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Original Article

The Protective Effect of Chicoric Acid on the Mancozeb-induced Male Reproductive Damage in Mice

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Abstract

Background and Aim: This study aimed to evaluate the protective effect of chicoric acid (CA) on mancozeb-induced male reproductive damage in mice.

Methods: 65 NMRI male mice were randomly divided into 7 groups (n=8); 1: control group, 2: MZB–induced toxicity 3: MZB+ atropine (0.25 mg/kg daily), groups 4, 5, and 6: MZB+25, 50 and 100 mg/kg of CA respectively. 7: CA (100 mg). The mice were sacrificed thirty-five days later and blood and testis samples were obtained. Testosterone levels, sperm parameters, protamine deficiency, and sperm chromatin dispersion (SCD) were used to evaluate the reproductive system function.

Results: The sperm count and sperm viability decreased in the MZB-intoxicated group; the sperm DNA fragmentation and protamine deficiency increased in this group. Head and neck deformity decreased in MZB+ CA groups ($p<0.05$). In MZB+ CA groups, the sperm motility type A, and B increased than the MZB group, abnormal sperm morphology within 100 mg/kg CA groups was less than 50 and 25 mg/kg. The groups pretreated with CA showed a significant increase in Leydig cells.

Conclusion: The results revealed that chicoric acid has a protective effect on testis tissue damage induced by MZB. So, this is a promising therapeutic choice for the treatment of male infertility.

Keywords: Antioxidant; Chicoric acid; Mancozeb; Mice; Sperm.

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Introduction

The use of pesticides as the most important chemicals in agriculture has increased (to control pest animals) over the last decade. Their uncontrolled use and widespread application of these compounds cause many people to be daily exposed to these toxins, resulting in acute or chronic health side effects (1). Mancozeb (MZB) is a carbamate class of fungicide used to protect crops from a series of fungal diseases and it has importance over other carbamates (2).

Despite low reports of Mancozeb's acute side effects, their chronic complications such as chromosomal aberrations, tumor incidence, structural and functional alterations of gonads have been established (3, 4). Almost 15-30% of all couples suffer from infertility and the major parts of infertility are related to male reproductive system defects and sperm abnormality (5, 6). Disruptions in the nucleoproteins produce can modify the sperm chromatin structures and then affect male fertility (7). The origin and abundance of various types of male infertility are different. The excessive levels of reactive oxygen species (ROS) can be involved in infertility; although the existence of ROS is essential for sperm's normal development as capacitation, hyperactivation, head acrosome reactions, and oocyte adhesion during fertilization. The ROS elevation caused sperm DNA damage and chromatin defects that disturb the sperm's normal function, morphology, and motility. The indirect

valuation of protamine deficiencies in sperm chromatin is Chromomycin A3 (CMA3), which used for the estimate of the percentage of spermatozoa protamination to DNA binding (8). Several studies described that natural substance from plants source, exhibit strong antioxidant activity could protect the reproductive system damage. Many plants have various antioxidant compounds including polyphenols; mainly flavonoid compounds that have strong protective effects, against oxidative stress and their damages caused by drugs and toxins (9-12).

The Cichorium intybus is one of the plants used in traditional medicine which is a member of the Asteraceae family and possessing various biological activities; Chicory can lower the blood glucose, lipid and uric acid, and hepatic-protection (13, 14). The alcoholic and aqueous extracts of Cichorium intybus showed significant protection against DNA damage and protein oxidation due to the presence of phenolic compounds in chicory that eliminate ROS (15, 16). chicoric acid is the dominant phenolic reported in Cichorium intybus which has been reported to have potential antioxidant, anti-inflammatory, antiviral and immune-stimulating properties (17).

The main component of Cichorium intybus is chicory acid, which acts as an anti-oxidant, which might have a good effect for removing of MZD toxin's side effects; so in the present study, we aimed to find out the protective effect of the Cichoric acid on mancozeb-induced male reproductive damage in mice.

Methods

Chemicals

Chicoric acid (CA), Phosphate-buffered saline (PBS), and Chromomycin A3 (CMA3) were purchased from Sigma-Aldrich chemical company (St Louis, MO, USA). All chemicals and reagents used were analytical grades.

Animals

In this study, 65 Adult NMRI male mice (10-12 weeks) weighing between 25±30 gr were used. The animals were procured from an animal house located at Gerash University of Medical Sciences, Gerash, Iran. They were housed in well-ventilated stainless-steel cages at room temperature (22±2 ºC) in the hygienic condition under 12 hours light and dark schedule and were fed by standard laboratory diet. Food and water were given ad libitum.

Experimental design

To induce mancozeb poisoning in mice, the mancozeb (500 mg/kg) is gavage orally once every two days in olive oil. The animals were divided into seven groups (n=8). Animals in group 1 (NS) received 5ml/kg/PO normal saline for 35 days, group 2 (MZB) received, mancozeb (500 mg/kg) for 35 days, Groups 3 (MZB- atropine) received mancozeb+atropine (0.25 mg/kg daily) for 35 days. Animals in groups 4, 5, and 6 (MZB- CA) received mancozeb+25, 50, and 100 mg/kg of chicoric acid, respectively, for 35 days. Group 7 (CA) received only 100 mg/kg/PO of chicoric acid for 35 days (3, 18). All administration was done orally (PO). Twenty-four hours after the last administration, the animals were sacrificed.

Sample collection

At the end of the study, the animals were anesthetized and sacrificed with an intraperitoneal injection of ketamine-xylazine. Blood samples were collected by cardiac puncture. The blood samples have remained at room temperature for 30 min, and then serum was separated by centrifugation at 2000 rpm at 22 ℃ for 10 min. The serum sample was kept at -20 ∘C for the evaluation of testosterone levels. The right testes and vas deferens were removed for the assessment of sperm analysis and testicular parameters(19).

Assessment of serum testosterone level

Evaluation of testosterone levels was done by using the ELISA kit (IBL Company, Japan).

Assessment of sperm parameters

After euthanasia, testis, epididymis, and vas deferens were measured and weighted. The right testis, caudal epididymis, and vas deferens were removed for evaluation of sperm parameters.

Semen collection

The epididymis sperms were collected through slicing the caudal region of the epididymis, then transferred into 5 mL Ham's F10 medium supplemented with 8mg/mL bovine serum albumin ((BSA) Sigma Company, St. Louis, USA)), then incubated for 30 min at (37 ◦C and 5% CO_2) in the

humidified air to allow sperm to swim out from epididymis tubules. After semen collection, the sperm motility, count, morphology, viability, and chromatins integrity were estimated using conventional methods (19).

Sperm motility

To assess sperm motility, 10 µL sperm suspension was placed on a (pre-heated) slide and covered with a slip, then the sperm motility evaluated under a light microscope (Nikon TS100, Tokyo, Japan) via using a 400x magnification, and almost 200 spermatozoa were evaluated for each specimen and reported as WHO protocols, in which: A (Direct path fast progressive sperm motility), B (tortuous path fast progressive sperm motility). C (slow progressive sperm), D (immotile sperm) per total sperms. The morphological sperm parameters are evaluated as a deformity in the head, neck, and tail (8).

Sperm count

For assessment of sperm count, immediately after dissection of the epididymis, sperm fluid was dipped (1:20 dilution was prepared in a 1 cc microtube by adding10 µL sperm mixture in 190 µL of distilled water. consequently, 10 µL of this mixture was dropped on a Neubauer chamber and count. We were sperm counted in four large squares as the mean was multiplied by 104; finally, the result was described as the sperm's number in milliliter (20).

Assessment of morphology and viability

Sperm viability was evaluated using the eosin stain then at least 100 spermatozoa were counted. The staining was done with a (10 µL) of sperm suspension and (20 µL) of eosin stain. Dead sperm cells were stained because of damaged membranes and the sperm head seemed purple to red but the live sperms were unstained(21).

Testicular parameters

The width, thickness, and length also the volume of testis were measured by using a standard digital caliper. The histological parameter such as the thickness of the germinal epithelium, the number of Leydig cells, and the diameter of seminiferous tubules was measured by a light microscope and counted in 10 randomly selected fields after the testes were fixed and embedded in paraffin (19).

Protamine deficiency assessment

Protamine deficiency was evaluated using Chromomycin A3 (CMA3) staining according to Fortes·sc(2014) methods (22). After semen samples were washed with PBS, the sample 3 times centrifuged (3000 rpm for 5 min).

The fixations Carnoy's solution (Methanol/Glacial acetic: 3:1) was added to the washed sperms and placed in the refrigerator (at 4 °C for 10 min). These sample slides were stained with 100 µL CMA3 (0.25 mg/mL in McIlvaine buffer, pH 7.0, with 10 mM $MgCl^2$) (Sigma-Aldrich Corp., St. Louis) in a dark room for 20 min. after the slides were PBS washed, mounted with buffered glycerol.

Then almost 200 spermatozoa per slide were counted with a fluorescence microscope(Japan-Nikon-Eclipse600) under filter (460-470 nm) and magnification (100 X) to reporting positive and negative CMA3 sperms, in which normal protamine spermatozoa is called CMA3-negative and seems dull green color after staining while Protamine deficient spermatozoa are categorized CMA3-positive seems bright yellow color after staining (8).

Statistical analysis

The results obtained were expressed as mean±SEM and using one-way ANOVA followed by using Tukey's post hoc test for comparison of data between these groups. The Statistical significance was set at $p<0.05$ by using SPSS (Version 20; SPSS Inc., Chicago, USA) software.

Results

Serum testosterone level

The highest blood testosterone level in the CA (100 mg) group and the lowest level of Testosterone seen in MZB – intoxicated (P-value: 0.061); their Serum Testosterone Level was (0.9-0.48 ng/mL), respectively.

Sperm's viability

The number of live sperms in the positive control group (Mancozeb) was significantly different from the normal group. The highest number of live sperm was seen in the group that received 100 mg/kg of chicoric acid, which was significant (p-value: 0.011) (Figure 1).

Structural sperm's abnormalities

In the Mancozeb group, the most abnormalities were seen in the sperm head, and in the atropine group, the defects of the sperm head were less than in the Mancozeb group. Head malformations at the group that received 100 mg/kg of chicory acid were less than 50 and 25 mg/kg (p-value: 0.003) (Figure 1).

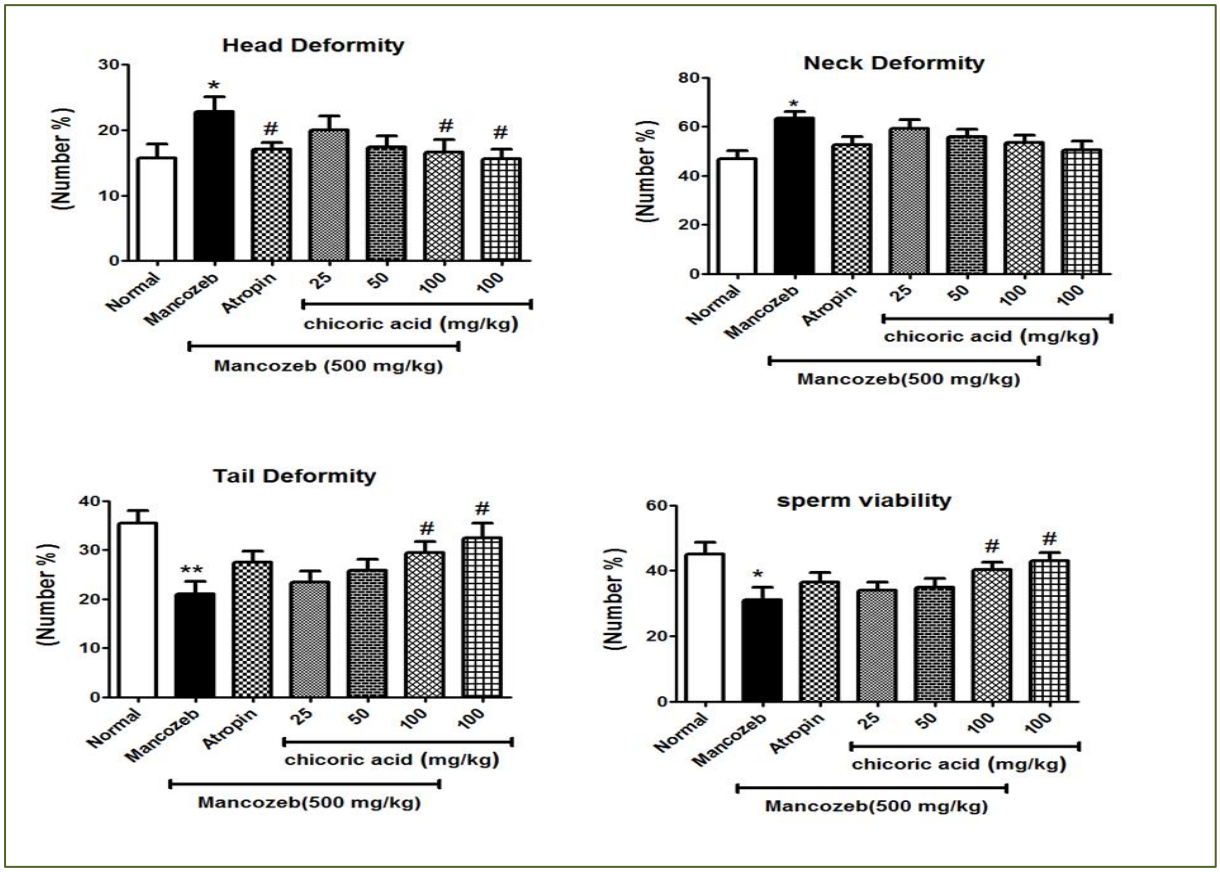

Figure 1. Effect of chicoric acid (CA) on sperm viability and kind of abnormal morphology in MZB-intoxicated male mice. Data were analyzed by a one-way ANOVA test followed by Tukey's post hoc test.
*Significantly different from the control group (*p< 0.05).
#Significantly different from the MZB group (#p< 0.05).

The most common malformations in the neck were seen in the Mancozeb group. The atropine group had lower neck deformity than the chicory acid receiving groups (p-value: 0.04) (Figure 1).

In the Mancozeb group, the tail anomaly was significantly less than normal. The highest tail anomaly was seen in the 100 mg/kg chicory group (significant). The lowest tail malformations were in chicory at a dose of 25 mg/kg (p-value: 0.903) (Figure 1).

Types of sperm's motility

The lowest amount of sperm with the Type A movement was seen in the Mancozeb group. The highest numbers of sperm with the Type A movement were seen in the 100 mg/kg chicory group (p-value: 0.000) (Figure 2).

The lowest amount of this type of movement was seen in the Mancozeb group. It increased in atropine compared to the toxin group. At chicory treated groups, it increased significantly compared to the Mancozeb group (p-value: 0.000) (Figure 2).

Motility type C significantly increased in the Mancozeb group. At chicory treated groups, these movements at doses of 25 mg/kg were greater than 50 and 100 mg/kg, respectively (P-value: 0.06) (Figure 2). Motility type D was lowest in the

normal group, whereas it was highest in the Mancozeb group (significant). In chicory 100 and Mancozeb-chicory 100 groups significantly decreased (p-value: 0.000) (Figure 2).

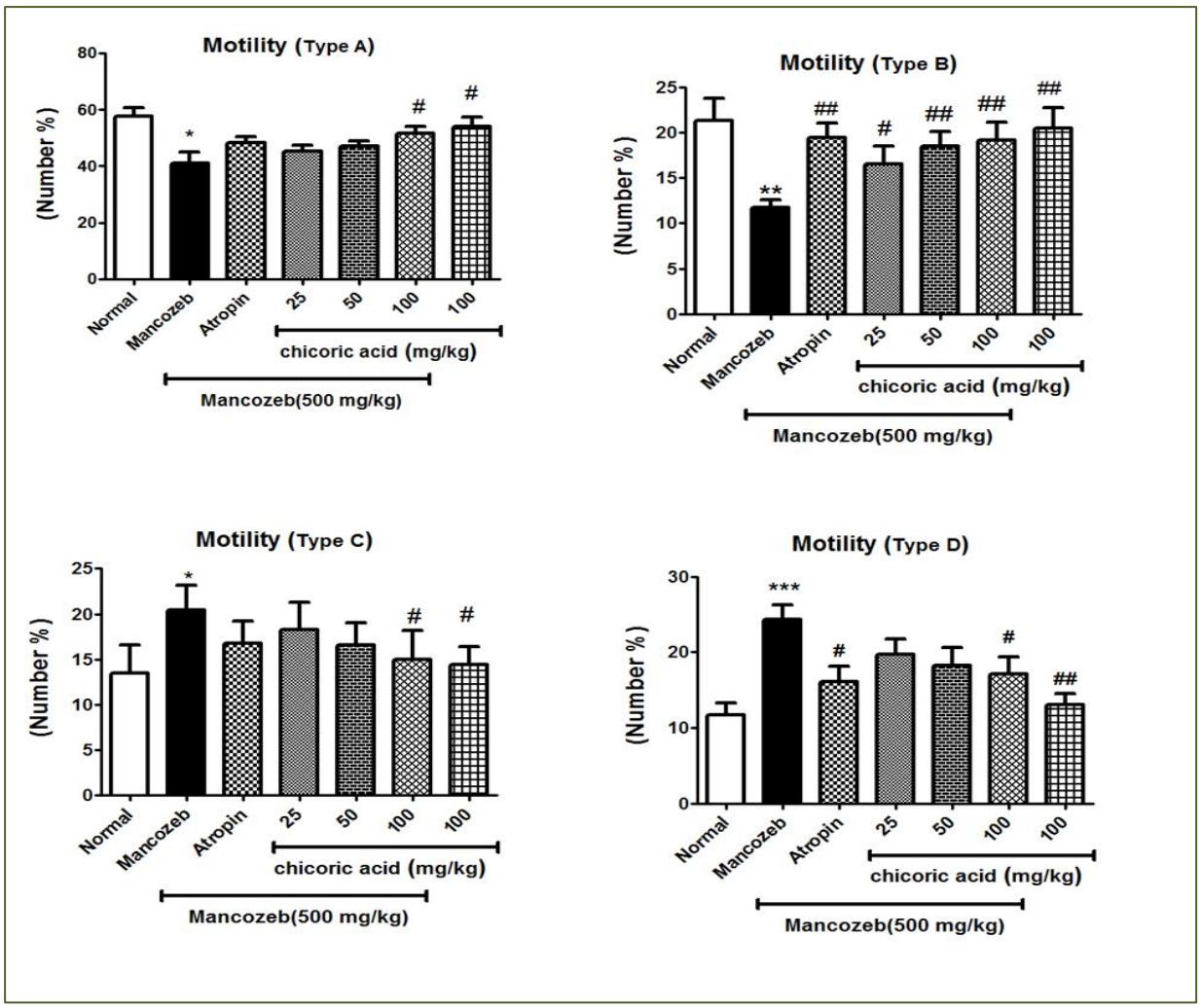

Figure 2. Effect of chicoric acid (CA) on Types of sperm motility in MZB-intoxicated male mice. Data were analyzed by a one-way ANOVA test followed by Tukey's post hoc test.
*Significantly different from the control group (*p< 0.05).
Significantly different from the MZB group (#p< 0.05).

Protamine deficiency

In the Mancozeb group, it was significantly higher than normal. In chicory treated groups, it was significantly reduced at a dose of 100 (p-value: 0.001) (Table 1).

Testicular study

The lowest Leydig cell was seen in the Mancozeb group which was significantly different from the normal group (p<0.05). In the chicory group 100 and the meknozeb +100 mg/kg, chicoric acid group the number of Leydig cells was higher than in the Mancozeb group (p-value: 0.063) (Figure 3). Testicular volume, length, testicular width, and weight of mice in different groups, did not show any significant difference (p-value: 0.274). The weight of the rats was significantly lower in the Mancozeb group. At the dose of 100 mg chicoric acid, the weight of mice was significantly higher (p-value: 0.18).

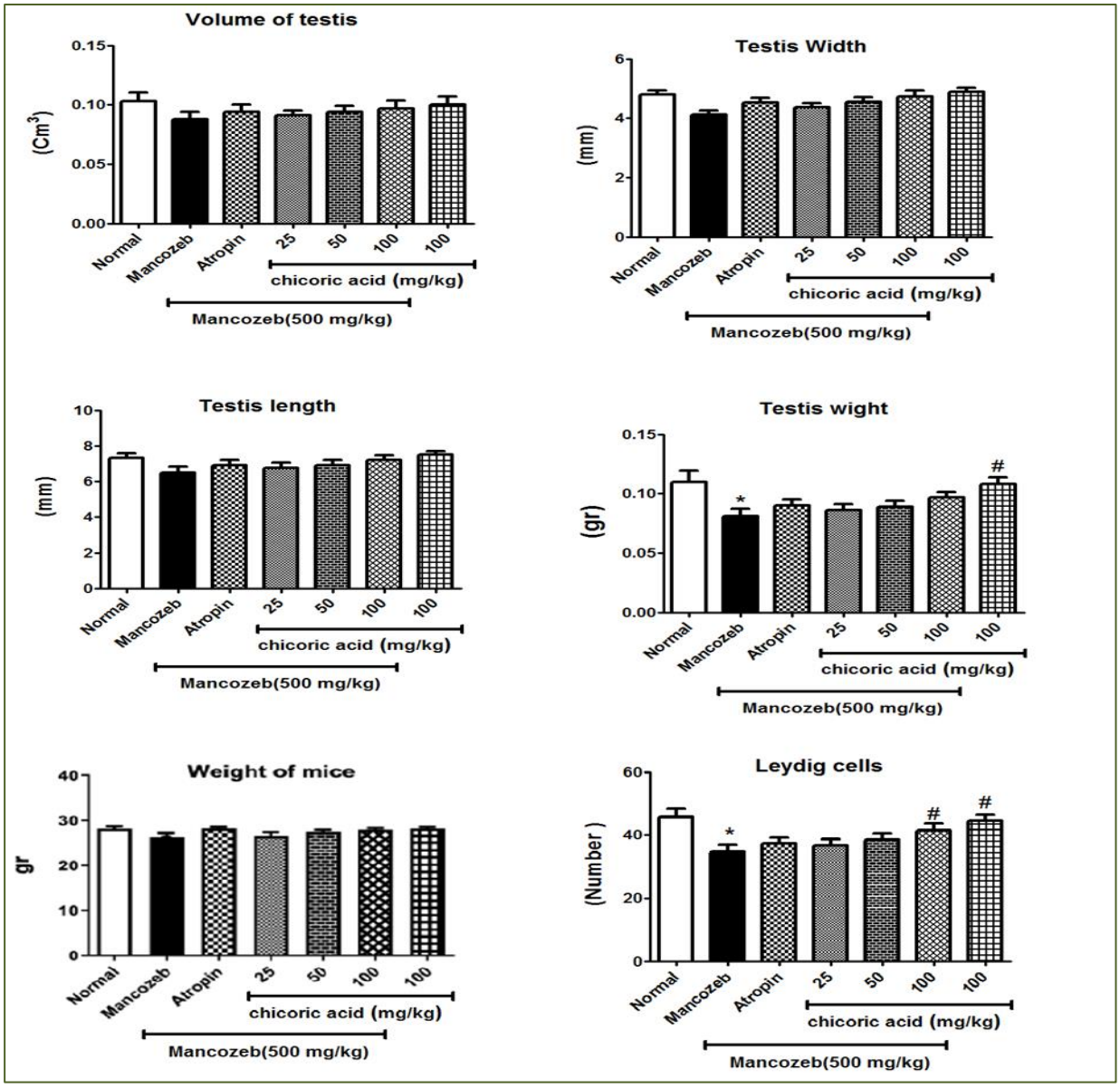

Figure 3. Effect of chicoric acid (CA) on testicular parameters in MZB- intoxicated male mice. Data were analyzed by a one-way ANOVA test followed by Tukey's post hoc test.
*Significantly different from the control group (*p<0.05). #Significantly different from the MZB group (#p<0.05).

Table 1. Effect of chicoric acid (CA) on Protamine deficiency and testosterone level in MZB-intoxicated male mice. Data were analyzed by a one-way ANOVA test followed by Tukey's post hoc test.

Groups	Protamine deficiency (Number %)	Testosterone level(ng/mL)
Normal	**29**	**0/83**
Mancozeb	**51**	**0/48**
Atropin + Mancozeb	**34**	**0/50**
25mg/kg CA + Mancozeb	**40**	**0/61**
50mg/kgCA + Mancozeb	**36**	**0/68**
100mg/kg + Mancozeb	**33**	**0/74**
100mg/kg CA	**26**	**0/9**

Discussion

Our findings showed that after MZB - intoxicated, the percentage of abnormal sperms such as DNA damaged sperms and protamine impairment was increased significantly. During impaired spermatogenesis, sperm chromatin deficiency and DNA damage occur and lead to reduce the quality of sperm parameters and sperm chromatin packing; Also elevated free radicals especially reactive oxygen species (ROS) induce sperm protamine deficiency and nuclear immaturity (8, 20).

The oxidative stress decreases the number of Leydig cells through enzymatic and non-enzymatic manner, which causes a reduction of testosterone level (23). On the other hand, there is a negative correlation between normal spermatogenesis and the elevated ROS level (24). Normal spermatogenesis depends on cooperation between the Leydig cells and Sertoli cell's endocrine activity. According to this study, we can conclude that CA can promote testosterone biosynthesis, sperm quality, and decrease tubular seminiferous atrophy with antioxidants properties. The sperm count is increased in CA-MZD rather than MZD, in which the highest sperm alive number is seen in CA 100 mg that indicated the functions of the sertoli cell and within CA-MZD groups, the testosterone levels higher than MZD-induced shown the Leydig cell activity that affected the male reproductive system.

In the current study the sperm motility, viability, and DNA integrity significantly decreased in MZB-intoxicated mice, this was in agreement with Sakr and Okdah (2004) that indicated the degeneration of the Spermatogenic cells and reduction in the diameter of the seminiferous tubules (4). Besides, in this study, the histopathological alterations in the testis were seen but did not affect significantly in weight, volume, and height of the testis. The improvement of spermatogenesis criteria after administration of

chicoric acid (CA), which might be due to antioxidant properties and also androgenic activities; on the other hand's fructose in seminal fluid is the main source of energy for sperms motility and since fructose is androgen-dependent then affected to sperm parameters such as sperm motility, count and viability because the Chemical agents can penetrate from the blood-testis barriers (25).

In the present study, we considered a significant decrease in the diameter of the seminiferous tubules in MZB-intoxicated mice compared with control and CA which was in agreement with Dorostghoal et al.(26). Hormonal abnormalities due to exposure to pesticides were reported by many studies, the serum testosterone level in MZB-intoxicated mice reduced in agreement with ours, the level of testosterone hormone and luteinizing hormone decreased in MZB – intoxicated and increased this level within CA treatment; which was in contrast with Soliman et al(25) study reported that administration of C. intybus extracts at doses of (250 and 500 mg/kg) showed a significant decrease in serum levels of FSH, LH, and testosterone. Also, Yahia et al. (2015)(27) reported that exposure to pesticides induced malformations in androgen-dependent tissues. The sperm parameters associated with the spermatogenic function of Sertoli cells in the seminiferous tubules, while the testosterone hormone levels related to the testicular steroidogenic functions of Leydig cells; on the other hand, the seminiferous tubules and Leydig cells were decreased in MZB-intoxicated mice then might lead to the low quality of sperm parameters and testosterone levels. Our findings showed that after administration of CA against the MZB exposures mice, the testosterone levels and quality of sperm parameters increased which emphasizes the CA protection effects. The current study confirmed that chicory administration improved the sperm protamine deficiency and the number of DNA fragmentation; this may be due to the chicory antioxidant components such as chicoric acid and abundant polyphenol compounds(28). Sperm analysis is a valuable criterion for the determination of male fertility and estimates the level of the hormones such as testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH)(29). Chicory increased testosterone production by increased the release of LH and FSH and prevents aromatase enzyme activity (30). The generation of reactive oxygen species (ROS) induces an adverse change in chromatin quality and sperm parameters

(31). Protamine is a necessary protein for hydrodynamic property and compaction of sperm's head which promotes the sperm's normal motility. An increase in ROS level leads to lower protamine in chromatins, consequently, sperm head compaction is disrupted (8, 32). Normal sperm motility is affected by protamine levels and function. The chicoric acid decreased protamine deficiency and promotes sperm motility. In the current study, the ideal dosage of CA that protects the MZD- intoxicated was 100 mg. Among sperm malformation, the head deformity is the highest effect on motility that maximum head deformity was seen in MZD- intoxicated groups and minimum head malformation realized in CA 100 mg. The oxidative stress reduces the Leydig cells in the enzymatic and non-enzymatic manner and causes a reduction of testosterone levels(33). On the other hand, there is a negative correlation between normal spermatogenesis and increased ROS levels (34). Normal spermatogenesis depends on increased Leydig and Sertoli cells endocrine activity and decreased oxidative stress, and then according to this study, we can conclude that CA can promote testosterone biosynthesis, sperm quality and decrease tubular atrophy with antioxidants effects. Mancozeb accelerates ROS production, so it affects sperm parameters and testicular functions. The present study confirmed that chicoric acid administration is capable to largely reduce the damage caused by MZB, and the dose of 100 (mg/kg) can stimulate the sperm fertility criteria.

Conclusion

In conclusion, since chicoric acid has powerful anti-inflammatory and antioxidant effects, therefore; this substance probably decreased the toxic effects of MZB on testis cells due to the antioxidant effects and eliminating free radicals.

Our findings support the traditional use of C. intybus as a solution for male reproductive problems, it is hoped that based on the present study and other studies on different animal models as well as subsequent studies on human models this substance, could one day be used as a safe drug. Accordingly, because of chicoric acid’s androgenic activities, antioxidant therapy probably improves the sperm chromatin and reduced DNA damage due to MZD induced.

Conflict of Interest

The authors declared that they have no conflict of interest.

Acknowledgment

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Ethics

This study was approved by the Ethical Committee of the Gerash University of Medical Sciences guidelines for animal use by referee number GERUMS REC.1396.1071.

Author contributions

Conceptualization: HF. Formal analysis: HA. Methodology: IA. Project administration Writing - original draft: IA. Writing - review & editing: HA.

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