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Original Research

Fertility enhancing efficacy of *Cicer arietinum* in male albino mice

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Abstract: The present study was conducted to investigate the effect of incorporating *Cicer arietinum* (*C. arietinum*) in the diet on the testicular functions of the male mice. Seventy-two mice were divided equally into four groups that were daily fed a diet containing 0, 20, 30 and 50% of *C. arietinum* seeds, respectively. After 7, 14 and 21 days of starting the experiments, the mice were anesthetized and euthanized to collect the blood, testes, epididymis and seminal vesicles. The present results showed that the increased percentage of *C. arietinum* in the diet caused significant elevations in the serum levels of testosterone and luteinizing hormone (LH), sperm concentration, sperm motility as well as the testicular levels of antioxidants including glutathione (GSH), glutathione peroxidase (GPx) and catalase (CAT), in comparison to the controls. On the other hand, marked reductions in the sperm abnormality, testicular levels of malondialdehyde (MDA), the percentage of DNA damage in tail and tail moment (TM) were observed in the mice that received a diet containing *C. arietinum* as compared to the controls. Both the sperms and testes of the mice fed a diet containing *C. arietinum* in the diet showed a normal intact appearance of the electrophoresed genomic DNA on agarose, as those of the controls. In conclusion, *C. arietinum* is not only a safe ingredient in the fast-food but also an enhancer of the testicular functions.

Key words: *Cicer arietinum*; Mice; Fertility; Testis; Antioxidants; Genetic safety.

Introduction

Male infertility has become an international problem in the last decades. According to the World Health Organization (WHO), about 10-15% of the young human couples suffer from the infertility, with 50% of the infertility cases were related to the male factors (1). Normally, estrogen is necessary for the normal activity of the male reproductive tract (2). But, the intake of an exogenous estrogen or the inhibition of the endogenous estrogen production prompts the structural and functional alterations in the male reproductive system. Atanassova *et al*. (3) and Akondi *et al*. (4) clarified that the exposure of neonatal rats to the exogenous estrogenic compounds caused a significant decline in the plasma level of testosterone, sperm concentration, Sertoli cell number, rete tubule distension leading to the loss of fertility.

Today, soya and soy products are widely consumed by humans especially in the fast-food due to their edible taste and high protein contents (5). Unfortunately, soy foods are rich in phytoestrogens (isoflavones) that can bind to and activate estrogen receptors leading to negative effects on the male reproductive system (6). Indeed, Robertson *et al*. (7) demonstrated that the consumption of phytoestrogen-rich food could influence the process of spermatogenesis. Modaresi *et al*. (8) reported that a diet containing soy with the percentages of 20, 30 and 50% caused significant declines in the serum testosterone levels and sperm count of mice. One of the main causes of male infertility is the oxidative stress (OS) phenomena that is induced by the high consumption of a diet containing phytoestrogens (9,10). This may be attributed to their adverse effects on the testicular antioxidant system and consequently, the sperms become more susceptible to the damage by the reactive oxygen species (4). Aitken and Roman (11) demonstrated that the OS disrupted the spermatogenesis via the oxidation of proteins, lipids and DNA of germ cells lineage. These critical alterations eventually led to the DNA mutation, germinal cell apoptosis, development of persistent testicular atrophy that ultimately resulted in long-lasting azoospermia and infertility (12). Thus, there is an urgent demand to explore new safe source(s) of protein to replace the soy products in junk foods.

While many studies have demonstrated the effects of soy or soy derivatives on testicular function and fertility of the adult male mice or rats (4,5,8,13,14), little or no studies have investigated the effect of *C. arietinum* on the testicular function and spermatogenesis of the adult male mice. *C. arietinum,* chickpea is a member of pulse crop that belongs to Fabaceae (Leguminosae) family and named as Hummus (15). It is widely used in traditional medicines in the management of wounds and dropsy as well as in the treatment of diabetes and osteoporosis due to its antidiabetic and anti-osteoporotic activities (16, 17). Therefore, the present work aimed to evaluate the effect of *C. arietinum* seeds on the testicular function of male mice for many reasons. Firstly, *C. arietinum* is rich in proteins (25-29%) as recorded by Sajja *et al*. (18). Secondly, it contains isoflavones which have estrogenic and androgenic activities (17, 19, 20). Thirdly, it has been evidenced that *C. arietinum* has an aphrodisiac activity that may enhance semen quality and therefore provoke the fertility potential of males. Fourthly, its bioactive constituents such as saponins, phytosterols, terpenoids, flavonoids, alkaloids and tannins which have antioxidant activity (17). Additionally, Oyedeji and Bolarinwana (21) mentioned that most of

the herbal aphrodisiacs have little or no side effects. It was reported that *C. arietinum* methanolic extract is safe up to 5000 mg/kg body weight (17). Therefore, the present study was designed to investigate the effects of *C. arietinum* on testicular functions of adult male mice.

Materials and Methods

Chickpea

Cicer arietinum seeds were obtained from cultivar 'Giza 1', Agricultural Research Center, Giza, Egypt.

Diet preparation

The seeds of *C. arietinum* were grounded and mixed with the commercial feed of mice with the following percentiles: 0, 20, 30 and 50% w/w.

Diet components

The ash, moisture, crude fiber, crude protein, carbohydrate and lipid contents were measured according to the method described by the Association of Official Analytical Chemists, AOAC (22).

Animal care and housing

The protocol of the present study was approved by the Institutional Animal Care and Use Committee (IACUC) of Cairo University, Egypt under the IACUC Permit Number of CUFS/F/PHY/23/15. All the experimental procedures were carried out in accordance with the international guidelines for the care and use of laboratory animals.

In the present work, adult healthy male albino mice were used as an experimental model. Seventy-two mice weighting 22±3 g were obtained from the animal house of National Research Center (NRC), Giza, Arab Republic of Egypt (ARE). Before the beginning of the experiments, mice were kept in the animal house of Zoology Department, Faculty of Science, Cairo University, in polyacrylic cages with six animals per cage. Animals were supplied with feed and water *ad libitum*. In the animal house, mice were subjected to a 12h/12h light-dark cycle at room temperature (22–25°C). The acclimatization period of animals lasted for 7 days before commencement of the main experiments.

Experimental design and treatment regime

The experimental design of the present study is demonstrated in figure (1).

Autopsy schedule and sample collection

At the end of each experimental week, 6 mice were randomly taken from each experimental group. Mice were weighted and then euthanized using an overdose of sodium pentobarbital. Blood samples were collected into dry test tubes and allowed to stand for 30 minutes at room temperature then centrifuged at 3000 rpm for the separation of serum. The serum was kept at -20°C for further hormonal assay. Mice were immediately dissected for the removal of testes, seminal vesicles and epididymis. The removed tissues were weighted, and the weights were expressed in mg.

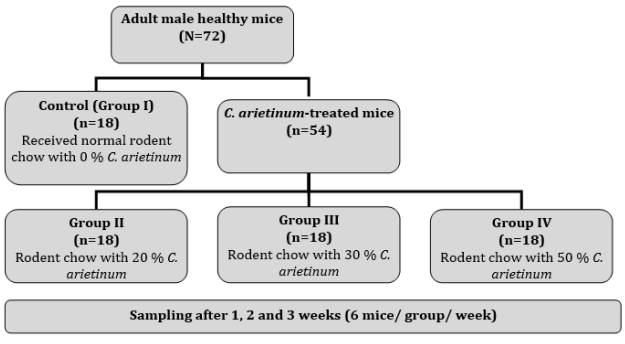

Figure 1. The experimental design of the present study.

Determination of the reproductive hormones

The serum levels of testosterone, FSH and LH were assayed through enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The concentrations of testosterone, FSH and LH were recorded automatically by a microplate reader (ELISA Reader, BioTek ELx 808, USA) and were expressed as ng/mL, mU/mL and mU/mL, respectively.

Determination of seminal vesicle fructose content

Seminal vesicles were homogenized in 0.1 M acetate buffer at pH 6.2 and centrifuged at 10,000 rpm. The levels of fructose were measured in the supernatant according to the method described by Foreman *et al*. (23).

Sperm collection and sperm quality analysis

The left cauda epididymis was removed and minced in Petri dish containing 2mL saline to liberate sperms. A drop of the resultant sperm suspension was placed in the cell counting chambers of the hemocytometer and was examined using a light microscope. The total number of sperms and the number of motile sperms were counted as described by Watanabe and Endo (24). The sperm concentration and motility rate were calculated using the following equations:

Sperm concentration = The total number of sperm / ($4 \times 10^4 \times 2$).

Sperm motility rate = (The number of motile sperm / the total number of sperm) × 100.

To assess sperm abnormality, the epidydimal filtrate was smeared on a slide and then was fixed with methanol for 10 min. Slides were stained with 1% eosin for 1 hour and then were washed with water. One thousand sperms were counted to determine the proportion of malformed sperm using a high-magnification microscope:

Sperm abnormality rate= (The number of malformed sperms / 1000) × 100.

Determination of the testicular lipid peroxidation and endogenous antioxidants

For the determination of malondialdehyde (MDA) content, a small part of testes was homogenized in 50 mM cold potassium phosphate buffer (PBS, pH 7.5), and then was centrifuged at 4000 rpm for 15 minutes. The level of MDA was measured in the testicular supernatant as described by Ohkawa *et al*. (25).

Another portion of testes was used for assaying ascorbic acid (AA) after homogenization in 5mM cold PBS (pH 7.4) containing 0.9% sodium chloride and 0.1%glucose, then centrifugation at 9000 rpm for 15 minutes at 4°C. The AA content was estimated in the resultant supernatant according to the method designated by Harris and Ray (26).

The remaining part of testes was homogenized in 50mM cold PBS (pH 7.5) containing 1mM EDTA, then centrifugated at 9000 rpm for 15 minutes at 4°C. The produced supernatant was used for measuring the glutathione (GSH) content and the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) according to the methods described by Beutler *et al*. (27), Nishikimi *et al*. (28), Paglia and Valentine (29) and Aebi (30), respectively.

The levels of MDA, AA and GSH as well as the activities of SOD, GPx and CAT were assayed using Biodiagnostics kits (Dokki, Giza, Egypt).

Comet assay

The alkaline comet assay was executed to detect single-strand breaks in both the sperms and the testicular cells. Sperms obtained from epididymis were suspended into the mincing solution, while a small piece of the testis (20 mg) was homogenized gently into the mincing solution. According to the method described by Tice *et al*. (31), about 10,000 cells were mixed with 75 µL of 0.5% low melting point agarose (Sigma) and were spread on a fully frosted slide pre-dipped in a normal melting agarose (1%). After solidification, cells were lysed in a cold lysis buffer (2.5M NaCl, 100mM EDTA, and 10mM Tris buffer, pH 10) with freshly added 10% DMSO and 1% Triton X-100 for 24 h at 4°C in dark. Subsequently, the slides were incubated in a fresh alkaline buffer (300mM NaOH and 1mM EDTA, pH 13) for 20 min. The unwinding DNA was subjected to electrophoresis for 20 minutes at 300 mA and 25V (0.90 V/cm), then neutralization in 0.4M Trizma base (pH7.5), followed by fixation in 100% cold ethanol, air-drying and finally storage at room temperature until the scoring. The extent of DNA migration for each sample was determined by simultaneous image capture and scoring of 100 cells stained with ethidium bromide at ×400 magnification using Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK). The extent of DNA damage was evaluated using tail length (TL), % tail DNA, and tail moment (TM) as DNA damage endpoints.

Laddered DNA fragmentation assay

DNA fragmentation assay in the sperms and testes was applied for the estimation of the apoptotic cells. According to the protocol of Sriram *et al*. (32), cells were lysed in Tris-EDTA (TE) lysis buffer containing 0.5% sodium dodecyl sulfate. A 0.5 mg/mL RNase A was added to the resultant lysate and then was incubated at 37°C for 1 h. Finally, a 0.2 mg/mL proteinase K was added to the sample and then incubated at 50°C overnight. Phenol extraction of DNA was followed by the precipitation reaction using 7.5 M ammonium acetate and isopropanol. DNA was separated by electrophoresis in 1% agarose gel at 70 V and visualized using a UV trans-illuminator and photography.

Statistical analyses

The present data were statistically analyzed by IBM Statistical Package for the Social Sciences version 22 (copyright by IBM SPSS software, US). The raw data were normally distributed as manifested by Shapiro-Wilk and Kolmogorov-Smirnov tests (33). Thus, the parametric statistical analyses were recommended. One-way analysis of variance (ANOVA) was used to study the effect of *C. arietinum* content in the mice feed on its proximate chemical composition. Two-ways ANOVA was applied to test the effect of experimental periods, *C. arietinum* intake and their interaction on the studied parameters of male albino mice. Post-*hoc* Dunnett's test was applied to illustrate the significant differences in the studied parameters. To fit the relationships among the studied variables, Pearson's correlation coefficient and regression analyses were applied. Data were expressed as a mean ± standard error of the mean (SEM).

Results

Effect of *Cicer arietinum* on the chemical composition of mice feed

Table (1) showed that the amount of *C. arietinum* in the diet significantly affected its contents of the ash, crude fibres, carbohydrates and lipids whereas insignificantly influenced the moisture and crude proteins contents. The levels of *C. arietinum* in the diet were correlated positively with the diet content of the carbohydrates and lipids whereas inversely with the crude fibres and ash contents.

Effect of *Cicer arietinum* on the weights of the whole body and sex organs

The weights of the whole body, testes, epididymis and seminal vesicle were displayed in table 2. In comparison to the controls, there were insignificant changes in the total body weight as well as in the weights of the reproductive organs of groups II, III and IV. In all the experimental groups, after two and three weeks of starting the experiments, the weights of the whole body, testes, epididymis and seminal vesicle were insignifi-

Table 1. The proximate chemical composition of the mice feed after the inclusion of 0, 20, 30 and 50% of *C. arietinum* (% on dry weight basis).

Parameters	Percentage of *C. arietinum* in feed				One-way ANOVA (Effect of *C. arietinum*)
	0%	20%	30%	50%	
Moisture (%)	8.82±0.55	8.78±0.59	9.33±0.19	9.42±0.48	$F_{3,8}$=0.49, P>0.05, r^C=+0.36
Ash (%)	13.04±0.60	11.60±0.46	11.25±0.43	9.13±0.15*	$F_{3,8}$=13.26, P<0.01, r^C =-0.88
Crude Fibers (%)	21.67±0.96	19.40±0.58	16.87±0.56*	15.69±0.46*	$F_{3,8}$=15.94, P<0.01, r^C =-0.92
Crude Proteins (%)	14.88±0.57	15.13±0.30	16.10±0.64	16.98±0.68	$F_{3,8}$=2.90, P>0.05, r^C =+0.70
Carbohydrates (%)	37.99±1.15	43.54±0.87*	44.55±1.45*	47.51±1.67*	$F_{3,8}$=9.09, P<0.01, r^C =+0.84
Lipids (%)	1.50±0.06	1.87±0.08	1.95±0.13	3.14±0.16*	$F_{3,8}$=40.92, P<0.001, r^C =+0.88

P<0.01 and P<0.001: significant effects. *: represents significant difference (P<0.05) as compared to the normal feed with 0% *C. arietinum*. r: the correlation coefficient of the concentrations of the studied parameter with the *C. arietinum* content (^{C}).

Table 2. The weights of whole body, testes, epididymis and seminal vesicles of group I, II, III and IV, throughout the three weeks of experiments.

Parameter	Time (days)	Experimental group				Two-ways ANOVA		
		I	II	III	IV	Time	*C. arietinum*	Interaction
Total body weight (g)	7	21.14±0.60	21.79±1.55	22.92±1.19	22.32±0.87	$F_{2,60}$=1.576, P>0.05, r^E= +0.20	$F_{3,60}$=1.582, P>0.05, r^C= +0.21	$F_{6,60}$=0.034, P>0.05 ---
	14	21.64±0.50	22.06±0.78	23.22±1.21	23.17±0.89			
	21	22.68±1.61	22.76±0.40	24.32±0.68	23.62±1.38			
Testes weight (mg)	7	42.91±1.21	43.96±3.30	47.29±2.49	45.32±1.76	$F_{2,60}$=1.434, P>0.05, r^E= +0.20	$F_{3,60}$=2.013, P>0.05, r^C= +0.21	$F_{6,60}$=0.040, P>0.05 ---
	14	43.50±0.89	44.78±1.59	47.14±2.46	47.04±1.80			
	21	45.72±3.08	46.35±0.70	49.37±1.39	47.95±2.81			
Epididymis weight (mg)	7	20.41±0.58	20.91±1.57	22.42±1.18	21.56±0.84	$F_{2,60}$=1.387, P>0.05, r^E= +0.20	$F_{3,60}$=1.906, P>0.05, r^C= +0.21	$F_{6,60}$=0.032, P>0.05 ---
	14	20.85±0.50	21.30±0.76	22.42±1.17	22.38±0.86			
	21	21.72±1.45	21.98±0.39	23.48±0.66	22.81±1.34			
Seminal Vesicle weight (mg)	7	18.30±0.52	18.75±1.41	20.10±1.06	19.33±0.75	$F_{2,60}$=1.459, P>0.05, r^E= +0.20	$F_{3,60}$=1.766, P>0.05, r^C= +0.21	$F_{6,60}$=0.039, P>0.05 ---
	14	18.70±0.45	19.10±0.68	20.11±1.05	20.07±0.77			
	21	19.64±1.40	19.71±0.35	21.06±0.59	20.46±1.20			

r: the correlation coefficient of the concentrations of the studied parameter with the experimental time (^{E}) and the *C. arietinum* (^{C}).

cantly differed from their values at the first week.

Effect of *Cicer arietinum* on the serum levels of testosterone, FSH and LH.

The serum levels of testosterone, LH and FSH of all the experimental groups were shown in table 3. The experimental time and *C. arietinum* content had remarkable effects on the levels of testosterone and LH in the serum of mice. On the other hand, the FSH levels were insignificantly affected by either the experimental periods or the amount of *C. arietinum* in the diet. The levels of testosterone and LH of groups II, III and IV were meaningfully elevated as compared to those of group I. The amount of *C. arietinum* in the diet showed positive correlations with the levels of testosterone (+0.70) and LH (+0.64). At the third week, the levels of testosterone and LH of groups II, III and IV were markedly higher than those at the first week of experiments.

Table 3. The levels of testosterone, FSH and LH in serum, the level of fructose in seminal vesicle and the concentration, motility and abnormality of sperms of group I, II, III and IV, throughout the three weeks of experiments.

Parameter	Time (days)	Experimental group				Two-ways ANOVA		
		I	II	III	IV	Time	*C. arietinum*	Interaction
Testosterone (ng/mL)	7	2.62±0.05	2.75±0.07	2.93±0.02*	3.12±0.10*	$F_{2,60}$=161.57, P<0.001, r^E= +0.55	$F_{3,60}$=181.78, P<0.001, r^C = +0.70	$F_{6,60}$=23.272, P<0.0001 ---
	14	2.63±0.05	2.88±0.02*	3.42±0.08*#	3.61±0.05*#			
	21	2.60±0.06	3.72±0.03*#	3.78±0.01*#	4.03±0.03*#			
Follicle stimulating hormone (FSH, mU/mL)	7	0.47±0.08	0.49±0.02	0.50±0.03	0.50±0.03	$F_{2,60}$=0.006, P>0.05, r^E= -0.01	$F_{3,60}$=0.069, P>0.05, r^C = +0.06	$F_{6,60}$=0.087, P>0.05 ---
	14	0.50±0.07	0.48±0.01	0.49±0.02	0.50±0.001			
	21	0.48±0.04	0.49±0.03	0.49±0.02	0.49±0.02			
Luteinizing hormone (LH, mU/mL)	7	0.09±0.01	0.10±0.01	0.12±0.01	0.14±0.01*	$F_{2,60}$=8.757, P<0.001, r^E= +0.34	$F_{3,60}$=23.502, P<0.001, r^C = +0.64	$F_{6,60}$=1.76, P>0.05 ---
	14	0.09±0.01	0.13±0.01*	0.14±0.01*	0.15±0.02*			
	21	0.08±0.01	0.15±0.01*#	0.17±0.01*#	0.19±0.02*#			
Fructose (mg/g vesicle)	7	35.08±1.38	36.23±1.22	36.94±1.20	37.88±0.96	$F_{2,60}$=3.257, P>0.05, r^E= +0.23	$F_{3,60}$=3.248, P<0.05, r^C = +0.34	$F_{6,60}$=0.497, P>0.05 ---
	14	35.10±1.43	36.44±0.57	36.64±1.28	36.86±2.94			
	21	35.09±1.33	38.87±0.98	39.22±1.65	41.61±1.56*			
Sperm concentration (x10^6/mL)	7	1.75±0.10	2.05±0.13	2.31±0.14*	2.44±0.11*	$F_{2,60}$=94.27, P<0.001, r^E= +0.50	$F_{3,60}$=114.59, P<0.001, r^C = +0.67	$F_{6,60}$=11.16, P<0.0001 ---
	14	1.78±0.09	3.08±0.15*#	3.52±0.12*#	3.79±0.05*#			
	21	1.72±0.10	3.37±0.15*#	3.86±0.15*#	3.96±0.09*#			
Sperm motility (%)	7	80.72±0.92	84.48±2.71	89.15±2.21*	93.46±1.20*	$F_{2,60}$=0.886, P>0.05, r^E= +0.09	$F_{3,60}$=40.80, P<0.001, r^C = +0.79	$F_{6,60}$=0.413, P>0.05 ---
	14	80.70±0.87	86.04±1.73	91.10±2.36*	94.46±0.94*			
	21	80.91±1.10	84.16±2.44	92.46±1.13*	96.42±1.01*			
Sperm abnormality (%)	7	5.67±0.37	4.98±0.67	5.57±0.40	4.99±0.47	$F_{2,60}$=2.317, P>0.05, r^E= -0.23	$F_{3,60}$=4.720, P<0.01, r^C = -0.27	$F_{6,60}$=1.00, P>0.05 ---
	14	5.66±0.38	4.53±0.40	5.60±0.63	4.20±0.19			
	21	5.63±0.39	3.94±0.39*	4.34±0.22*	4.73±0.34			

*: represents significant difference (P<0.05) as compared to the values of group I. #: represents significant difference (P<0.05) as compared to the values at the first week. P<0.05, P<0.01 and P<0.001: significant effects. r: the correlation coefficient of the concentrations of the studied parameter with the experimental time (^{E}) and the *C. arietinum* content (^{C}).

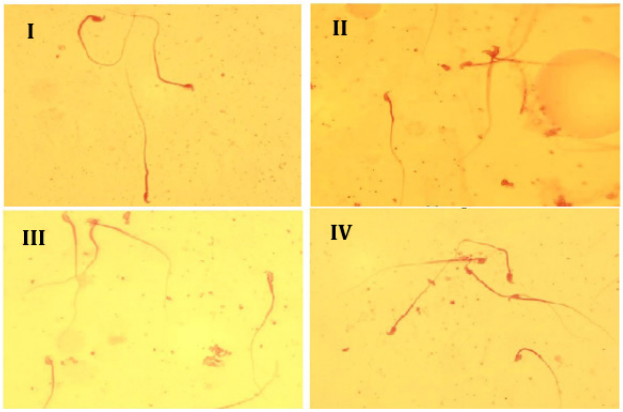

Figure 2. Representative photos for the examined sperms of group I, II, III and IV, after the three weeks of experiments.

Effect of *Cicer arietinum* on the fructose level, sperm concentration, motility and abnormality

The levels of fructose, sperm concentration, motility and abnormality were presented in table 3. The levels of fructose in the mice fed a diet containing *C. arietinum* were insignificantly differed from those of controls except for a significant elevation in group IV, at the third week. Sperm concentrations of group II, III and IV were markedly higher than group I and were positively correlated with the experimental time (r= +0.50) as well as the *C. arietinum* content (r= +0.67). Sperm motility of group III and IV was significantly higher than the controls, at all the time intervals and showed a direct correlation (r= +0.79) with the amount of *C. arietinum* in the diet. Sperm abnormalities of groups II, III and IV did not exhibit any significant changes as compared to the controls except for marked reductions at the third week in groups II and III. As shown in figure 2, the sperms of all the experimental groups had the same normal appearances.

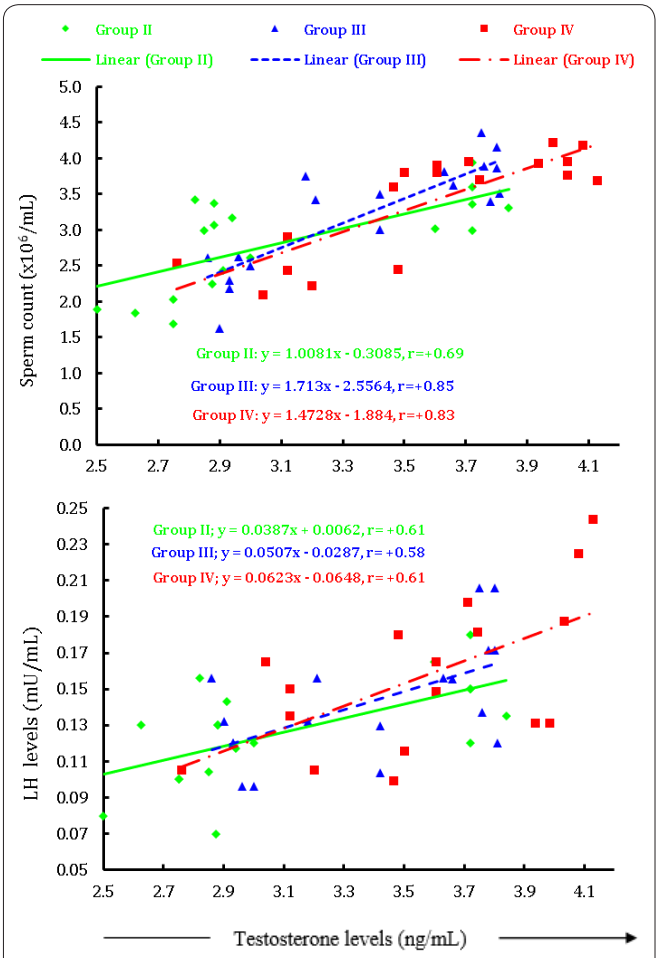

Figure 3. The relationships of testosterone with the levels of LH and sperm count of group II, III and IV.

In figure 3, regression analysis showed that the se-

Table 4. The levels of MDA, AA and GSH as well as the activities of SOD, GPx and CAT in the testes of group I, II, III and IV, throughout three weeks of experiments.

Parameter	Time (days)	Experimental group				Two-ways ANOVA		
		I	II	III	IV	Time	*C. arietinum*	Interaction
Malondialdehyde (MDA, nmol/mg testis)	7	2.28±0.16	1.89±0.05	1.81±0.17	1.30±0.08*	$F_{2,60}$=0.824, P>0.05	$F_{3,60}$=20.77, P<0.001	$F_{6,60}$=0.180, P>0.05
	14	2.11±0.19	1.88±0.12	1.74±0.15	1.30±0.05*			
	21	2.07±0.19	1.72±0.12	1.71±0.14	1.29±0.07*	r^E= -0.12	r^C = -0.70	---
Ascorbic acid (AA, mg/g testis)	7	38.76±0.68	40.77±1.57	41.71±1.69	41.75±0.28	$F_{2,60}$=3.049, P>0.05	$F_{3,60}$=1.737, P>0.05	$F_{6,60}$=0.413, P>0.05
	14	38.19±2.32	38.60±0.99	43.33±3.04	43.60±2.99			
	21	43.50±3.21	43.51±2.65	44.03±1.01	44.81±1.59	r^E= +0.38	r^C = +0.20	---
Glutathione (GSH, mg/g testis)	7	1.80±0.07	1.81±0.13	2.13±0.02	2.20±0.26	$F_{2,60}$=9.220, P<0.001	$F_{3,60}$=11.490, P<0.001	$F_{6,60}$=0.730, P>0.05
	14	1.97±0.14	2.01±0.08	2.41±0.05	2.76±0.23*			
	21	2.00±0.03	2.41±0.25#	2.52±0.18#	2.83±0.11*#	r^E= +0.39	r^C = +0.53	---
Superoxide dismutase (SOD, U/mg testis)	7	4.28±0.29	4.51±0.08	4.62±0.29	4.74±0.25	$F_{2,60}$=0.050, P>0.05	$F_{3,60}$=0.530, P>0.05	$F_{6,60}$=0.093, P>0.05
	14	4.38±0.27	4.55±0.20	4.68±0.48	4.75±0.56			
	21	4.50±0.30	4.52±0.06	4.54±0.22	4.56±0.21	r^E= +0.01	r^C = +0.16	---
Glutathione peroxidase (GPx, U/mg testis)	7	0.43±0.08	2.20±0.32*	2.25±0.28*	2.49±0.03*	$F_{2,60}$=5.965, P<0.01	$F_{3,60}$=59.824, P<0.001	$F_{6,60}$=0.835, P>0.05
	14	0.57±0.06	2.17±0.22*	2.35±0.20*	2.64±0.23*			
	21	0.45±0.02	2.92±0.50*	2.99±0.21*	3.16±0.21*#	r^E= +0.20	r^D= +0.73	---
Catalase (CAT, U/mg testis)	7	2.73±0.13	3.03±0.43	3.35±0.38	3.52±0.17	$F_{2,60}$=28.837, P<0.001	$F_{3,60}$=16.520, P<0.001	$F_{6,60}$=2.467, P<0.05
	14	3.03±0.34	4.23±0.20*#	4.36±0.19*#	4.39±0.04*			
	21	3.18±0.25	4.58±0.14*#	5.71±0.25*#	5.91±0.69*#	r^E= +0.56	r^C = +0.49	---

*: represents significant difference (P<0.05) as compared to the values of group I. #: represents significant difference (P<0.05) as compared to the values at the first week. P<0.05, P<0.01 and P<0.001: significant effects. r: the correlation coefficient of the concentrations of the studied parameter with the experimental time (^{E}) and the *C. arietinum* content (^{C}).

rum levels of testosterone had positive relationships with both the sperm concentration and LH levels in group II, III and IV.

Effect of *Cicer arietinum* on testicular lipid peroxidation and endogenous antioxidants

The levels of MDA, AA, GSH as well as the activities of SOD, GPx and CAT in the testes of all the studied groups were displayed in table 4. The testicular levels of MDA were significantly affected by the *C. arietinum* content in the diet in an inverse relationship (r= -0.70). In comparison to controls, the MDA levels of group IV showed a marked reduction at all the time intervals.

Two-ways ANOVA revealed that the experimental time and the amount of *C. arietinum* in the diet markedly influenced the testicular levels of GSH as well as the activities of GPx and CAT whereas insignificantly affected the AA level and SOD activity. At the second and third weeks, the GSH content of group IV was remarkably increased as compared to the group I. In the testes of groups II, III and IV, at the third week, the GSH contents were significantly higher than those at the first week. The GPx activities of groups II, III and IV were remarkably higher than those of group I, at all the time intervals and showed a positive correlation with the *C. arietinum* content of the diet (r= +0.73). In groups II, III, IV, the CAT activities were significantly enhanced as compared to controls, at the second and third weeks of experiments. By the third week, the CAT activities of groups II, III and IV were significantly higher than those at the first week.

Effect of *Cicer arietinum* on the Comet assay parameters

In table 5, the comet assay parameters including TL, DNA% in tail and TM in nuclei of sperms and testis of groups I, II, III and IV were presented. In both the testes and sperms, none of the studied factors significantly affected any of the studied comet parameters except for the amount of *C. arietinum* in the diet that markedly affected the DNA% and TM in the testicular cells. In groups III and IV, the DNA% in the tail of the testicular cells were markedly reduced as compared to the group I, at the third week. In the testes of group IV, the TM showed a significant decline in comparison to the group I, after three weeks of experiment. In the testis, the percentages of *C. arietinum* in the diet were inversely correlated with the DNA% (r= -0.56) and TM (r= -0.46). In figure 4, the comet nuclei from the sperms and testes of groups II, III and IV showed the same normal appearance of group I.

Effect of *Cicer arietinum* on the DNA fragmentation

The electrophoresed pattern of genomic DNA ex-

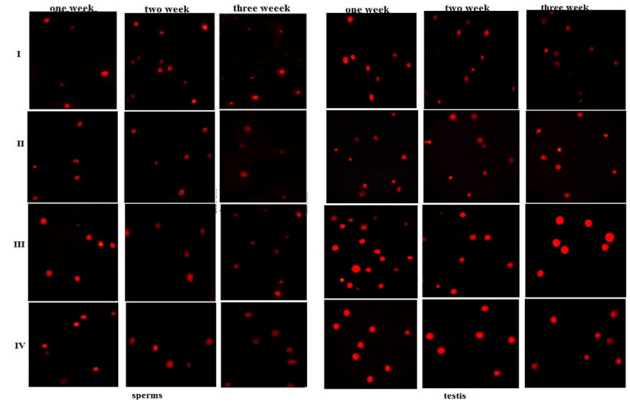

Figure 4. Representative photos of the observed comet nuclei in the sperms and testis of group I, II, III and IV, throughout the three weeks of experiments.

Table 5. The TL, DNA% and TM in the testes and sperms of group I, II, III and IV, throughout three weeks of experiments.

Parameter		Time (days)	Experimental group				Two-ways ANOVA		
			I	II	III	IV	Time	*C. arietinum*	Interaction
Testis	Tail length (TL, µm)	7	1.79±0.10	1.77±0.14	1.74±0.11	1.71±0.10	$F_{2,60}$=0.638, P>0.05	$F_{3,60}$=0.986, P>0.05	$F_{6,60}$=0.084, P>0.05
		14	1.79±0.09	1.68±0.15	1.66±0.15	1.63±0.11			
		21	1.79±0.09	1.65±0.14	1.62±0.05	1.59±0.04	r^E= -0.14	r^C = -0.21	---
	DNA% in tail	7	33.95±1.78	33.42±0.38	31.73±0.41	30.86±1.94	$F_{2,60}$=6.559, P<0.01	$F_{3,60}$=14.879, P<0.001	$F_{6,60}$=2.304, P<0.05
		14	33.80±1.85	32.17±2.23	30.83±2.30	25.95±1.61*			
		21	33.79±1.82	31.69±1.70	28.11±1.21*	19.51±1.25*#	r^E= -0.31	r^C = -0.56	---
	Tail moment (TM)	7	0.62±0.06	0.59±0.05	0.55±0.04	0.53±0.05	$F_{2,60}$=3.003, P>0.05	$F_{3,60}$=6.312, P<0.01	$F_{6,60}$=0.665, P>0.05
		14	0.62±0.06	0.55±0.07	0.52±0.08	0.43±0.05			
		21	0.61±0.06	0.52±0.06	0.46±0.02	0.31±0.02*#	r^E= -0.26	r^C = -0.46	---
Sperm	Tail length (TL, µm)	7	1.23±0.12	1.21±0.06	1.21±0.03	1.21±0.06	$F_{2,60}$=0.582, P>0.05	$F_{3,60}$=0.741, P>0.05	$F_{6,60}$=0.123, P>0.05
		14	1.22±0.13	1.20±0.07	1.13±0.05	1.10±0.06			
		21	1.23±0.12	1.18±0.11	1.10±0.11	1.08±0.09	r^E= -0.13	r^C = -0.18	---
	DNA% in tail	7	24.32±1.53	24.27±0.18	24.01±1.42	23.52±0.95	$F_{2,60}$=0.025, P>0.05	$F_{3,60}$=0.12, P>0.05	$F_{6,60}$=0.024, P>0.05
		14	24.39±1.57	24.14±1.58	24.28±1.80	24.13±2.19			
		21	24.37±1.55	24.34±2.46	24.09±2.09	23.14±1.77	r^E= -0.01	r^C = -0.07	---
	Tail moment (TM)	7	0.30±0.04	0.30±0.01	0.29±0.02	0.28±0.02	$F_{2,60}$=0.134, P>0.05	$F_{3,60}$=0.597, P>0.05	$F_{6,60}$=0.075, P>0.05
		14	0.30±0.04	0.29±0.03	0.28±0.03	0.27±0.03			
		21	0.30±0.04	0.30±0.05	0.27±0.04	0.25±0.03	r^E= -0.06	r^C = -0.16	---

*: represents a significant difference (P<0.05) as compared to the values of group I. #: represents a significant difference (P<0.05) as compared to the values at the first week. P<0.05, P<0.01 and P<0.001: significant effects. r: the correlation coefficient of the concentrations of the studied parameter with the experimental time (^{E}) and the *C. arietinum* content (^{C}).

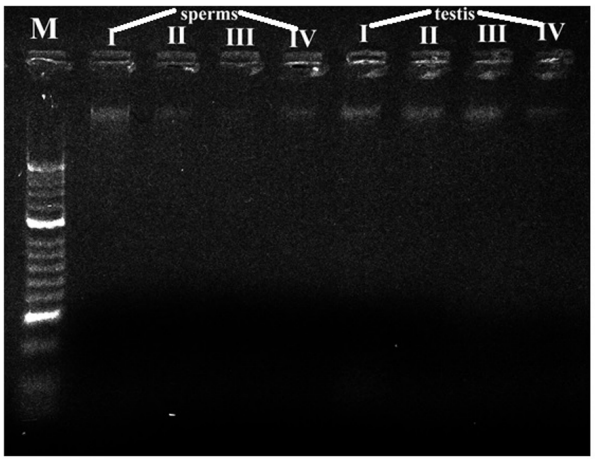

Figure 5. Electrophoresed pattern of genomic DNA extracted from the sperms and testis of group I, II, III and IV, after three weeks of the experiments. M: Marker.

tracted from the sperms and testis of groups II, III and IV showed intact bands as compared to the electrophoresed pattern of genomic DNA of group I (figure 5).

Discussion

Human spermatogenesis is mainly affected by the changes in the life and diet styles. Currently, the fast-foods are highly consumed by humans due to their delicious taste and high protein content (5). Meanwhile, fast-foods have negative effects on the male reproductive system due to their high contents of phytoestrogen (14). Therefore, the search for safe sources of protein becomes necessary to avoid the adverse effects of fast-food consumption on the human health. Jukanti *et al*. (34) recorded that *C. arietinum* was a good source of proteins and carbohydrates. Thereby, this study aimed to investigate the effect of *C. arietinum* on the fertility indices in male mice. In the current study, the chemical analysis of the provided diets showed that the inclusion of *C. arietinum* to the rodent chow preserved their protein contents. Furthermore, *C. arietinum* significantly elevated the levels of the carbohydrates and lipids that represent vital sources of energy (35).

Reproduction can be badly affected by the change in the weight of the reproductive organs (36). In the present investigation, no statistical variations were recorded in the weights of either the whole body or the reproductive organs between the control rats and those received *C. arietinum* in the diet. Accordingly, the addition of *C. arietinum* to the diet did not exert any remarkable adverse effects on either the reproductive organs or the whole body. In the same line, Yang *et al*. (37) found no significant changes in the body weight as well as the adipose tissue of the epididymis of rats after inclusion of *C. arietinum* in a high-fat diet. The constancy of the body weight may be linked to the ability of *C. arietinum* to induce satiation and fullness (38).

In the present study, the inclusion of *C. arietinum* in the diet led to significant elevations in the serum levels of testosterone and LH, in a dose-dependent manner, as compared to the controls. Similarly, Sajja *et al*. (18) reported a significant increase in the serum level of testosterone in male albino rats after the administration of the methanolic extract of *C. arietinum*. Testosterone is secreted from Leydig cells in the testis after stimulation by LH secretion from the anterior pituitary (39). This was confirmed, in the current results, by the recorded strong positive relationships between the levels of testosterone and LH in the serum of mice fed a diet containing *C. arietinum*. This may reflect the capability of *C. arietinum* to enhance the steroidogenesis resulting in an improved synthesis of LH and consequently an increased secretion of testosterone. These findings could be linked to the presence of bioactive components such as flavonoids, saponins, alkaloids in *C. arietinum* seeds (17). Flavonoids were reported for their androgenic activities as well as their ability to modulate both male and female gonadal dysfunctions (40). Moreover, Gauthaman *et al*. (41) found that saponins significantly increased testosterone levels by enhancing the biosynthesis of its dehydroepiandrosterone (DHEA) precursor. Alkaloids were reported for their ability to trigger steroidogenesis through their ergogenic properties (18). On the other hand, the ability of *C. arietinum* to keep normal levels of FSH, in the current data, could be helpful in optimizing the production of germ cells (42). Moreover, the increased levels of testosterone associated with the inclusion of *C. arietinum* in the diet reflected its aphrodisiac activity. As the natural agent that has aphrodisiac potential should be able to increase the concentration of testosterone, LH and FSH (43).

The present study evidenced that the fertility of the mice fed a diet containing *C. arietinum* was enhanced, in comparison to the controls. This finding was manifested by significant elevations in the sperm concentration and motility accompanied by a marked reduction in the sperm abnormality. The alleviation of the sperm abnormality, in the mice fed a diet containing *C. arietinum*, confirmed its efficacy to preserve cell structural components. Moreover, the increased concentration of sperms can be attributed to the elevated level of testosterone that is responsible for initiation and maintenance of spermatogenesis (39). This was evidenced by the strong positive relationships between the level of testosterone and the sperm concentration, in the current data. The enhanced sperm motility and concentration as well as the reduced sperm abnormality reported, in the present results, after the inclusion of *C. arietinum* in the diet, confirmed its efficacy not only to preserve sperms but also to boost their number and activity resulting in an enhanced male fertility. As a sufficient number of normal active sperms is essential to achieve successful fertilization (44). Furthermore, the present investigation suggested that *C. arietinum* may influence the mitochondria of the spermatozoon that supply the sperms with the energy required for their active motion. This suggestion was strengthened by Li *et al*. (45), who demonstrated that sperm motility is somewhat dependent on mitochondrial function. This may be attributed to the ability of *C. arietinum* to provide mitochondria with a high content of carbohydrate that acts as a good source of energy. The present findings revealed that *C. arietinum* improved the quality of sperms in a dose-dependent manner. As the grade of sperm quality depends on sperm count, motility, and normal morphology (46).

The level of fructose represents an essential marker for the function of the seminal vesicle. The present results showed that the levels of seminal fructose were

kept in the normal level in all the experimental groups except for a marked increase, at the third week, in the mice fed a diet containing 50% of *C. arietinum*. The sperm quality can be improved by increasing the seminal fructose concentration that supplies the sperms with the energy required for their activity (47). This may explain the increased motility of sperms recorded in the mice that received a diet containing 50% *C. arietinum*. This may be attributed to the presence of about 0.25 g of fructose per 100 g of *C. arietinum* seeds (34). Furthermore, the elevated level of seminal fructose may be linked to the reported increase in the level of testosterone, in the current results. This assumption is in the same line with Desai *et al*. (47) who reported that the seminal vesicle function is regulated by androgen secretion.

Gupta *et al*. (48) and Kianifard (49) reported that the male germ cells were adversely affected by the oxidative stress resulting in an incomplete functional maturation and capacitation of spermatozoa that led to the sterility. Consequently, antioxidants are necessary to protect and improve the process of spermatogenesis. The present study revealed that the testicular levels of MDA of mice fed a diet comprising *C. arietinum* were markedly reduced whereas the GSH content, GPx and CAT activities were significantly increased in comparison to the controls. The decreased level of testicular MDA may reflect the protective effect of *C. arietinum* on the cell membrane lipids against the oxidative damage via blocking the free radical-induced lipid peroxidation (10). Moreover, the reduced levels of MDA as well as the increased GSH content and CAT activity may be attributed to the presence of GSH, δ- tocopherol and CAT in the seeds of *C. arietinum* (50). Jukanti *et al*. (34) reported that *C. arietinum* seeds contain considerable amounts of iron. This may explain the increased activity of CAT that depends on iron as a cofactor (51). As the GSH is necessary for suppling GPx with the electrons required for its action (52). Thereby, the reported increase in GPx activity may be linked to the elevated content of GSH. Oliveira *et al.* (46) reported that the enhanced antioxidant system can retain a normal morphology of cell. This finding rationalized the role of *C. arietinum* in minimizing the number of malformed sperms, in the present study. The ability of *C. arietinum* to boost intrinsic antioxidants may be attributed to its content of flavonoids, saponins, and alkaloids. Accordingly, the boosted antioxidant levels and reduced lipid peroxidation can afford for the improved spermatogenesis reported in the present study. Similarly, Mittal *et al* (53) reported that the inclusion of *C. arietinum* seed coat fiber in the diet of rats remarkably ameliorated the toxic effects of N-nitrosodiethylamine on the levels of lipid peroxidation as well as the activities of antioxidants.

Recently, the nuclear DNA investigations of sperm and testicular cells were considered promising tools for the diagnosis of male sterility/fertility (54). It is well known that the maintenance of the testicular and sperm DNA integrity is critical for the normal reproduction. As the chief purpose of sperms is the delivery of their intact DNA that carry the genetic information. Thus, the damage in the sperm DNA is one of the causes of male subfertility. The present study exhibited that *C. arietinum* preserved the integrity of testicular and sperm DNA and prevented their damage. Furthermore, the alkaline type of comet assay revealed significant reductions in the comet TM and DNA% in the tail of testicular cells of mice fed a diet containing *C. arietinum* as compared to the controls. This indicated that *C. arietinum* not only has no genotoxic effect on the sperms and testicular cells but also has a protective effect on the DNA. This was manifested by the gel electrophoresis of spermatic and testicular cells which showed that all the experimental groups had the same normal pattern of the controls. The ability of *C. arietinum* to preserve DNA confirmed its safety on the molecular level. Thus, the present study explained the supportive role of *C. arietinum* in testicular function by the molecular mechanisms. The oxidative damage is one of the mechanisms that can elicit sperm DNA fragmentation/oxidation (55). Thus, the protective effect of *C. arietinum* on the testicular and sperm DNA may be attributed to its antioxidative properties. The protective efficacy of *C. arietinum* against apoptosis and DNA damage is required for successful fertilization. Since the integrity and stability of the condensed sperm chromatin structure preserve the paternal genome to be delivered to the oocyte successfully (56).

Based on the above-mentioned findings, unlike soy fast-food products, *C. arietinum* not only exerted no toxic effect on sperms but also boosted their quantity and quality, at least under the present experimental conditions. Furthermore, *C. arietinum* can facilitate DNA packaging of testis and sperms. Thereby, *C. arietinum* can be a promising fertility enhancer. Consequently, it can improve the reproductive status and quality of life. These encouraging results may pave the way to find a new approach in the alleviation of male infertility. However, the present results were achieved for male mice. Thus, more investigations should be performed and extended to human beings.

Conflict of interest

Authors declared no conflict of interest.

Author's contributions

All authors have equal efforts and contribution in this manuscript.

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