YALE JOURNAL OF BIOLOGY AND MEDICINE 96 (2023), pp.455-465.

ORIGINAL CONTRIBUTION

YALE JOURNAL OF BIOLOGY AND MEDICINE
YJBM
CELEBRATING 95 YEARS 1928–2023

Protective Effects of Chia Seeds and Omega-3 Fatty Acid against Cyclophosphamide-Induced Oligospermia in Male Wistar Rats: Potential Risks of Adverse Drug Interaction with Chia Seeds

Prince B. Vaghela, Archana M. Navale*, Chirangi B. Patel, Nishant H. Patidar, Prachi D. Nahar, Farmi Patel, Zainab Pathan, and Barsha Kumari

Department of Pharmacology, Parul Institute of Pharmacy, Parul University, Gujarat, India

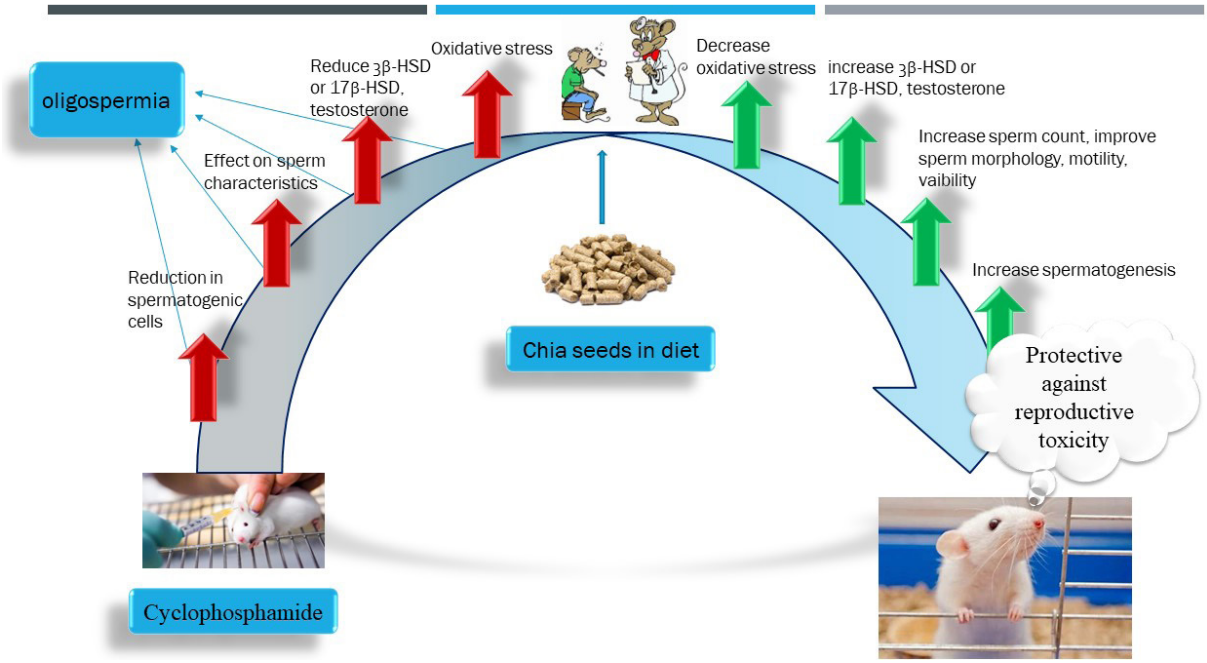
Objectives: The aim of this study was to investigate whether chia (*Salvia hispanica*) seeds, which are rich in omega-3 fatty acids, amino acids, and vitamins with antioxidant properties, can mitigate the negative effects on male reproductive function caused by cyclophosphamide, a frequently used chemotherapeutic agent. **Methods**: Male wistar rats are divided into seven groups (n=6). All groups except the normal control (NC) received cyclophosphamide (30mg/kg, i.p.) for the first 5 days. The standard group received clomiphene citrate (0.25 mg/kg, p.o.). Treatment groups T1%, T5%, T10%, and ω-3 received 1%, 5%, and 10% chia seeds in the diet, and 880 mg/kg omega-3 fatty acid (p.o) respectively for 15 days. The effect on the reproductive system was evaluated by analysis of epididymal sperm characteristics, biochemical parameters, and serum testosterone level. **Results**: Clomiphene citrate improved oligospermia via hormone mediated effect. Chia seeds and omega-3 fatty acid treatment also showed improvement in reproductive parameters including oxidative stress and histological features of the testes. Omega-3 fatty acid treatment was more effective for the prevention of cyclophosphamide toxicity on testes as compared to chia seeds. Nasal bleeding was noted in several animals subjected to chia seed treatment. This occurrence might be attributed to chia seeds' impact on coagulation and/or platelet function, potentially heightened due to chemotherapy associated bone marrow suppression. **Conclusions**: In our study, chia seeds as well as omega-3 fatty acid treatment were found to be protective against cyclophosphamide-induced reproductive toxicity in rats. However, the adverse effect of hemorrhage associated with drug interaction of chia seeds with cytotoxic chemotherapeutic drugs needs careful attention and further investigation.

*To whom all correspondence should be addressed: Dr. Archana M. Navale, Ph.D. Associate Professor, Department of Pharmacology, Parul Institute of Pharmacy, Parul University, Gujarat, India; Email: archanachavan_83@yahoo.co.in; ORCID: 0000-0003-3604-477X.

Abbreviations: WHO, World Health Organization; CP, cyclophosphamide; WBC, white blood cell; CAT, Catalase Activity; GSH, reduced glutathione; HSD, hydroxysteroid dehydrogenase; NAD, nicotinamide adenine dinucleotide; DC, disease control; NC, normal control; Std, Standard treatment; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); PBS, phosphate buffer solution; SD, standard deviation.

Keywords: Chia seeds, Cyclophosphamide, omega-3 fatty acid, oligospermia, drug interaction

Author Contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.



Graphical Abstract

INTRODUCTION

According to the the World Health Organization, (WHO) the total number of cancer cases worldwide was 19.3 million in 2020 and it is estimated that the number of new cases will increase to 30.2 million by 2040. The estimated number of cancer-related deaths from 2020 to 2040 is 9.96 million to 16.3 million. The number of cancer cases in India in 2020 was around 1.32 million, while the number of cancer-related fatalities was nearly 0.852 million, as per the WHO [1].

Chemotherapeutic agents are used to treat cancer in patients but they cause significant adverse effects. The seminiferous epithelium of the testicles is severely damaged in more than 50% of male patients receiving chemotherapy for a variety of solid tumors and in some cases results in irreversible damage [2].

Cyclophosphamide (CP), a cyclic phosphoramide ester, is a potent antineoplastic and immunosuppressive drug widely used for cancer chemotherapy [3]. Alkylating agents' primary pharmacological action is to interfere with cell division, development, and function, primarily by cross-linking DNA strands. The therapeutic effects of CP and many of its toxic properties are based on its capacity to interfere with all rapidly proliferating tissues [4].

The presence of rapidly dividing cells makes the reproductive system extremely sensitive to this medication [5]. CP treatment has been observed to result in biochemical and structural disruption in the testes and epididymis of male rats. Apart from these structural and functional changes, oligospermia and azoospermia are also common consequences of CP exposure as observed in experimental animals [6-8]. Male patients receiving CP showed abnormal gonadotropin secretion and reduced testosterone levels linked to testicular damage [9].

Chemotherapy-induced infertility has a significant negative effect on the quality of life; as a result, special attention must be paid to protecting germ cells during and after chemotherapy [10].

Chia (*Salvia hispanica*) seeds contain phytochemicals, such as chlorogenic acid, caffeic acid, myricetin, quercetin, and kaempferol [11]. All of these phytoconstituents are proven to be potent antioxidants [12-16]. As oxidative stress plays an important role in the pathogenesis of oligospermia [17], the antioxidant effect provided by chia seeds may play a protective role in this process. Omega-3 fatty acid has been shown to improve semen quality parameters and increase testosterone levels in male volunteers in several earlier studies [18]. Chia seeds due to the high content of omega-3 fatty acids may also have a beneficial effect on testicular physiology.

Many dietitians claim that chia seeds are good for the male reproductive system, but there is no scientific evidence available. In this study, we plan to evaluate the effect of chia seed and omega-3 fatty acid supplementation on epididymal sperm characteristics, oxidative stress parameters, and hormonal parameters including blood testosterone levels.

MATERIALS AND METHODS

Experimental Animals

All the animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) (PIPH 02/22) and animals were used according to the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) guidelines. Experiments were performed on healthy adult male Sprague-Dawley rats (200-300 g). The rats were supplied by Jai Research Foundation, Vapi. Animals were kept at controlled environmental conditions with room temperature (22 ± 2 ◦C), humidity (50 ± 10%), and a 12-hour light-dark cycle. Rats were allowed to access the food and water ad libitum and acclimatized at least for 1 week in the experimental conditions prior to the start of the experiment.

Chemicals and Materials

Cyclophosphamide (Zydus Oncoscience); Nicotinamide adenine dinucleotide (Loba chem., CAS No 53-84-9); DTNB, Ellman's Reagent (Sigma Aldrich, CAS No 69-78-3); Dehydroepiandrosterone (Loba chem., CAS No 53-43-0); GSH (HiMedia, CAS No 70-18-8); Testosterone (HiMedia, CAS No 58-22-0); Bovine serum albumin (HiMedia, CAS No 9048-46-8); and Trypan blue solution (Sigma Aldrich, CAS No 72-57-1).

Experimental Design

Animals were randomly categorized into seven groups, with six animals in each group to ensure sufficient statistical power. Groups 2-7 received cyclophosphamide (30mg/kg, i.p.) for day 1-5 [19] and respective treatments (from day 1 to 15) as mentioned below.

Group 1 (normal control (NC): Saline (5 ml/kg, day 1 to 15);

Group 2 (disease control (DC)): Cyclophosphamide (30mg/kg, i.p., day 1 to 5)+ saline (5 ml/kg, day 1 to 15);

Group 3 (standard treatment): Cyclophosphamide (30mg/kg, i.p., day 1 to 5)+ Clomiphene citrate (0.25 mg/kg, i.p., day 1 to 15) [20];

Group 4 (T1%): Cyclophosphamide (30mg/kg, i.p., day 1 to 5) + chia seeds (1% in diet, oral, day 1 to 15);

Group 5 (T5%): Cyclophosphamide (30mg/kg, i.p., day 1 to 5) + chia seeds (5% in diet, oral, day 1 to 15);

Group 6 (T10%): Cyclophosphamide (30mg/kg, i.p., day 1 to 5) + chia seeds (10% in diet, oral, day 1 to 15);

Group 7 (ω-3): Cyclophosphamide (30mg/kg, i.p., day 1 to 5) + omega-3 fatty acid (880 mg/kg, i.p., day 1 to 15).

The omega-3 fatty acid dose for group 7 was selected to replicate the omega-3 fatty acid content present in a 5% chia seed diet, aligning with the chia seed dosage administered to group 5. All animals were euthanized 24 h after the last day of treatment (ie, day 16) by anesthesia with ketamine (60 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). Dosing was done between 9 to 10 am every day. Determination of parameters was done in the morning on specified days. The dosing and testing were carried out in a randomized manner, with each animal handled in a different sequence each day. On the day of final assessment the animals were assigned random numbers and the samples for analysis were identified with these numbers to eliminate bias during assessment of parameters.

ANALYSIS OF EPIDIDYMAL SPERM CHARACTERISTICS

Total Sperm Count

Took a small piece of cauda from the right epididymis in a petri plate containing 0.9% normal saline (5 ml) at room temperature. Allowed spermatozoa to ooze out in the petri plate (2-5 min). The resultant solution was referred to as suspension A from here onwards. To derive the total sperm count, took a 100 µL sample from suspension A and added 1.9 mL of 0.9% normal saline (20 times dilution) in a microcentrifuge tube. Mixed contents gently by rotating the tube on the palm. Charged hemocytometer with a properly mixed sample. Kept the hemocytometer chamber at room temperature for 2-3 minutes for proper distribution of the sample. Total sperm from white blood cells (WBC) counting squares under 10x objective was counted. Calculated the total sperm count as follows:

$$Total\ sperm\ count\ (million/ml) = \frac{N x 20 x 1000 x dilution\ volume}{0.4}$$

Where N = total sperm counted in four WBC counting squares.

Sperm Viability

For observation of sperm viability, we took 10 µL from suspension A on a clean glass slide. Added 10 µL of 0.9% w/v normal saline and 10 µL of trypan blue solution. Mixed contents by swift swirl action and with the help of a micropipette tip. Applied coverslip and observed viable/non-viable sperm under 40x objective on a microscope. Viable sperm remained non-stained/colorless whereas non-viable sperm took pink/faint red color. A total of 200 sperm (viable and non-viable) were counted and sperm viability (%) calculated, as follows:

$$Sperm\ viability\ (\%) = \frac{Viable\ sperm}{Total\ sperm} x 100$$

Sperm Motility

For observation of sperm motility, the hemocytometer was charged with 10 µL from suspension A. Motile and non-motile sperm were present in all four WBC counting squares and were counted under 40x objective. If sperm concentration was very high or low, which may obscure observation of motility, then the hemocytometer was loaded with a fresh sample.

$$Sperm\ motility\ (\%) = \frac{Motile\ sperm}{Total\ sperm} x100$$

Sperm Morphology

For sperm morphology, we took a 100 µL sample from suspension A and 1 mL 10% neutral buffered formalin (NBF) where added in a microcentrifuge tube. Gently mixed contents. Added 2-3 drops of 1% eosin added and mixed contents gently. Kept the tube at room temperature for 30-45 minutes to allow staining of sperm. Added 1-2 drops of the stained sample added on a clean glass slide. Appropriate size coverslip was applied. A minimum of 100 sperm from different 4-5 fields on a glass slide were observed. Recorded abnormalities like tailless sperm, headless sperm, no hooked head sperm, double tail, coiled tail, double head, bent tail, and any other abnormality. Calculated the % abnormal sperm as below:

$$Abnormal\ sperm\ (\%) = \frac{Total\ abnormal\ sperm}{Total\ observed\ sperm} x100$$

BIOCHEMICAL ASSAYS

Preparation of Tissue Homogenate

Testes were immediately removed, cleared of adhering connective tissue, washed in 0.9% saline, blotted on filter paper, and weighed. One testis was set aside for histological processing and immersed in a 10% neutral formalin solution. The whole tissue was homogenized in saline at a concentration of 1 ml saline/100 mg tissue and centrifuged at 10,000g for 30 min at 4°C. The clear supernatant was used for quantitative estimation of catalase (CAT) activity and reduced glutathione (GSH) level.

Quantification of Reduced GSH Content in Testes

Mixed the tissue homogenate (100 µL) with 400 µL of phosphate buffer solution (PBS) (0.2 M, pH 7.4). To this 1.5 mL of DTNB (0.1 mM) solution was added. The mixture was incubated at room temperature for 10 min followed by recording absorbance at 412 nm. Total GSH concentration in the samples was determined based on the standard curve and used linear regression. The total GSH concentration was expressed as µM mg_{-1} protein. The background rate was determined used a sample blank lacking GSH or sample [21].

Determination of Catalase Activity in Tissue Homogenate

Mixed the tissue homogenate (0.1 mL) with 1.9 mL of PBS. One mL of H_2O_2 solution was added to the buffer. The decrease in extinction at 240 nm at 1 min with an interval of 3 min measured. Added 0.1 ml of tissue homogenate and 2.9 mL of the buffer added in the reference cuvette as ample control. Utilized the molar extinction coefficient at 43.6 to determine the catalase activity. In terms of millimoles of H_2O_2 consumed/min/mg of protein sample, we defined the specific activity at 25°C [22].

$$Millimoles\ of\ H2O2 consumed/min/mg\ of\ protein = \frac{mM\ H2O2 decomposed/min/mg\ protein\ (U/mg\ protein)\ DA/min x10}{43.6 x mg\ protein\ in\ sample}$$

Assay of Testicular 3β-hydroxysteroid dehydrogenase (HSD) Activity

The testicular activity of 3β-HSD was measured biochemically. At a tissue concentration of 100 mg/mL, the tissue was carefully homogenized while keeping a chilling condition at 4°C in 20% spectroscopic-grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA, and centrifuged at 10,000 g for 30 min at 4°C. The incubation mixture was made up of the supernatant (1 mL), 1 mL of 100 µM sodium pyrophosphate buffer (pH 8.9), 0.04 mL of ethanol containing 30 µg of dehydroepiandrosterone, and 0.96 mL of 25 milligram% bovine serum albumin. After adding 0.1 mL of 0.5 µM nicotinamide adenine dinucleotide to the tissue supernatant mixture in a spectrophotometer cuvette at 340 nm, enzyme activity was measured against a control (without nicotinamide adenine dinucleotide (NAD)). A change in absorbance of 0.001/min at 340 nm is equal to one unit of enzyme activity [23].

Assay of Testicular 17β-HSD Activity

The testicular activity of 17β-HSD was measured biochemically. For the evaluation of 17-HSD activity, the same supernatant as used for the 3β-HSD test (above) was used. This supernatant (1 mL) was combined with 3 ml of the incubation mixture, which included 0.96 milliliters of 25 mg % BSA, 0.04 ml of ethanol containing 0.3 µM testosterone, and 1 ml of 0.44 mM sodium pyrophosphate buffer (pH 10.2). After 0.1 mL of 0.5 µM NAD was added to the stirring mixture, the enzyme activity was assessed at 340 nm against a control. (without NAD). A change in absorbance of 0.001/min at 340 nm is equal to one unit of enzyme activity [23].

Serum Testosterone Assessment

Blood was collected by retro-orbital plexus route under light anesthesia. Measurement of serum testosterone level was done by chemiluminescence immunoassays (CLIA).

Histopathological Examinations of Testes

Testes tissue was taken and fixed in 10% formalin, embedded in paraffin wax. Sections were cut at 5 m thickness and stained with hematoxylin and eosin (H&E). The sections were then viewed under a microscope for histopathological changesin structures such as, seminiferous tubules (T), the presence of different kinds of germ cells, including spermatogonia (SG) primary spermatocytes (Pc), spermatids (St), and Sertoli cells (Sc), spermatozoa (SZ) in the lumen, interstitial tissue (IT) containing interstitial cells, and Leydig cells (Lc).

Statistical Analysis

All values are expressed as mean ± SD. One-way ANOVA with Dunnett's post-test was performed using GraphPad Prism version 8. Statistical values with $P < 0.05$ were considered statistically significant.

RESULTS

Effect on Testis Weight

Cyclophosphamide treatment resulted in a significant reduction in testis weight normalized against the body weight of the rats. Treatment with omega-3 fatty acid could preserve the testis's weight in the animals as indicated in Table 1.

Effect on Epididymal Sperm Characteristics

Epididymal sperm count in the DC group (28.33 ± 13.38×10^6/mL) was significantly lower in comparison with the NC group (138.00 ± 12.31×10^6/mL) indicating oligospermia associated with cyclophosphamide exposure. Sperm count in the T5%, T10%, and ω-3 group (59.00 ± 12.313×10^6/mL, 68.00 ± 11.815×10^6/mL, and 97.66 ± 17.918×10^6/mL, respectively) were significantly higher than the DC group, Standard treatment group (61.66 ± 18.545×10^6/mL) also demonstrated a significant improvement in sperm count as compared to disease control group as indicated in Figure 1A.

The percentage of abnormal sperm in the DC group (15.83 ± 2.639) increased significantly in comparison to the NC group (5.16 ± 0.753), while that in all the other treated groups were significantly improved as compared to the disease control group (6.33 ± 1.506, 11.33 ± 4.274, 10.16 ± 2.927, 6.83 ± 1.472 and 6.00 ± 1.414 in standard, T1%, T5%, T10%, and ω-3 group respectively) (Figure 1B).

Cyclophosphamide treatment resulted in a significant reduction in epididymal sperm motility and viability as observed in the rats of the DC group (32.00 ± 30.232% and 42.16 ± 21.507% respectively) compared to the NC group (87.16 ± 3.764% and 94.66 ± 1.211% respectively). All other treatment groups except T1% demonstrated significant improvement in motility (82.83 ± 2.701, 62.83 ± 8.519, 76.66 ± 6.022, 86.33 ± 4.719 in standard, T5%, T10%, and ω-3 treated groups respectively) as well as in viability (83.00 ± 5.177, 75.66 ± 7.448, 80.33 ± 8.017, 91.00 ± 3.286 in standard, T5%, T10%, and ω-3 treated groups respectively). (Figure 1 (C, D)).

EFFECT OF TEST TREATMENTS ON BIOCHEMICAL PARAMETERS

Effect on Oxidative Stress Parameters

The reduced glutathione (0.38 ± 0.081 in NC vs 0.13 ± 0.042 in DC) and catalase (7.32 ± 0.482 in NC vs 1.08 ± 0.148 in DC) levels in testicular tissue were reduced in disease-control rats pertaining to cyclophosphamide treatment. Only T5%, T10%, and omega-3 fatty acid treatment were able to reduce the oxidative stress associated with cyclophosphamide-induced oligospermia (GSH: 0.23 ± 0.054, 0.26 ± 0.040, 0.35 ± 0.080, respectively), (CAT: 4.31 ± 1.602, 5.35 ±1.499, 7.53 ±1.853, respectively). (Figure 2A, 2B).

Effect on Hormonal Parameters

A remarkable decrease in serum testosterone level was observed in the DC group as compared to the normal rats. A significant increase in testosterone levels was seen in the standard as well as ω-3 fatty acid-treated group and T10% group. A notable inhibition was seen in the testicular 3β-HSD and 17β-HSD enzyme activities in the DC group, as compared to that in the NC group. Both standard and ω-3 fatty acid treatment significantly increased these enzyme activities in respective animals as well as T10% group also increase the enzyme activities, but not as much as Std or ω-3 (Table 1).

Histopathological Examinations of Testes

The testes from the NC group of rats shows normal tissue architecture with seminiferous tubules (T), different kinds of germ cells, including spermatogonia (SG), primary spermatocytes (Pc), spermatids (St), and Sertoli cells (Sc), interstitial tissues (IT) containing interstitial cells and Leydig cells (Lc), and spermatozoa (SZ) present in the lumen. The testes section from DC rats shows vacuolation (V), a reduced number of germ cells, or oligospermia, and germ cells with pyknotic nuclei (PN). The testes section from the standard treatment group and

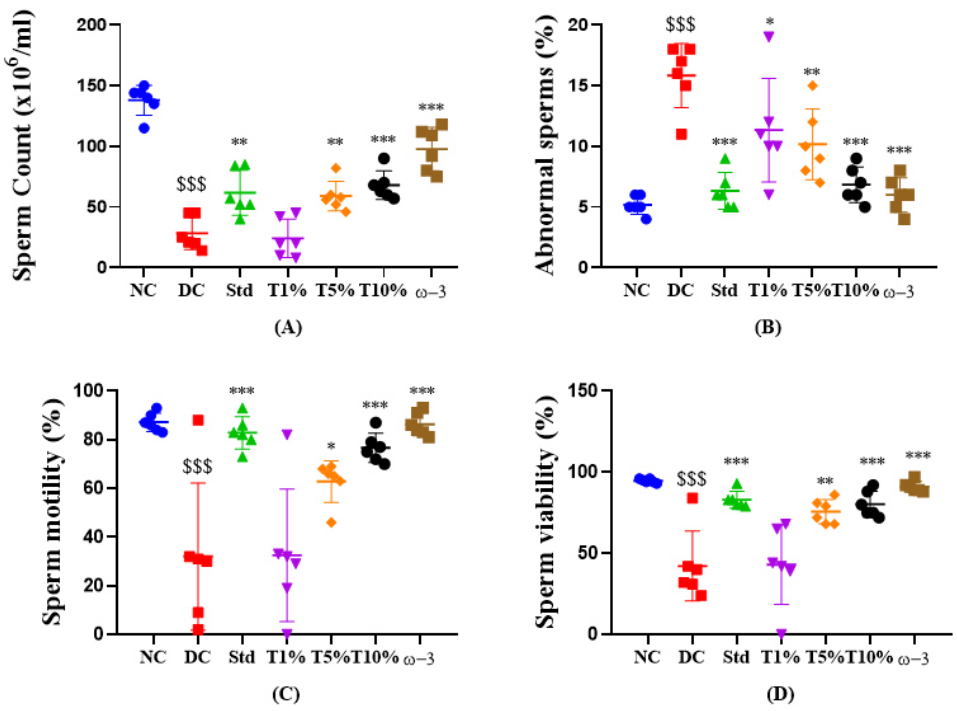


Figure 1 A,B,C,D. **Analysis of epididymal sperm characteristics**. Each value is the mean ± SD of six observations in each group. Data were analyzed by One-way ANOVA followed by Dunnet's Post hoc Analysis. $$$. (P<0.001, when compared to NC), *** (P<0.001, when compared to DC), ** (P<0.01), * (P<0.05) when compared to DC), NC: normal control group, DC: disease control group, Std: standard treatment, T1%: 1% chia seeds in diet, T5%: 5% chia seeds in diet, T10%: 10% chia seeds in diet, ω-3: omega-3 fatty acid.

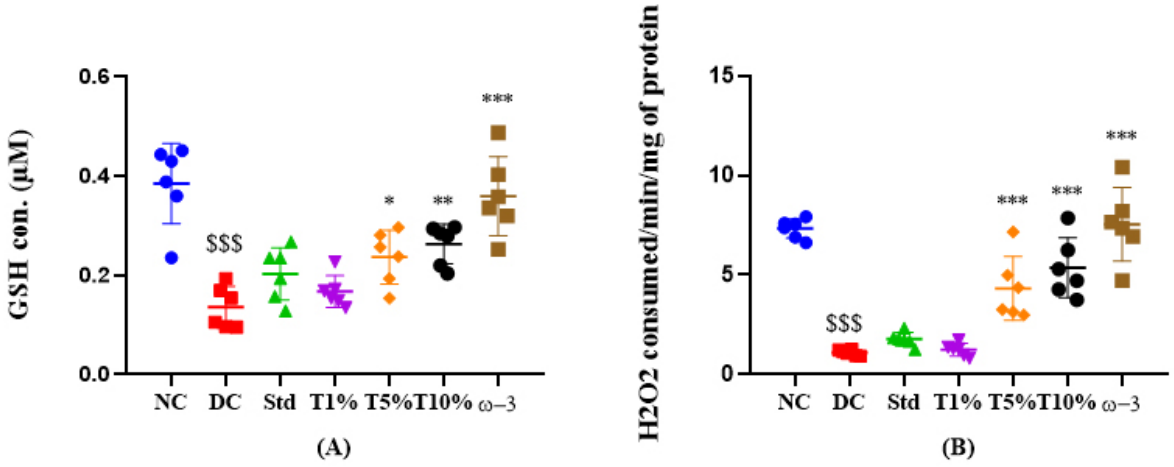


Figure 2 A,B. **Effect of test treatments on oxidative parameters (Reduced Glutathione and Catalase Activity) in rats**. Each value is the mean ± SD of six observations in each group. Data were analyzed by One-way ANOVA followed by Dunnet's Post hoc Analysis. $$$. (P<0.001, when compared to NC), *** (P<0.001, when compared to DC), ** (P<0.01), * (P<0.05) when compared to DC), NC: normal control group, DC: disease control group, Std: standard treatment, T1%: 1% chia seeds in diet, T5%: 5% chia seeds in diet, T10%: 10% chia seeds in diet, ω-3: omega-3 fatty acid.

Table 1. Effect of Test Treatments on Reproductive Organ Weight, Testosterone, 3β-HSD, and 17β-HSD Activity

Parameter	NC	DC	Std	T1%	T5%	T10%	ω-3
Testis (g)/body weight ratio	0.65±0.049	0.48±0.026^{aaa}	0.57±0.034^{b}	0.44±0.102	0.45±0.045	0.57±0.026^{b}	0.59±0.005^{bb}
Testosterone level (ng/mL)	1.95±0.015	0.62±0.239^{aaa}	1.51±0.153^{bbb}	0.65±0.165	0.40±0.068	0.97±0.160^{bb}	1.25±0.244^{bb}
3β-HSD (Units/mg tissue/h)	4.68±0.785	1.61±0.412^{aaa}	3.15±0.877^{bbb}	1.70±0.325	2.16±0.285	2.69±0.274^{bb}	2.98±0.319^{bbb}
17β-HSD (Units/mg tissue/h)	4.34±1.054	1.60±0.395^{aaa}	3.31±0.760^{bbb}	2.05±0.602	2.58±0.271^{b}	2.98±0.396^{bb}	3.39±0.351^{bbb}

Each value is the mean±SD of six observations in each group. Data were analyzed by One-way ANOVA followed by Dunnet's Post hoc Analysis. ^{aaa}(P<0.001, when compared to NC), ^{bbb}(P<0.001, when compared to DC), ^{bb}(P<0.01), ^{b}(P<0.05) when compared to DC), NC; normal control group, DC; disease control group, Std; standard treatment, T1%; 1% chia seeds in diet, T5%; 5% chia seeds in diet, T10%; 10% chia seeds in diet, ω-3; omega-3 fatty acid.

omega-3 fatty acid-treated group animals demonstrate well-preserved histological features as compared to the disease group. Similarly, test treatment also shows the preservation of histological structure in a dose-dependent manner. It would be important to note that omega-3 fatty acid-treated rat testes shows notably better histological features as compared to that of the 5% chia seed treatment group (Figure 3).

GENERAL OBSERVATIONS AND MORTALITY

The general health parameters were regularly observed throughout the period of the experiment. Signs of nasal and ocular bleeding were observed in several animals treated with chia seeds. Bleeding was observed in four animals of T1% chia seed-treated animals out of which mortality was observed in three animals. Bleeding and mortality were observed in animals belonging to the T5% and T10% test groups in two and three animals, respectively. The lost numbers of animals were replaced with new animals in the respective treatment groups to fulfill the minimum sample size of six animals per group. A total 50 animals were used in study.

DISCUSSION

Chemotherapy drugs, specifically alkylating agents, have been linked to reproductive toxicity due to their genotoxic nature. These drugs target spermatogenic cells, which have high mitotic activity and can cause damage during the early stages of spermatogenesis. As a result, strategies to minimize the side effects of cancer medications while maintaining their chemotherapeutic efficacy are highly anticipated.

CP is an anti-cancer and immunosuppressive therapy. The use of CP is limited by testicular toxicity, resulting in reproductive organ atrophy and reduced fertility in male patients receiving CP chemotherapy [24]. CP has been shown to cause defective spermatogenesis and altered histology of the testes, reducing sperm viability and motility in animals treated with CP [4,5,25].

CP treatment in our experiment caused a significant reduction in testes weight to body weight ratio. This reduction in testes weight is likely to be due to atrophy of seminiferous tubules, as observed in previous studies [26,27]. Treatment with 10% chia seed showed a significant improvement in this ratio, indicating a preserved testicular structure as compared to CP-treated animals.

The control of male gonadal function is governed by the hypothalamic–pituitary–gonadal axis. This begins with the hypothalamus secreting GnRH, prompting the pituitary to release FSH and LH. These hormones also regulate Leydig cell activity, which generates testoster-

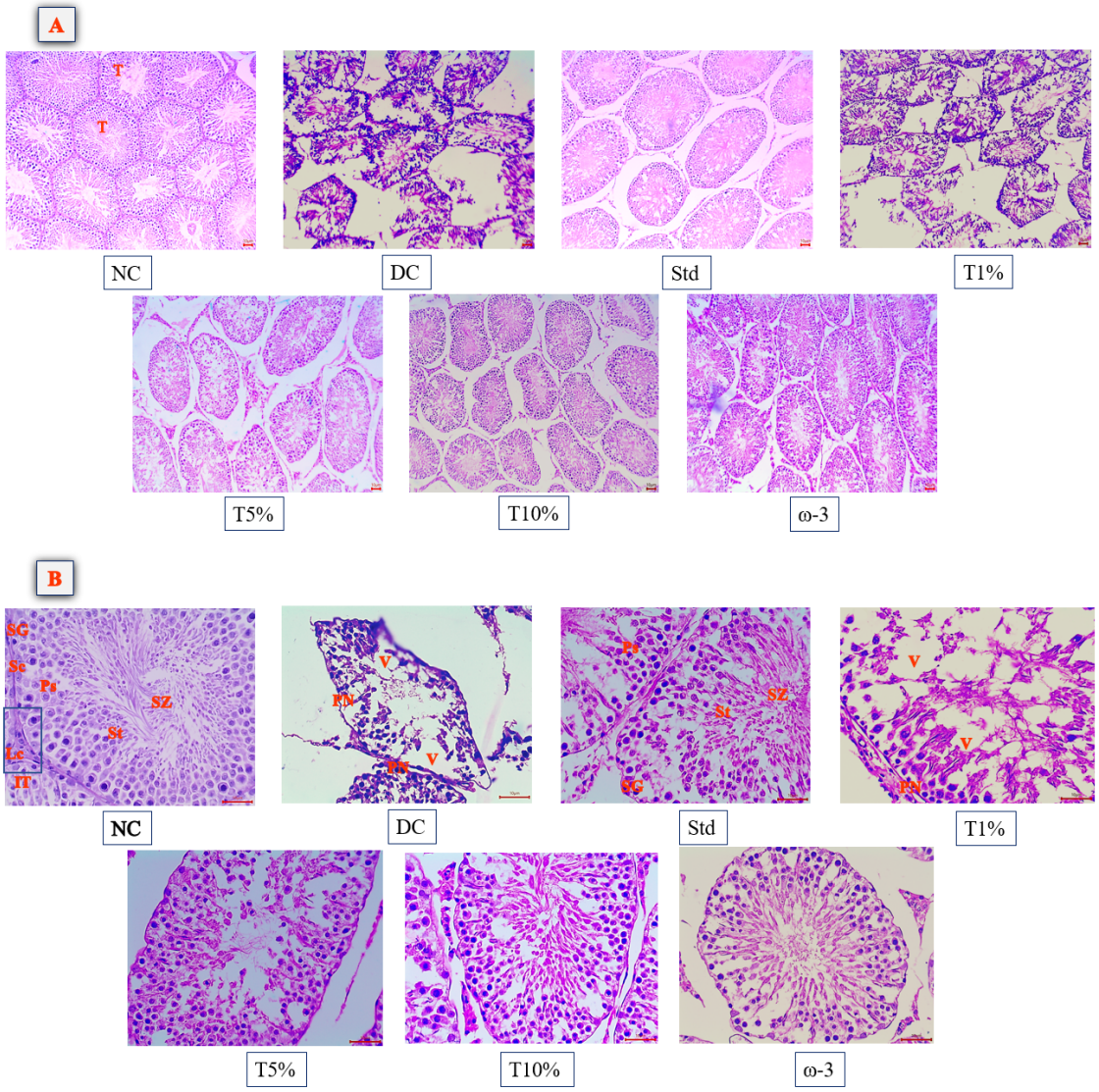


Figure 3 A,B. **Effect of test treatments on the histological aspects of testes in rats**. (**A**) H & E stain. Magnification 10X. Seminiferous tubules indicated as T (**B**) H & E stain. Magnification 40 X. Different kinds of germ cells, including spermatogonia (SG) primary spermatocytes (Pc), spermatids (St), and Sertoli cells (Sc). Interstitial tissue (IT) containing interstitial cells and Leydig cells (Lc). Spermatozoa (SZ) are present in the lumen, vacuolation (V), and Germ cells with pyknotic nuclei (PN).The red bar at right lower corner represents 10μM.

one in males [28]. CP substantially lowers testosterone levels, even at a single dose of 100 mg/kg [29,30]. Our investigation also unveiled CP's effects on both spermatogenesis and the pituitary–testicular axis as indicated by significant reduction in testosterone level in CP-treated group. Chia seed therapy significantly increased serum testosterone levels along with 17- and 3β-HSD activity. Similarly, quercetin [30], morin [31,32], rutin [33], and omega-3 fatty acids [34] are found to augment testosterone levels, backed by prior animal research. Thus, chia seeds' protective influences likely stem, at least partially, from their flavonoids and omega-3 fatty acids.

CP-treated animals showed elevated oxidative stress levels in the testes in our experiment, which is in line with previous studies showing oxidative damage induced by CP [35]. It interferes with the body's antioxidant defenses by generating significant quantities of reactive oxygen species along with its detrimental metabolite, acrolein. This leads to elevated oxidative stress, resulting in a notable decrease in total antioxidant capacity, glutathione peroxidase levels, and rise in malondialdehyde levels [36]. In our study, chia seed-treated rats especially higher dose group demonstrated a lower oxidative stress level as compared to that in the disease control group animals. Previous studies conducted with chia seeds have also demonstrated a strong antioxidant effect [37-39].

CP treatment disrupts the sperm characteristics of the testes pertaining to aforementioned mechanisms [40]. In our study, similar effects were observed in animals treated with CP. This group had lower sperm count, a higher number of abnormal sperm, and decreased sperm viability and motility. On the other hand, rats treated with chia seeds in their diet demonstrated improved sperm characteristics. This effect on sperm characteristics was also reflected in histopathological analysis. Omega-3 fatty acids administration as food component has been demonstrated to have reduced risk of asthenozoospermia in men [41,42]. Apart from this, flavonoids and polyphenols are also known to improve sperm quality and characteristics, as per previous studies [43-46].

As an attempt to determine, whether the protective effect of chia seeds is solely due to omega-3 fatty acids or other constituents, we treated one group of animals with a dose of omega-3 fatty acid equivalent to the medium dose (5% in diet) of chia seeds in our study. The omega-3 fatty acid group showed improvement in all parameters related to sperm characteristics. However, the effect observed with omega-3 fatty acid was far better than that observed in the equivalent dose of chia seeds (5% in diet). The reason for this observation may be associated with the adverse effect of bleeding observed in the chia seed-treated animals. Bleeding was observed in some of the animals treated with chia seeds. A similar adverse effect was observed with omega-3 fatty acid-treated animals, albeit with less severity. Upon literature review, it was noted that omega-3 fatty acids are known to have anti-platelet effects [47-49]. In addition to omega-3 fatty acids, chia seeds have several other phenolic compounds such as Hydroxytyrosol acetate, Danshensu, Ferulic acid, *p*-Coumaric acid derivative, Rosmarinic acid, and Carnosol, which are demonstrated to have anti-platelet effects in previous studies [50-56]. Thus, the anti-platelet effect associated with chia seeds was found to be more potent than that observed in omega-3 fatty acid-treated animals.

CP causes bone marrow depression increasing the risk of bleeding. Chia seed use in rats treated with CP resulted in an adverse drug interaction manifested as bleeding. Thus, although, chia seeds demonstrated a good effect on oligospermia in animals, they cannot be recommended for use by patients undergoing CP treatment (or any other cytotoxic chemotherapeutic drug) without further investigation. Further studies assessing the safety of chia seed use with respect to its anti-platelet effect are needed to be done.

CONCLUSIONS

In our study, chia seeds as well as omega-3 fatty acids treatment were found to be protective against cyclophosphamide-induced reproductive toxicity in male rats. However, the adverse effect of hemorrhage associated with drug interaction of chia seeds with cytotoxic chemotherapeutic drugs need careful attention and further investigation.

REFERENCES

1. Global Cancer Observatory - Tomorrow's Cancer Incidence and Mortality Estimates, Worldwide, 2025 [Internet]. Global Cancer Observatory. [cited 2023 Jun 12]. Available from: [https://gco.iarc.fr/tomorrow/en/dataviz/isotype?types=1&single_unit=500000&years=2025].
2. Howell SJ, Shalet SM. Testicular function following chemotherapy. Hum Reprod Update. 2001;7(4):363–9.
3. Ahmed AR, Hombal SM. Cyclophosphamide (Cytoxan). A review on relevant pharmacology and clinical uses. J Am Acad Dermatol. 1984 Dec;11(6):1115–26.
4. LiverTox: Clinical and Research Information on Drug-Induced Liver Injury [Internet]. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012–. Alkylating Agents. 2015 Mar 10.
5. Aguilar-Mahecha A, Hales BF, Robaire B. Acute cyclophosphamide exposure has germ cell specific effects on the expression of stress response genes during rat spermatogenesis. Mol Reprod Dev. 2001 Nov;60(3):302–11.
6. Trasler JM, Hales BF, Robaire B. A time-course study of chronic paternal cyclophosphamide treatment in rats: effects on pregnancy outcome and the male reproductive and hematologic systems. Biol Reprod. 1987 Sep;37(2):317–26.
7. Trasler JM, Hermo L, Robaire B. Morphological changes in the testis and epididymis of rats treated with cyclophosphamide: a quantitative approach. Biol Reprod. 1988 Mar;38(2):463–79.
8. Trasler JM, Robaire B. Effects of cyclophosphamide on selected cytosolic and mitochondrial enzymes in the epididymis of the rat. J Androl. 1988;9(2):142–52.
9. Hoorweg-Nijman JJ, Delemarre-van de Waal HA, de Waal FC, Behrendt H. Cyclophosphamide-induced disturbance of gonadotropin secretion manifesting testicular damage. Acta Endocrinol (Copenh). 1992 Feb;126(2):143–8.
10. Connolly KJ, Edelmann RJ, Cooke ID, Robson J. The impact of infertility on psychological functioning. J Psychosom Res. 1992 Jul;36(5):459–68.
11. Khalid W, Arshad MS, Aziz A, Rahim MA, Qaisrani TB, Afzal F, et al. Chia seeds (Salvia hispanica L.): A therapeutic weapon in metabolic disorders. Food Sci Nutr. 2022 Dec;11(1):3–16.
12. Tan H, Zhen W, Bai D, Liu K, He X, Ito K, et al. Effects of dietary chlorogenic acid on intestinal barrier function and the inflammatory response in broilers during lipopolysaccharide-induced immune stress. Poult Sci. 2023 May;102(5):102623.
13. Khan FA, Maalik A, Murtaza G. Inhibitory mechanism against oxidative stress of caffeic acid. Yao Wu Shi Pin Fen Xi. 2016 Oct;24(4):695–702.
14. Taheri Y, Suleria HA, Martins N, Sytar O, Beyatli A, Yeskaliyeva B, et al. Myricetin bioactive effects: moving from preclinical evidence to potential clinical applications. BMC Complement Med Ther. 2020 Aug;20(1):241.

15. Xu D, Hu MJ, Wang YQ, Cui YL. Antioxidant activities of quercetin and its complexes for medicinal application. Molecules. 2019 Mar;24(6):1123.
16. Sharma N, Biswas S, Al-Dayan N, Alhegaili AS, Sarwat M. Antioxidant role of kaempferol in prevention of hepatocellular carcinoma. Antioxidants. 2021 Sep;10(9):1419.
17. Abbott K, Burrows TL, Acharya S, Thota RN, Garg ML. Dietary supplementation with docosahexaenoic acid rich fish oil increases circulating levels of testosterone in overweight and obese men. Prostaglandins Leukot Essent Fatty Acids. 2020 Dec;163:102204.
18. Falsig AL, Gleerup CS, Knudsen UB. The influence of omega-3 fatty acids on semen quality markers: a systematic PRISMA review. Andrology. 2019 Nov;7(6):794–803.
19. Xiaotong M, Libin L, Yixuan M, Kadier A, Shengbin B. Immunohistochemical Identification of GM-CSF in Testis of Rats with Oligozoospermia with the Intervention of Cuscuta chinensis Flavonoids. Int J Morphol. 2020 Jun;38(3):761–5.
20. Watcho P, Mpeck IR, Deeh Defo PB, Wankeu-Nya M, Ngadjui E, Bonsou Fozin GR, et al. Cyclophosphamide-induced reproductive toxicity: beneficial effects of Helichrysum odoratissimum (Asteraceae) in male Wistar rats. J Integr Med. 2019 Sep;17(5):366–73.
21. Rahman I, Kode A, Biswas SK. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nat Protoc. 2006;1(6):3159–65.
22. Parimelazhagan T, Thangaraj P. In Vivo Antioxidant Assays. Pharmacol Assays Plant-Based Nat Prod; 2016. pp. 89–98.
23. Mukhopadhyay PK, Dey A, Mukherjee S, Pradhan NK. The effect of coadministration of α-tocopherol and ascorbic acid on arsenic trioxide-induced testicular toxicity in adult rats. J Basic Clin Physiol Pharmacol. 2013;24(4):245–53.
24. Stork CM, Schreffler SM. Cyclophosphamide, Editor(s): Philip Wexler, Encyclopedia of Toxicology (Third Edition), Academic Press, 2014, Pages 1111-1113, ISBN 9780123864550.
25. Özbilgin MK, Demirören S, Üçöz M, Oztatlici M. Cyclophosphamide suppresses spermatogenesis in the testis of mice through downregulation of miR-34b and miR-34c. Andrologia. 2021 Aug;53(7):e14071.
26. Higuchi H, Nakaoka M, Ozaki K, Kawamura S, Okuno Y, Matsuo M, et al. Evaluation of recovery from cyclophosphamide testicular toxicity in rats. J Toxicol Pathol. 1997 Sep;10(3):165–73.
27. Abd El Tawab AM, Shahin NN, AbdelMohsen MM. Protective effect of Satureja montana extract on cyclophosphamide-induced testicular injury in rats. Chem Biol Interact. 2014 Dec;224:196–205.
28. Okon UA, Utuk II; Nigerian Medical Journal. Ascorbic acid treatment elevates follicle stimulating hormone and testosterone plasma levels and enhances sperm quality in albino Wistar rats. Niger Med J. 2016;57(1):31–6.
29. Akomolafe SF, Aluko BT. Protective effect of curcumin on fertility in cyclophosphamide exposed rats: involvement of multiple pathways. J Food Biochem. 2020 Jan;44(1):e13095.
30. Ebokaiwe AP, Obasi DO, Njoku RC, Osawe S. Cyclophosphamide-induced testicular oxidative-inflammatory injury is accompanied by altered immunosuppressive indoleamine 2, 3-dioxygenase in Wister rats: influence of dietary quercetin. Andrologia. 2022 Apr;54(3):e14341.
31. Arisha AH, Ahmed MM, Kamel MA, Attia YA, Hussein MM. Morin ameliorates the testicular apoptosis, oxidative stress, and impact on blood-testis barrier induced by photo-extracellularly synthesized silver nanoparticles. Environ Sci Pollut Res Int. 2019 Oct;26(28):28749–62.
32. Hussein MM, Gad E, Ahmed MM, Arisha AH, Mahdy HF, Swelum AA, et al. Amelioration of titanium dioxide nanoparticle reprotoxicity by the antioxidants morin and rutin. Environ Sci Pollut Res Int. 2019 Oct;26(28):29074–84.
33. Abarikwu SO, Otuechere CA, Ekor M, Monwuba K, Osobu D. Rutin ameliorates cyclophosphamide-induced reproductive toxicity in male rats. Toxicol Int. 2012 May;19(2):207–14.
34. Uygur R, Aktas C, Tulubas F, Uygur E, Kanter M, Erboga M, et al. Protective effects of fish omega-3 fatty acids on doxorubicin-induced testicular apoptosis and oxidative damage in rats. Andrologia. 2014 Oct;46(8):917–26.
35. Ekeleme-Egedigwe CA, Famurewa AC, David EE, Eleazu CO, Egedigwe UO. Antioxidant potential of garlic oil supplementation prevents cyclophosphamide-induced oxidative testicular damage and endocrine depletion in rats. J Nutr Intermed Metab. 2019 Dec;18:100109.
36. Salimnejad R, Rad JS, Nejad DM. Protective effect of ghrelin on oxidative stress and tissue damages of mice testes followed by chemotherapy with cyclophosphamide. Crescent J Med Biol Sci. 2018 Jan;5:138–43.
37. Capitani ME, Spotorno V, Nolasco SM, Tomás MC. Physicochemical and functional characterization of by-products from chia (Salvia hispanica L.) seeds of Argentina. Lebensm Wiss Technol. 2012 Jan;45(1):94–102.
38. Reyes-Caudillo E, Tecante A, Valdivia-Lopez MA. Dietary fibre content and antioxidant activity of phenolic compounds present in Mexican chia (Salvia hispanica L.) seeds. Food Chem. 2008 Mar;107(2):656–63.
39. Creus A, Chicco A, Álvarez SM, Giménez MS, de Lombardo YB. Dietary Salvia hispanica L. reduces cardiac oxidative stress of dyslipidemic insulin-resistant rats. Appl Physiol Nutr Metab. 2020 Jul;45(7):761–8.
40. Khamis T, Hegazy AA, El-Fatah SS, Abdelfattah ER, Abdelfattah MM, Fericean LM, et al. Hesperidin Mitigates Cyclophosphamide-Induced Testicular Dysfunction via Altering the Hypothalamic Pituitary Gonadal Axis and Testicular Steroidogenesis, Inflammation, and Apoptosis in Male Rats. Pharmaceuticals (Basel). 2023 Feb;16(2):301.
41. Eslamian G, Amirjannati N, Rashidkhani B, Sadeghi MR, Baghestani AR, Hekmatdoost A. Adherence to the Western pattern is potentially an unfavorable indicator of asthenozoospermia risk: a case-control study. J Am Coll Nutr. 2016;35(1):50–8.
42. Eslamian G, Amirjannati N, Rashidkhani B, Sadeghi MR, Baghestani AR, Hekmatdoost A. Dietary fatty acid intakes and asthenozoospermia: a case-control study. Fertil Steril. 2015 Jan;103(1):190–8.
43. Yelumalai S, Giribabu N, Karim K, Omar SZ, Salleh NB. In vivo administration of quercetin ameliorates sperm ox-

idative stress, inflammation, preserves sperm morphology and functions in streptozotocin-nicotinamide induced adult male diabetic rats. Arch Med Sci. 2019 Jan;15(1):240–9.
44. Cheraghi E, Sajadi SM, Soleimani Mehranjani M. The effect of Quercetin on the quality of sperm parameters in frozen-thawed semen of patients with Asthenospermia. Andrologia. 2021 Oct;53(9):e14167.
45. Ye RJ, Yang JM, Hai DM, Liu N, Ma L, Lan XB, et al. Interplay between male reproductive system dysfunction and the therapeutic effect of flavonoids. Fitoterapia. 2020 Nov;147:104756.
46. Tvrdá E, Benko F, Slanina T, du Plessis SS. The role of selected natural biomolecules in sperm production and functionality. Molecules. 2021 Aug;26(17):5196.
47. Gao LG, Cao J, Mao QX, Lu XC, Zhou XL, Fan L. Influence of omega-3 polyunsaturated fatty acid-supplementation on platelet aggregation in humans: a meta-analysis of randomized controlled trials. Atherosclerosis. 2013 Feb;226(2):328–34.
48. Adili R, Hawley M, Holinstat M. Regulation of platelet function and thrombosis by omega-3 and omega-6 polyunsaturated fatty acids. Prostaglandins Other Lipid Mediat. 2018 Nov;139:10–8.
49. Gong Y, Lin M, Piao L, Li X, Yang F, Zhang J, et al. Aspirin enhances protective effect of fish oil against thrombosis and injury-induced vascular remodelling. Br J Pharmacol. 2015 Dec;172(23):5647–60.
50. González-Correa JA, Navas MD, Muñoz-Marín J, Trujillo M, Fernández-Bolaños J, de la Cruz JP. Effects of hydroxytyrosol and hydroxytyrosol acetate administration to rats on platelet function compared to acetylsalicylic acid. J Agric Food Chem. 2008 Sep;56(17):7872–6.
51. Yu C, Qi D, Lian W, Li QZ, Li HJ, Fan HY. Effects of danshensu on platelet aggregation and thrombosis: in vivo arteriovenous shunt and venous thrombosis models in rats. PLoS One. 2014 Nov;9(11):e110124.
52. Shuai S, Yue G. Ferulic Acid, A Potential Antithrombotic Drug. J Lung Health Dis. 2018 May;2(2):25–8.
53. Ekowati J, Diyah NW, Syahrani A. Synthesis and antiplatelet activities of some derivatives of p-coumaric acid. Chem Chem Technol. 2019;13(3):296–302.
54. Chen C, Ma J, Xu Z, Chen L, Sun B, Shi Y, et al. Rosmarinic Acid Inhibits Platelet Aggregation and Neointimal Hyperplasia In Vivo and Vascular Smooth Muscle Cell Dedifferentiation, Proliferation, and Migration In Vitro via Activation of the Keap1-Nrf2-ARE Antioxidant System. J Agric Food Chem. 2022 Jun;70(24):7420–40.
55. Song Y, Yan H, Chen J, Wang Y, Jiang Y, Tu P. Characterization of in vitro and in vivo metabolites of carnosic acid, a natural antioxidant, by high performance liquid chromatography coupled with tandem mass spectrometry. J Pharm Biomed Anal. 2014 Feb;89:183–96.
56. Lee JJ, Jin YR, Lim Y, Hong JT, Kim TJ, Chung JH, et al. Antiplatelet activity of carnosol is mediated by the inhibition of TXA2 receptor and cytosolic calcium mobilization. Vascul Pharmacol. 2006 Sep;45(3):148–53.