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Original Article

The Ameliorative Impact of *Cichorium intybus* L. Distillate on Reproductive Parameters in Male Mice

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Abstract

Background: *Cichorium intybus* L. (Kasni) distillate is widely used in Eastern countries as a safe herbal drink to improve male fertility. However, the potential effects on fertility parameters and possible adverse effects have not been studied experimentally. The current study aims to evaluate the impact of *Cichorium intybus* L. distillate (CD) on male mice fertility.

Methods: In the present study (Shiraz, Iran), 30 male mice (30-35 g) were divided into three groups. 10 mice received distilled water (DW) for five weeks as the control group. Another 10 mice, named group CD1/2, received chicory distillate of 1/2 dilution, and the other 10 mice received chicory distillate of CD1/4 dilution as CD1/4 group, *ad libitum* for three weeks, and they received DW for two weeks afterward. Experimental mice were sacrificed on day 35, and sperm analysis and sera collection were performed for further investigation of FSH, LH, testosterone, and some liver and kidney function parameters. We used the left testis for stereological analysis, and the right one was excised to investigate the expression of the androgen receptor gene. For statistical analysis using SPSS 18.0, mean±SD values were analyzed by one-way analysis of variance (ANOVA) with Dunnett's analysis as *post hoc* to compare between groups. In stereological investigations, the Kruskal-Wallis method was used for pairwise comparisons to compare groups. The P value was considered statistically significant at $P<0.05$.

Results: Treatment with CD1/2 resulted in the elevation of serum FSH (P=0.002), LH (P=0.009), testosterone (P=0.034), seminiferous tubule epithelium volume (P=0.029) and length (P=0.028), and Leydig cells number (P=0.009) in comparison with the control group. Administrating CD1/2 (P=0.038) and CD1/4 (P=0.013) significantly increased sperm count compared to the control group.

Conclusion: The results revealed that using chicory distillate can improve hormone levels and sperm count in male mice.

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Keywords ● Chicory ● Fertility agents, male ● Gonadotropins, testosterone, semen analysis

What's Known

- Infertility of couples worldwide causes high treatment charges and disappointing side effects, which increases the tendency toward complementary medicine. According to folk medicine, *Cichorium intybus* distillate is being used as a safe herbal drink for the treatment of infertility. However, the potential ameliorative effects and possible adverse effects have not been yet studied experimentally.

What's New

- The administration of *Cichorium intybus L* distillate boosted reproductive parameters in male mice and increased serum FSH, LH, and testosterone hormones, sperm count, number of Leydig cells, and seminiferous tubule epithelium volume and length with no evidence of hepatotoxicity and/or nephrotoxicity in this experimental study.

Introduction

Infertility affects 3-16% of couples with an average prevalence of 9% with equal distribution between males and females.[1] Receiving infertility care results in high financial costs for

couples trying to conceive.[2] Psychological and emotional effects of infertility lead couples to a stressful crisis.[3] Reactive oxygen species (ROS) produced in metabolic pathways are essential for spermatogenesis and fertilization. While, elevated ROS levels lead to the damage of DNA and cell membrane in spermatozoa.[4, 5] Many reports indicated that the increasing rate of obesity, psychological stress, unhealthy diet, smoking, and COVID-19 pandemic complications have decreased male fertility.[6, 7] High treatment charges and disappointing side effects have increased the inclination towards complementary and alternative medicine (CAM).[8]

Based on the World Health Organization (WHO) announcement, 60% of people worldwide and 80% of people in developing countries rely on herbal medicines for their health issues, which are believed to be safe and more affordable.[9] The ameliorative effects of herbal plant extracts on sperm parameters,[10] androgen status,[11] and fertility index[12] have been revealed in many studies. In traditional Iranian medicine (TIM), extracts and distillates are the most favorite preparations of medicinal plants, which have gained much attention in some previous studies.[13] Due to easy preparation and use, distillates are very popular drinks in Eastern countries, especially in Iran.

Various preparations of Chicory or *Cichorium intybus* L., a member of the Asteraceae family and genus *Cichorium L.*, can be utilized to improve fertility.[14] *Cichorium intybus* L. distillate (CD) is widely used as a fertility-boosting drink among people in the Middle East especially in Iran. However, the scientific mechanisms and potential side effects are not investigated yet. The current study was designed to investigate the impact of CD on mice fertility, testosterone level, androgen receptor (AR) gene expression, sperm parameters, and the stereological aspects of the testis. Considering that *Cichorium intybus L.* extract is approved to be hepatoprotective,[15] the possible hepatotoxicity and nephrotoxicity effects of its distillate were evaluated in this study.

Materials and Methods

Chicory Distillate Preparation

Chicory (*C. intybus* L.) was harvested from lands located in Sepidan (Fars, Iran). The species was confirmed by a professor of the Department of Biology as an herbal expert (voucher number 561) at Shiraz University, Shiraz, Iran. To prepare 1 L of the distillate, a boiler was used for the distillation of 0.175 Kg of dried plant with 1.75 L of water, as previously described.[16] The condenser tube cooled the outgoing steam to produce CD, which was kept light-protected and stored at 4 ºC until use.

Animals

In this study, adult male mice (30-35 g) were provided by Shiraz Comparative and Experimental Medicine Center, Shiraz, Iran, and were kept at 12:12 h light:dark period and temperature of 20±2 °C.[17] All methods were performed according to the guidelines of the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1393.s7389).

Experimental Design

In the current study, the animals were randomly separated into three groups (n=10). The control group was administrated with distilled water (DW) for five weeks. Two treatment groups received the diluted CD in a 1:1 (CD1/2) or 1:3 proportion with DW (CD1/4), *ad libitum* for three weeks and DW for two weeks afterward, *i.e.*, a total duration of five weeks as mice spermatogenesis duration.[18] Daily consumption of CD per Kg body weight was calculated and the animals were weighed weekly. After anesthetization on day 35, the sera obtained from the blood samples were kept at -80 °C for biochemical tests. The left cauda epididymis was dissected for sperm analysis, and the left testes were separated for further stereological investigations. The right testes were kept at -80 °C for evaluating the androgen receptor (AR) gene expression.

Biochemical Analysis

Hormone Analysis: Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were evaluated by ELISA kit for mice (Hangzhou Eastbiopharm Co., China). Testosterone level was analyzed by Biospes mice ELISA kit (Biospes, China).

Liver/Kidney Function Analysis: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) analysis were carried out to evaluate the potential hepatotoxicity of CD by Biorexfars kit (Biorexfars, Iran). Blood creatinine and blood urea nitrogen (BUN) were analyzed as kidney function parameters, using the Biorexfars kit (Biorexfars, Iran) and Mancompany kit (Mancompany, Iran), respectively.

The Expression of the Androgen Receptor Gene: To evaluate the level of AR gene expression, quantitative Real-Time Polymerase Chain Reaction (RT-PCR) was carried out. Total RNA was extracted, using RNA extraction mini kit (Yekta Tajhiz Azma, Iran), and cDNA synthesis was carried out by

reverse transcriptase (Fermentas, Canada). Real-time PCR apparatus, ABI 7500 (Applied Biosystems, USA), and Master Mix containing SYBR Green (Amplicon, Denmark) were applied for RT-PCR. As the internal control gene, beta-actin was used for normalizing the CT values of AR gene expression by the $2^{-\Delta\Delta CT}$ method. The primer pairs were designed for AR and beta-actin genes by the software of AlleleID 7.73 (PREMIER Biosoft International, USA). The sequences were as follows, respectively:
Forward: 5' ATGACAACAACCAACCAGATT 3'
Reverse: 5' TTAGTGAAGGACCGCCAAC 3'
Forward: 5' CACACCCGCCACCAGTTCG 3'
Reverse: 5' ACCCATTCCCACCATCACACC 3'

***Sperm Analysis*:** Left cauda epididymis was immediately cut after sacrifice and kept in 37 °C normal saline for three min. A hemocytometer was used to count the sperm heads using a light microscope. To evaluate sperm motility, they were categorized as different motile types or immotile in 10 random microscope fields.[19] Sperm smears were stained by eosin Y to evaluate sperm morphology as normal or abnormal subgroups.[20]

***Stereological Analysis*:** In each group, for stereological analysis of the left testes, five mice were randomly chosen. Scherle's immersion method was used to calculate testis primary volume.[21]

To calculate the volume of the testis, isotropic uniform random (IUR) sections were prepared by Cavalieri's method using an orientation.[22, 23] Consecutively, sections with 5 and 25 µm thicknesses and a constant interval were obtained by a microtome to prepare 8-12 sections from each testis. Then, 5 µm thick sections were stained with azan to estimate tissue volume, and 25 µm thick sections were stained with hematoxylin and eosin to calculate cell number.

Testis Cavalieri's volume was evaluated with the final magnification of 13×:

$$V(testis) = \sum_{i=1}^{n} A \times T$$

V (testis) equals the accurate testis volume, A stands for tissue section area, and T stands for the interval between the adjacent sections.

The volume of tubules as well as interstitial tissue was calculated by point counting. To obtain the volume of the seminiferous tubules and interstitial tissue, their volume density was estimated by 280× magnification:
Vv (structure)=ΣP (structure)/ΣP (total).

Vv (structure) equals the structure volume density and ΣP (structure) and ΣP (total) stand for all points covering the target structure and the testis area, respectively.

To evaluate the structure volume, the value of the structure volume density was multiplied by the testis volume:
V (structure)=Vv (structure)×V (testis)

To estimate the total number of spermatogonia A, spermatogonia B,[24] Sertoli and Leydig cells, and round and long spermatids and spermatocytes, a light microscope (Nikon E200, Japan) with a 60× objective lens and the final magnification of 1,640× was used. A counting frame was located on the tissue sections. Each cell number was evaluated by the method of "optical dissector" using a microcator (MT12, Germany) for evaluating the z-axis of each section. The following formula was used to calculate the numerical density of the cells.[25]
Nv (cells)=[ΣQ/(ΣP×(*a/f*)×h)]×(t/BA)

Nv (cells) stands for the numerical density of cells. ΣQ is the number of nuclei for each type of cell, and *a/f* stands for the area of each counting frame. ΣP represents the number of frames that are counted for each tissue. h stands for the optical dissector height, which was evaluated by the microcator. *t* represents the average thickness of the sections, and BA stands for block advance of the microtome.
N (cells)=N_V (cells)×V (structure)

N (cells) represents the cell number, V (structure) is the volume of epithelium for germinal layer cells, and interstitial tissue volume for Leydig cells.

To estimate the length of seminiferous tubules, we evaluated the length density of tubules by an unbiased counting frame[23] with 148x magnification:
Lv=2ΣP/(*a/f*)×ΣP

ΣQ equals the counted tubule profile number, *a/f* stands for the area of each counting frame, and ΣP represents the whole counted frame number in each tissue. To calculate the length of the tubule (L) the following formula was used:
L=L_V×V (tubule)

L_V is the length density, and V is the tubule's total volume.

Statistical Analysis

Statistical significance was calculated by SPSS 18.0 (SPSS Inc., USA), and the graphs were prepared by GraphPad Prism 5 software (San Diego, USA). To compare the groups, mean±SD values were analyzed by one-way analysis of variance (ANOVA) with Dunnett's analysis as *post hoc* (n=10). To compare groups in stereological investigations (n=5), the Kruskal-Wallis method was used for pairwise comparisons. The P value<0.05 was considered statistically significant.

Results

To obtain the average daily usage of CD, the daily consumption volume was divided by the body weight, which was calculated to be 150±2 for the CD1/2 group and 75±1 mL/Kg/day for the CD1/4 group.

Biochemical Tests

***Hormonal Analysis*:** The values for serum FSH, LH, and testosterone are demonstrated in figure 1. The results indicated that CD1/2 administration resulted in significantly higher levels of FSH (16.3±1.7 ng/mL, P=0.002), LH (7.8±1.3 mIU/mL, P=0.009), and testosterone (50.0±3.5 pg/mL, P=0.034) than the control group (FSH=13.9±1.8 ng/mL, LH=6.4±0.7 mIU/mL, testosterone=44.5±3.11 pg/mL). There were no significant changes observed in hormone levels in the CD1/4 receiving group.

Toxicity: The levels of ALT and AST, as hepatotoxicity indicators, and BUN and creatinine, as nephrotoxicity parameters, are exhibited in figure 2. As reported, CD consumption resulted in no significant hepato/nephro-toxicity.

Androgen Receptor Gene Expression

Our results revealed that administration of CD1/2 or CD1/4 indicated no significant effects on AR gene expression compared to the control group.

Semen Parameters

The level of sperm count and the ratio of sperm normality are demonstrated in table 1. As shown, the total number of sperms was significantly higher in the CD1/2 group (60.6±10.2, P=0.038) and CD1/4 group (63.2±18.4, P=0.013) than the control group (43.6±7.7).

The percentage of normal and abnormal sperms (big/small head sperms, short/coiled tail sperms, or sperms possessing only head or tail) showed no significant changes. The values of sperm motility, including fast progressive, slow progressive, and nonprogressive sperms indicated no significant changes in CD-consuming groups compared to the control group.

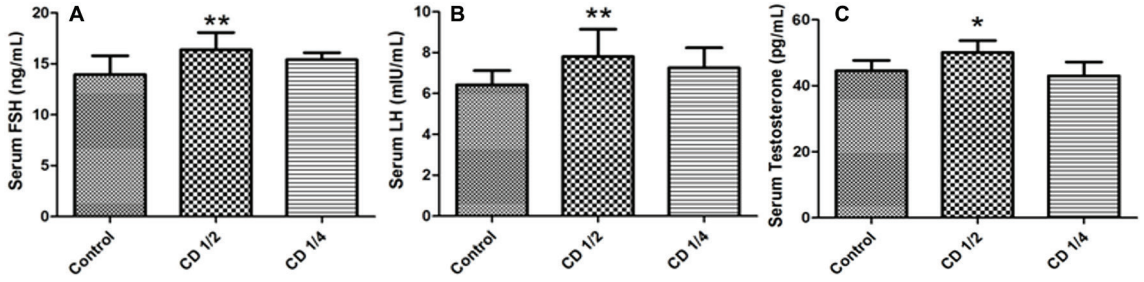

Figure 1: Serum concentrations of mice hormones are shown in groups of 10 mice each. P values are presented in CD1/2 and CD1/4 groups, respectively: FSH P=0.002, P=0.09 (A), LH P=0.009, P=0.018 (B), and testosterone P=0.03, P=0.85 (C). Distilled water was administrated to the control group, and diluted *Cichorium intybus* distillate was administrated to CD1/2 and CD1/4 groups in 1/2 and 1/4 ratios, respectively. *P<0.05 and **P<0.01 vs. control group.

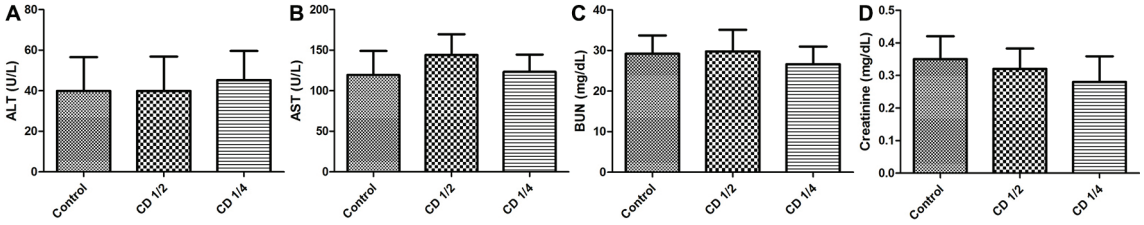

Figure 2: Serum hepatic enzyme activities are shown in groups of 10 mice each. P values are presented in CD1/2 and CD1/4 groups respectively: ALT P=0.99, P=0.89 (A) and AST P=0.10, P=0.99 (B) and kidney function parameters BUN P=0.99, P=0.43 (C), and Creatinine P=0.73, P=0.09 (D) in male mice (n=10). Distilled water was administrated to the control group and diluted *Cichorium intybus* distillate was administrated to CD1/2 and CD1/4 groups in 1/2 and 1/4 ratios, respectively.

Table 1: Sperm count and the ratio of normality in sperm morphology

Groups	Sperm count (× 10^6/mL)	Normal%	Abnormal morphology					
			Small head%	Big head%	Short tail%	Coiled tail%	Tail only%	Head only%
Control	43.65±7.70	95.60±2.43	0.64±0.24	0.21±0.14	0	0.12±0.03	1.20±0.38	2.29±1.48
CD 1/2	60.62±10.26*	95.18±2.73	0.33±0.22	0.24±0.14	0.13±0.09	0.13±0.08	0.89±0.48	2.54±0.78
P value	0.03	0.99	0.09	0.99	0.83	0.98	0.52	0.98
CD 1/4	63.23±18.42*	95.16±2.88	0.53±0.20	0.21±0.15	0.28±0.19	0.15±0.09	0.78±0.44	2.83±1.77
P value	0.01	0.99	0.85	>0.999	0.07	0.89	0.26	0.81

The values are presented as mean±SD. Distilled water was administrated to the control group, and diluted *Cichorium. Intybus* distillate was administrated to CD1/2 and CD1/4 groups in 1/2 and 1/4 ratios, respectively. *P<0.05 compared to the control group.

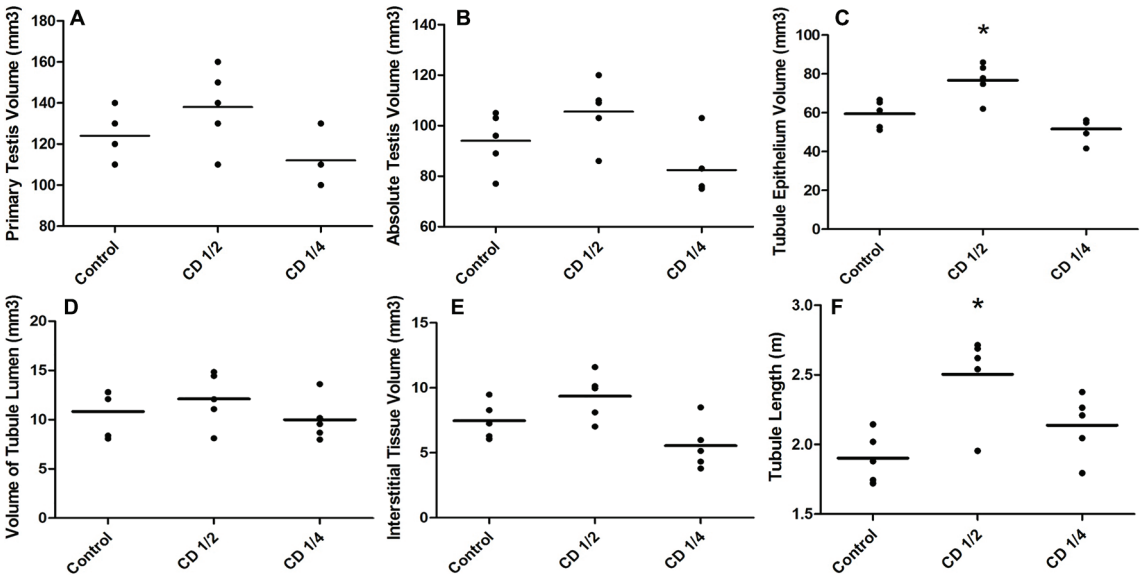

Figure 3: Stereological analyses of mice testis are shown in groups of five each. Distilled water was administrated to the control group, and diluted *Cichorium intybus* distillate was administrated to CD1/2 and CD1/4 groups in 1/2 and 1/4 ratios, respectively. P values are presented in CD1/2 and CD1/4 groups, respectively: Primary testis volume P=0.22, P=0.15 (A), testis volume by Cavaliries' method P=0.15, P=0.09 (B), the epithelium volume of seminiferous tubule P=0.029, P=0.22 (C), the lumen volume of seminiferous tubule P=0.69, P=0.84 (D), the volume of testis interstitial tissue P=0.15, P=0.09 (E) and seminiferous tubules length P=0.028, P=0.37 (F) are compared between groups. *P<0.05 compared to the control group.

Table 2: Testis cell count in 7 different cell types (×10^6)

Groups	Spermatogonia A	Spermatogonia B	Sertoli cell	Primary spermatocyte	Round spermatid	Long spermatid	Leydig cell
Control	0.83±0.06	0.16±0.07	2.70±0.29	12.12±0.98	31.85±2.49	30.91±3.02	2.21±0.02
CD1/2	0.85±0.06	0.17±0.06	2.73±0.27	12.55±0.95	32.85±2.72	31.94±2.88	2.67±0.39*
P value	P=0.92	P>0.999	P=0.99	P=0.86	P=0.90	P=0.92	P<0.01
CD1/4	0.79±0.06	0.18±0.06	2.63±0.16	11.65±0.78	30.50±2.24	29.94±1.97	2.19±0.02
P value	P=0.79	P=0.99	P=0.98	P=0.81	P=0.78	P=0.94	P=0.99

The values (n=5) are presented as mean±SD. Distilled water was administrated to the control group, and diluted *Cichorium intybus* distillate was administrated to CD1/2 and CD1/4 groups in 1/2 and 1/4 ratios, respectively. *P<0.01 compared to the Control group.

Stereological Parameters

The values for the primary volume of the testis, accurate testis volume (Cavalieri's), seminiferous tubule lumen and epithelium volume, the calculated volume for the interstitial tissue of the testis, and seminiferous tubules length are summarized in figure 3. As shown, CD consumption made a significant increase (P=0.029) in tubule epithelium volume in CD1/2 group (76.7±9.3 mm^3) compared to the control group (59.4±7.1 mm^3), as well as a significant increase (P=0.028) in the tubule length in CD1/2 group (2.5±0.3 m) compared to the control group (1.9±0.2 m). However, no significant effects were observed in other parameters. The numbers of different testis cell types are demonstrated in table 2. As shown, CD1/2 consumption significantly increased (P=0.009) Leydig cells number in comparison with the control group. There were no significant changes in the number of spermatogonia A/B, Sertoli cells, round and long spermatids, and spermatocytes in CD-treated groups.

Discussion

The results of the current study revealed that the administration of *C. intybus* distillate could increase the level of male reproductive hormones and sperm count. About 50% of common medications worldwide are derived from herbal medicine,[26] and various plant species with a diversity of preparations have been used for the treatment of male infertility in many countries,[27] including Iran.[28] The easily prepared form of distillate is especially used for such purposes. However, there is a lack of scientific evidence to verify their ameliorative impact or potential side effects on reproductive parameters. According to the literature, this research is the first scientific study to investigate the impact of *C. intybus* L. distillate, considering various fertility

parameters, histological analysis, and probable potential toxicity.

According to the results, CD1/2 consumption significantly elevated the serum concentrations of FSH, LH, and testosterone. Regulation of sperm production through the endocrine hypothalamic-pituitary-gonadal axis begins with the Gonadotropin-releasing hormone (GnRH), produced by the hypothalamus. GnRH induces FSH and LH secretion from the anterior pituitary. Leydig cells located in the interstitium of the testes are responsible for the secretion of testosterone, and Sertoli cells on the periphery of the seminiferous tubules, stimulate the spermatogenesis procedure.[29] In the current study, CD1/2 elevated LH levels, which in turn increased the secretion of testosterone as a result of a potential ameliorative effect of CD on this endocrine axis. On the other hand, an increase in FSH level led to a rise in sperm count. In line with our study, Dorostghoal and colleagues revealed that administration of *C. intybus* leaf extract increased FSH, LH, and testosterone levels in lead-treated rats compared to lead-treated control rats. However, there was no significant increase in hormone levels compared to the healthy control animals.[30] The difference in the results might be due to different preparations of the plant or different animal species.

In a study by Seghatoleslam and others, the composition and antioxidant properties of *C. intybus* L. distillate, used in our study, were investigated. The main components were found to be terpenes, flavonoids, and polyphenols. In the mentioned study, CD revealed antioxidant properties through the amelioration of glutathione peroxidase, glutathione reductase, and catalase enzyme activity, and reduced glutathione level in carbon tetrachloride treated rats.[14] Phytochemical analysis of *C. intybus* L. in previous studies has approved the existence of sesquiterpene lactones with antioxidant properties.[31, 32] The adverse effects of environmental toxins on FSH, LH, and testosterone levels can be improved by antioxidant compounds,[33] which are the main ingredients of CD.

To investigate the potential hepato/nephrotoxicity effects of CD, the levels of ALT, AST, BUN, and creatinine were evaluated, which revealed no differences between groups. However, hepatoprotective features of *C. intybus* L. have been approved in several studies.[34, 35]

According to the results of AR gene expression, no differences between groups were observed, which suggests that the observed improvement in fertility parameters could have resulted from the elevation of testosterone production rather than the expression of the receptors.

According to the results of the sperm analysis, it was shown that CD administration in both concentrations significantly increased sperm count, but it had no significant effects on the motility or normality of the sperm. It can be concluded that the increased serum FSH in the CD1/2 group might improve the level of spermatogenesis. On the other hand, the antioxidant properties of CD might prevent the peroxidation of polyunsaturated fatty acids (PUFA), which is the main cause of sperm injury. Studies have reported that the administration of terpene-containing plants such as *C. intybus* L. extract can improve sperm parameters via inhibition of PUFA peroxidation.[36, 37]

In the current study, the volume of seminiferous tubule epithelium was increased in the CD1/2 group together with an elevated tubule length, which was in agreement with the higher level of sperm production. Consumption of CD1/2 also increased the number of Leydig cells, followed by the elevation of testosterone concentration. However, an increase in the interstitial tissue volume was also expected. A study by Chaimontri and colleagues was in agreement with our finding, in which administration of a plant extract with antioxidant capacity and terpenoids, increased the volume of seminiferous tubule epithelium and increased sperm count.[38]

The limitation of the current study includes inadequate quantity of mice serum due to small body volume, which limited the serum-based tests, such as antioxidant enzymes, total antioxidant capacity, and the level of lipid peroxidation.

Conclusion

This experimental study demonstrated that *C. intybus* L. distillate consumption in male mice could improve the levels of hormones such as FSH, LH, and testosterone as well as sperm count. According to the ameliorative effects of CD and the promising results of liver and kidney function tests (ALT, AST, BUN, and Creatinine), it can be considered a safe complementary and/or alternative medicine to improve fertility in the male mice model. However, as an herbal supplement, the maintenance of the distillate, dosage, efficacy, and biochemical reactions leading to the observed results are yet to be investigated. At last, to extend the results in humans, it is suggested to evaluate the effects of CD in humans in a clinical trial, using a large sample population for a long duration.

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Authors' Contribution

Sh.F: Contributed in study design, performed the experimental procedure, statistical analysis, writing and revision of the manuscript; S.K.D: Contributed to the design and performance of the histological methods and semen analysis; A.N: Contributed to the microscopic study and the stereological calculations; A.S: Designed the study, supervising the experiments, writing, and revision of the manuscript. All the authors have substantial contributions to the final manuscript revisions and have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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