International Journal of Biochemistry Research & Review
14(1): 1-11, 2016, Article no.IJBCRR.28351
ISSN: 2231-086X, NLM ID: 101654445

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Oils of *Nigella sativa* L. and *Cinnamon zeylanicum* Inhibit the Testicular Cytotoxicity and Genotoxicity Induced by Mancozeb in Rats

Heba Barakat[1*] and Shimaa El-Masry[1]

[1]Department of Biochemistry and Nutrition, Women's College, Ain Shams University, Egypt.

Authors' contributions

This work was carried out in collaboration between both authors. Both of the authors designed the study, wrote the protocol, supervised the work, carried out all laboratories work, performed the statistical analysis, managed the analyses of the study, wrote the manuscript and edited it. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJBCRR/2016/28351
Editor(s):
(1) Richard A. Manderville, Departments of Chemistry and Toxicology University of Guelph, Canada.
Reviewers:
(1) Xiuhui Zhong, Agricultural University of Hebei, China.
(2) B. Ravishankar, SDM Centre for Research in Ayurveda and Allied Sciences, Udupi, India.
(3) Bechan Sharma, University of Allahabad, Allahabad, 211002, UP, India.
(4) T. Pullaiah, Sri Krishnadevaraya University, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/16344

Original Research Article

Received 15th July 2016
Accepted 22nd September 2016
Published 27th September 2016

ABSTRACT

Mancozeb, a fungicide of ethylene bisdithiocarbamate is widely used in agriculture. It leads to disturbances in many cellular processes. This study is aimed at elucidating the possible protection effects of *Nigella sativa* oil (NSO) and *Cinnamon zeylanicum* oil (CZO) in alleviating the toxicity of mancozeb on reproductive performance in adult male rats. Animals were orally administered with NSO (2 ml/kg/day) or CZO (100 mg/kg/day) either alone or with mancozeb (100 mg/kg/day) for 50 days. Results showed that groups administered each of the oils alone increased serum testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels, antioxidant enzymes and integrity of cellular and genetic status of testes compared to control group. However, the co-administration of mancozeb with each of these oils modulated the testicular toxicity exerted in the toxicated group. The results showed alleviation in the integrity of the testicular cells and the %DNA in tail and tail length, the hormone measurements and the serum oxidative stress compared

**Corresponding author: E-mail: dr_hebabarakat@yahoo.com;*

with the toxicated (non- treated) group. Our results indicated that NSO had higher protective effect than CZO against mancozeb-induced reproductive toxicity.

Keywords: Cinnamon zeylanicum; comet assay; mancozeb; Nigella sativa; testicular toxicity.

1. INTRODUCTION

The environmental exposure to pesticides has potential risks to food consumers, production workers, farmers and others as evidenced from the previous *in vitro* and *in vivo* experiments [1-4]. Using of pesticides is not only resulted in a high cost of production but also causes poisoning and potential toxicity to biological system [5].

The incidence of male fertility decay has increased alarmingly since 1980s as a result of multi-factorial events including environmental pollution such as that caused by agrochemicals. Agrochemicals are continuously used on a massive scale for global food production and persist as residues in food of both vegetable and animal origin as well as in the air and water [6].

Mancozeb (manganese ethylene bisdithiocarbamate complex with zinc) is one of the carbamates pesticides, that has significance over carbamates. It is used against a variety of foliar fungal diseases and seed treatment in Egypt. It shows its biological effects through its metabolites like ethylene thiourea (ETU) and carbon bisulfide (CS_2) [7]. Thus exposure of the general population to mancozeb and its metabolites cause endocrinal disruption [8] concomitantly with alterations in the antioxidant defense system [9].

Cell damaging effects of free radicals can be met out by antioxidants, flavonoids or other active compounds against diseases by acting as protective factors or increasing antioxidant activity [10]. There has been increased interest among phytotherapy researches to use medicinal plants with antioxidant activity for protection against toxicity. *Nigella sativa* L. (black cumin) and *Cinnamon zeylanicum* (Cinnamon), both are known for their antioxidant, anti-inflammatory and curing effects for different diseases [11-13].

Nigella sativa L. belongs to the botanical family of *Ranunculaceae* that is used as a flavoring agent in bread and pickles. *Nigella sativa* oil (NSO) contains over 100 bioactive molecules among which thymoquinone (30-48%), ρ-cymene (7-15%), carvacrol (6-12%), 4-terpineal (2-7%), t-anethol (1-4%), sesquiterpene longifolene (1-8%), thymohydroquinone, dithymoquinone and α-pinene are some of the predominant compounds [14].

Cinnamon zeylainicum, a medicinal plant belongs to *Luaraceae* family. This plant contains a variety of biological active chemicals which have immense medical potential, including essential oils that provide the specie`s flavor. *Cinnamon zeylanicum* oil (CZO) contains 65-80% cinnamldehyde, 5-10% eugenol and other compounds such as cinnamic acid, hydroxyl cinnamldehyde, cinnamyl alcohol, coumarin, cinnamyl acetate in lesser percentages [15].

Although in recent years, the knowledge on the toxic effects of carbamates markedly improved with doses not less than 200 mg/Kg body weight [16], that its testicular DNA damage due to its toxic radicals still need more investigations. Moreover the role of NSO and CZO against mancozeb-induced changes in male reproductive performance has not been studied so far. So this study was undertaken to investigate the degree of male reproductive toxicity induced by low dose of mancozeb and the protective role of each of the NSO and CZO administration in alleviating this toxicity.

2. MATERIALS AND METHODS

2.1 Chemicals

Mancozeb was purchased from Cam Company for agrochemicals, New City Nubaria, Beheira, Egypt.

2.2 Extracted Oils

Both of the NSO and CZO were purchased from the Department of Oils and Fats, Division of the Food Industry and Nutrition at the National Research Center, Dokki, Giza, Egypt.

2.3 Experimental Animals

Seventy two adult male Sprague-Dawley rats weighing 154-178 g were purchased from Research of Bilharzias Institute, Academic of

Scientific Research and Technology, Cairo, Egypt. Rats were acclimatized to the animal house laboratory conditions of the Medical Research Center, Ain Shams University, Cairo, Egypt for 7 days maintained at constant 24℃ with 12 h light-dark cycle and fed a standard purified control diet prepared according to AIN [17] and water *ad libitum*. After acclimatization, the rats were randomized into six experimental groups. The protocol of this study was approved by the research ethical approval committee of the Medical Research Center, Ain Shams University.

2.4 Experimental Design

Animals within the different treatment groups were treated for 50 consecutive days, because the spermatogenic cycle including spermatocytogenesis, meiosis and spermiogenesis takes about 48-52 days [18].

The mancozeb dose given was, a low as, 100 mg/Kg body weight/day orally by gavage [19] dissolved in corn oil [20].

NSO and CZO doses were homogenized with corn oil for daily oral administration by gavage in a dose of 2 ml/Kg body weight [21] and 100 mg/Kg body weight [22], respectively.

The study included the following experimental groups: control group, administered daily oral corn oil by gavage; toxicated group, administered mancozeb dose only; NSO and CZO groups, administered NSO and CZO doses only; and treated groups, administered simultaneously mancozeb and either of the NSO or CZO.

2.5 Body Weight Measurement

Body weight was recorded weekly beginning on zero time (the time prior to treatment) and continued until the end of the treatment.

2.6 Sample Collection

At the end of the experimental period, animals were sacrificed under ether anesthesia. Blood was collected from the hepatic portal vein, centrifuged (10 min, 3000 rpm, 4℃) for serum separation. Testes were excised, washed and dried for calculating its relative weight. Also representative samples of both testes were taken for comet assay and histological examination.

2.7 Sex Hormones Measurements

Serum testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) measurements were performed by radioimmunoassay using commercial kits from Radim (RadimSpA, Pomezia, Italy) according to Ismail [23], Beitens et al. [24] and Santner et al. [25], respectively.

2.8 Oxidant-antioxidant Status Assessment

Serum activities of catalase (CAT) and glutathione peroxidase (GPx) were determined according to the methods described by Luck [26] and Chiu et al. [27], respectively. Serum malondialdehyde (MDA) and nitric oxide (NO) levels were determined colorimetrically according to Draper and Hadley [28] and Berkels et al. [29], respectively.

2.9 Comet Assay

The alkaline (pH >13) comet assay was performed according to the method described by Tice et al. [30], with minor modifications for testes El-Ghor et al. [31]. The extent of DNA migration for each sample was determined by simultaneous image capture and scoring of 50 cells at ×400 magnification using Komet 5 image analysis software developed by Kinetic Imaging, Ltd (Liverpool, UK). The extent of DNA damage was evaluated according to the following endpoints measurements: Tail length: it is used to evaluate the extent of DNA damage away from the nucleus and expressed in μm; % DNA in tail: Intensity of all tail pixels divided by the total intensity of all pixels in the Comet; and tail moment: calculated as:

Tail moment = tail length x % DNA in tail / 100.

2.10 Histological Examination

Specimens from testes were fixed in 10% formal saline and processed routinely for embedding in paraffin. Sections of μm were stained with hematoxylin and eosin stain [32] and examined under the light microscope.

2.11 Statistical Analysis

Means were calculated for all data. Significant differences between means were evaluated using one way analysis of variance (ANOVA) and

Post Hoc Tukey. A difference was considered significant when p was less than 0.05. Data analysis was carried out using Microsoft Excel (2010) Microsoft Corporation and Statistical Package for Social science (SPSS) version 16 Inc.

3. RESULTS

3.1 Effect of NSO and CZO Administration on the Final Body Weight and Testis Weight in Mancozeb-toxicated Rats

Table 1 shows that administration of mancozeb to the experimental rats caused a highly significant (P<0.05) drop in the body weight and relative testes weight compared to control group. However, there were no significant differences between control and each of the NSO or CZO groups. On the other hand treatment of rats with NSO and CZO along with mancozeb caused a non- significant (P<0.05) increase in body weight and relative testes weight compared to mancozeb-toxicated group.

3.2 Effect of NSO and CZO Administration on the Sex Hormones in Mancozeb-Toxicated Rats

From Table 2, it is obvious that, treatment with low dose of mancozeb exhibited a marked decrease in the plasma total testosterone level and a significant increase (P<0.05) in the main gonadotropin hormones (FSH and LH) levels comparing to control group. While the toxicated groups treated with NSO or CZO showed a significant increase (P<0.05) in the total testosterone and decrease in the levels of FSH and LH compared to mancozeb-toxicated group. However, Rats administered NSO or CZO alone exerted a significant increase in the levels of testosterone, LH and FSH when compared to control group.

3.3 Effect of NSO and CZO Administration on the Oxidant-antioxidant Status in Mancozeb-toxicated Rats

Table 3 revealed that oral administration of mancozeb to experimental rats caused disturbance in the serum oxidant-antioxidant status as indicated by significant (P<0.05) reduction in the antioxidant enzymes (CAT and GPx), accompanied with significant (P<0.05) elevation in lipid peroxidation markers (MDA and NO) compared to control group. However the administration of either NSO or CZO alone showed the highest levels of serum CAT and GPx and the lowest levels of serum MDA and NO among all the experimental groups. The administration of NSO and CZO along with mancozeb reversed the adverse effect of mancozeb toxicity and significantly (P<0.05) ameliorated the oxidant-antioxidant status when compared to rats administered mancozeb only.

Table 1. Final body weight and testes weight of adult male albino rats classified on different experimental designs: (means ± S.D.)

Groups	Final body weight (g)	Relative testes weight (%)
Control	197.83 ± 7.16^{a}	3.28 ± 0.53^{a}
Mancozeb-toxicated	175.33 ± 11.12 ^{b}	2.26 ± 0.64 ^{b}
NSO	193.0 ± 4.79 ^{ace}	2.78 ± 0.38 ^{abd}
CZO	202.50 ± 4.505 ^{a}	3.21 ± 0.47 ^{ad}
Mancozeb-toxicated+NSO	183.16 ± 10.12 ^{bc}	2.61 ± 0.69 ^{cbd}
Mancozeb-toxicated+CZO	184.66 ± 10.01^{be}	2.76 ± 0.48 ^{abd}

P<0.05, There are no significant differences between means have the same letters in the same column

Table 2. Sex hormones values of adult male albino rats classified on different experimental designs: (means ± S.D.)

Groups	Total testosterone (ng/ml)	FSH (mIU/ml)	LH (mIU/ml)
Control	3.06 ± 0.61 ^{a}	0.135 ± 0.04 ^{a}	0.356 ± 0.03 ^{a}
Mancozeb-toxicated	1.06 ± 0.03 ^{b}	0.498 ± 0.06 ^{b}	0.838 ± 0.04 ^{b}
NSO	4.04 ± 0.37 ^{c}	0.151 ± 0.04 ^{a}	0.460 ± 0.04 ^{c}
CZO	5.54 ± 0.33 ^{d}	0.190 ± 0.05 ^{a}	0.471 ± 0.06 ^{c}
Mancozeb-toxicated+NSO	1.58 ± 0.05 ^{e}	0.265 ± 0.03 ^{c}	0.643 ± 0.03 ^{d}
Mancozeb-toxicated+CZO	1.87 ± 0.04 ^{e}	0.281 ± 0.03 ^{c}	0.716 ± 0.02 ^{e}

P<0.05, There are no significant differences between means have the same letters in the same column

Table 3. Oxidant-antioxidant status of adult male albino rats classified on different experimental designs: (means ± S.D.)

Groups	CAT (U / g)	GPX (U / g)	MDA (nmol/ml)	NO (µmol/L)
Control	45.08 ± 2.33 [a]	11.36 ± 1.14 [a]	2.75 ± 0.24 [a]	1.56 ± 0.25 [a]
Mancozeb-toxicated	24.43 ± 2.38 [b]	3.97 ± 0.56 [b]	7.98 ± 1.04 [b]	3.51 ± 0.30 [b]
NSO	80.48 ± 4.52 [c]	14.65 ± 1.28 [c]	0.99 ± 0.34 [c]	0.94 ± 0.24 [c]
CZO	63.06 ± 3.80 [d]	11.60 ±1.12 [a]	1.12 ± 0.41 [c]	1.0 ± 0.28 [c]
Mancozebtoxicated+NSO	35.85 ± 3.17 [e]	10.20 ± 0.82 [a]	3.69 ± 0.35 [d]	2.0 ± 0.14 [d]
Mancozeb-toxicated+CZO	33.81 ± 2.90 [e]	7.75 ± 1.04 [d]	4.30 ± 0.44 [d]	2.55 ± 0.20 [e]

P<0.05, There are no significant differences between means have the same letters in the same column.

3.4 Effect of NSO and CZO Administration on the Degree of Testicular DNA Damage Caused by Mancozeb (Comet Assay)

Administration of mancozeb produced increases in the levels of testicular DNA damage evidenced by significant increases in tail length, the percentage of DNA in tail as evidence from the comet assay by the migration of DNA from the nucleus into the tail, and the tail moment comparing to control (Table 4). However this elevation was significantly (P<0.05) alleviated by the administration of NSO or CZO for 50 consecutive days. The best results were observed in CZO group, which recorded lower levels of comet tail length, the percentage of tail, and the tail moment than the control group. Despite of the administration of mancozeb with CZO had more effect on amelioration of testicular DNA damage than administration of mancozeb with NSO comparing to rats administered mancozeb alone, that there was no significant difference recorded between the treated groups with respect to the % DNA in tail and tail moment.

3.5 Histological Examination

The histological studies revealed that treatment of rats with low dose of mancozeb was able to induce damage to the testes tissues as illustrated by degeneration and necrosis of spermatogoneal cells lining seminiferous tubules as well as interstitial edema (Fig. 2) in contrast to control group that showed normal appearance of testes tissues (Fig. 1). Each of the NSO and CZO administered alone to animals showed no histopathological changes and normal appearance of tissue (Figs. 3 and 4) respectively. The administration of NSO along with mancozeb approximately restored the histological architecture to normal and decreasing the alterations induced by mancozeb treatment (Fig. 5). However the treatment with CZO showed a slight degeneration of spermatogoneal cells lining seminiferous tubules (Fig. 6).

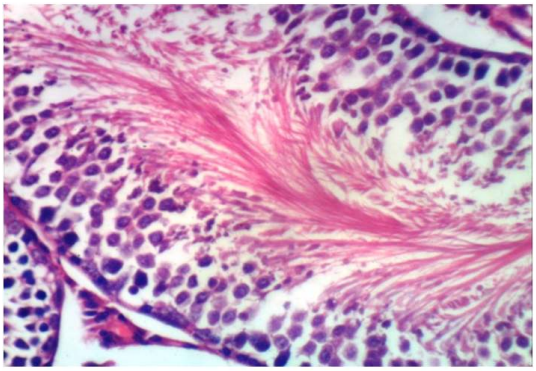

Fig. 1. Testis of rat from control group showing the normal histological structure of seminiferous tubules with normal spermatogoneal cells and complete spermatogenesis with sperm production (H & E X 400)

Table 4. Degree of testicular DNA damage of adult male albino rats classified on different experimental designs: (means ± S.D.)

Groups	Tail length (Mm)	% DNA in tail	Tail moment
Control	8.61 ± 0.70 [a]	28.30 ± 0.14 [a]	3.39 ± 0.15 [a]
Mancozeb-toxicated	12.31 ± 0.45 [b]	34.65 ± 1.53 [b]	4.72 ± 0.23 [b]
NSO	10.58 ± 0.18 [c]	20.01 ± 2.10 [c]	3.15 ± 0.63 [a]
CZO	7.71 ± 0.54 [d]	18.85 ± 2.54 [c]	1.92 ± 0.68 [c]
Mancozebtoxicated+NSO	12.08 ± 0.68 [b]	30.30 ± 1.54 [a]	4.61 ± 0.27 [b]
Mancozeb-toxicated+CZO	11.29 ± 1.01 [c]	29.42 ± 2.22 [a]	4.07 ± 0.39 [d]

P<0.05, There are no significant differences between means have the same letters in the same column

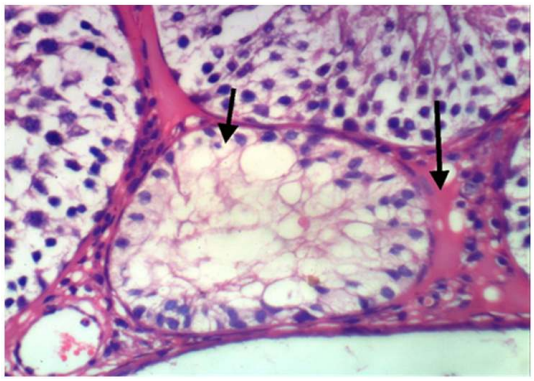

Fig. 2. Testis of rat from mancozeb-toxicated group showing degeneration and necrosis of spermatogoneal cells lining seminiferous tubules as well as interstitial edema (H & E X 400)

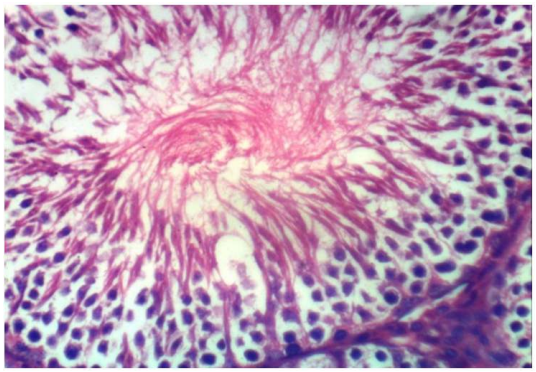

Fig. 3. Testis of rat from NSO group showing no histopathological changes (H & E X 400)

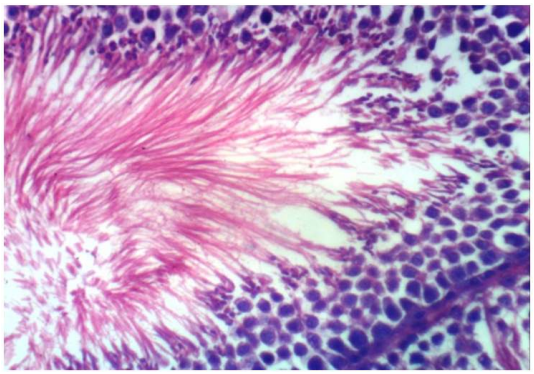

Fig. 4. Testis of rat from CZO group showing no histopathological changes (H & E X 400)

4. DISCUSSION

Mancozeb is an effective fungicide that has been in use for many years. It is characterized as the most agricultural chemical with respect to male reproductive toxicity [33]. The Carbon bisulfide (CS_2) which is the major mancozeb metabolite is the cause of the pro-oxidant processes in blood. CS_2 undergoes extensive bio-transformation by the microsomal monooxygenase in the liver leading to several thiol-containing products, which promote free radical production and lipid peroxidation. Also, aminothiols such as cysteine and homocysteine may function as pro-oxidant [34] by binding to and destructing cytochrome P450 [35], changing the cytosolic NAD(P)H level and/or interfering with the cellular redox status [36,37].

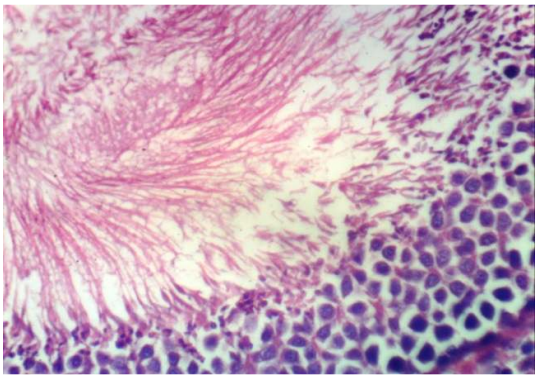

Fig. 5. Testis of rat from mancozeb-toxicated+NSO group showing no histopathological changes (H & E X 400)

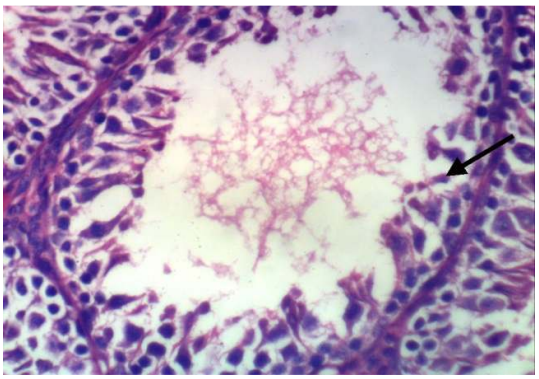

Fig. 6. Testis of rat from mancozeb-toxicateb+ CZO group showing slight degeneration of spermatogoneal cells lining seminiferous tubules (H & E X 400)

In the present investigation, the daily mancozeb oral administration with low dose (100 mg/Kg body weight) for 50 days decreased both of the body and testicular relative weight and altered the normal testicular atrophy. As the weight of testis is largely dependent on the mass and

integrity of the differentiated spermatogenic cells, so the reduction in the weight of testis may be due to the decreased number of germ cells and laceration of cells. CS_2 is considered the cause of the testicular and epididymal degeneration, and thus affects spermatogenesis [5]. That reduction in the spermatogenesis at the spermatid stage was explained as a result of the structural imbalance at the tubulin [38]. Moreover, the sperm analysis by Ksheerasagar and Kaliwal [7] revealed a significant decrease in sperm concentration and motility in albino mice treated with mancozeb compared to the control mice. This alteration is possibly due to a dysfunction of Sertoli cell responsible for the maintenance of spermatogenesis. In addition the decrease in sperm motility may be due to morphological defects in the intermediate piece and the flagellum which ensures spermatozoa`s movement and speed [5].

Serum total testosterone levels were significantly decreased in mancozeb-treated rats in the present study as compared to the control group. The lower plasma testosterone level could be attributed to the impairment in testis cells which is related to the spermatogenic inhibition. That among the Steroli cells which are responsible for the production of the nutritional fluid of the spermatozoa and also for forming the testes barrier to protect the germ blood-born toxicants [39]. Moreover, both of the serum LH and FSH levels were increased significantly in the toxicated rats compared with the control group. These alterations in LH and FSH levels may be due to the indirect effect of mancozeb on the pituitary gland. It has been reported that toxic agents as nitric oxide (NO) may act directly on the gonadotropins to alter its synthesis and secretion or indirectly by altering the pituitary cells responsiveness to gonadal steroids or to GnRH which destroy the endocrinological homeostasis via the feedback of the hypothalamus to the pituitary [7].

Besides hormonal alterations, the spermatogenic inhibition in the toxicated rats may also be due to the formation of free radical products which attack the testis. Lipid peroxidation is highly detected by the increased serum level of MDA which is consistent with significantly decreased serum activities of CAT and GPx. These observations indicated a disturbance in the oxidant-antioxidant defense system. Also, a high concentration of serum NO level was detected in the mancozeb treated rats. NO is an end-metabolic product directly related to peroxinitrite overproduction. This increased NO level could be the consequence of iNOS activation, which is induced especially under high oxidative stress conditions.

It is well known that the enzymatic antioxidants (GPx and CAT) play an important role in the cellular antioxidant defense system by scavenging free radicals and other ROS, preventing oxidation of biomolecules. That the disturbance in the antioxidant enzymes activities could be the reason of increased level of lipid peroxidation in rats treated with mancozeb. Domica et al. [40] demonstrated that mancozeb inhibited the mitochondrial respiration which in turn produced robust ROS generation in cells exposed to acute toxicity for 24h. The increased oxidative stress results in the lipid peroxidation, which affects the membrane integrity and fluidity. Since the mammalian spermatozoa membranes are rich in polyunsaturated fatty acids. So, they are very susceptible to oxygen-induced damage (superoxide, hydroxyl, nitric oxide, peroxide, peroxynitrite) which is mediated by lipid peroxidation. Moreover, the induction of ROS is associated with DNA fragmentation in sperm nuclei [41]. Further, the ROS mediated peroxidation of critical thiol groups in protein can alter the structure and function of spermatozoa [42].

In the present investigation, mancozeb induced germinal cell degeneration as evident from the testes comet assay which revealed the genotoxicity of mancozeb administration (tail length, tail moment and %DNA in tail). These results may be an indication of apoptosis induced by mancozeb in a stage specific manner in spermatogenia and spermatocyte of rat testes as a result of the increased ROS produced. This explanation is emphasized from Srivastava et al. [43] who examined an *in vitro* exposure of human lymphocytes to mancozeb by (5 µg/ml) for 24 h decreased significantly the levels of SOD and CAT and increased ROS generation and lipid peroxidation. Additionally its triggered apoptosis and activation of $NF\text{-}_kB$ with increased DNA damage expressed by p53 and p21 and reduction in DNA repair genes.

The present study indicates the beneficial effects of NSO and CZO against mancozeb induced testes toxicity in rats. NSO had been reported as a scavenging of the free-radical via its bioactive components. Thymoquinone, the main compound in NSO inhibits non-enzymatic lipid peroxidation in liposomes. Moreover, other

compounds in the NSO such as p-cymene, m-cymene, α-thujene and carvacrol have been reported to possess antioxidant effects and radical scavenging properties in rats [14,21,44-47]. Moreover, CZO has several biological activities including radical scavenging. The antioxidant, metal chelating and free radical scavenging activity of phenolic compounds as ellagic acid and other oil compounds have been reported in different experimental studies [22,48-50].

Furthermore, the beneficial scavenging effect of each of the oils administered was translated as a testicular DNA protective against the genotoxic effects of mancozeb (oxidative DNA damage) as indicated from the comet assay. Both of the NSO and CZO decreased the testicular tail length and % DNA in tail and accordingly tail moment. These results are supported by the decreased levels of serum MDA and NO and subsequently the increased serum activities of CAT and GPx. Elgawish and Abdelrazek [51] concluded that the cinnamon administered to male rats in the dose of 250 mg/Kg body weight had significantly inhibited the testicular apoptosis induced by lead acetate as indicated by lowering the testicular caspase-3 expression. Also, Yüce et al. [22] reported that the CZO consumption (100 mg/Kg body weight) for 10 weeks tended to decrease the apoptotic germ cells. On the other hand, El-Boghdady and Darwish [21] documented that NS served as potent DNA protective via inhibiting the purine-catabolizing enzymes, attenuating the lysosomal damage as well as COX-2 mediated inflammation signaling in renal tissue.

The administration of NSO or CZO alone increased significantly serum testosterone, LH and FSH levels. Furthermore, the co-administration of NSO or CZO with mancozeb showed a marked adjustment in hormone level imbalance induced by mancozeb and subsequent improved semen quality. The improvement of reproductive parameters after the NSO or CZO administration may be explained due to the presence of bioactive compounds in the oils which affect the hypothalamus-pituitary axis and has thus increased concentrations of these hormones [52,53]. Furthermore, Shagauo and Davidson [54] concluded that cinnamon is capable of releasing LH hormone by affecting hypothalamus axis and increasing the secretion rate of GnRH hormone which proliferate the sex cells by increasing the leyding cell activities in adult rats. In another explanation, Mosbah et al. [14] showed an increment in the steroidogenesis and testosterone production accompanied by an improvement in serum proliferation by NSO treatment. They explained these results by an increase in the testicular 17-ketosteroid reductase activity which is responsible in the transfer of cholesterol in mitochondria and testosterone biosynthesis.

5. CONCLUSION

The study revealed that the administration of NSO or CZO alone for healthy adult male rats improved their fertility and antioxidant status and the testicular cell and DNA integrity. However, the NSO recorded the higher benefit than CZO. Moreover, the co-administration of each of the NSO or CZO with mancozeb modulates its toxicity in testes.

ETHICAL APPROVAL

All authors hereby declare that “Principles of animal care” were followed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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