic high-dose ND and WP exposure caused heart muscle structural alterations in adult male albino rats, manifested by inflammatory and degenerative changes, fibrosis, and apoptosis, that were proved by histological and immunohistochemical methods. In addition, there was an alteration in the lipid profile, increased lipid peroxidation and disturbed redox system balance via promoting the oxidant agents and depleting the antioxidant enzymes (SOD and GPx). Also, both the histological structural changes and biochemical abnormalities were maximum in the combined group compared to either ND or WP alone so, caution should be taken when consuming AAS and a high protein diet, such as WP, over extended periods. Increased administration of fruits and vegetables as natural antioxidants is highly recommended while administering WP and ND supplements.

RECOMMENDATIONS

Further studies will be fruitful to detect the underlying mechanisms of WP and ND toxicity on the heart. Moreover, additional human research is required to modify WP and ND beneficial dose and duration of administration beside safety.

Study limitations:

One of the limitations of the study, there was no group of rats with exercise effect.

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