Metiram fungicide-induced histomorphological and biochemical changes in rat ovaries: Attenuation by *Nigella sativa* oil

Hanaa Zakaria Nooh

Anatomy and Embryology Department, Faculty of Medicine, Menoufia University
Faculty of medicine, Shebin El- Kom, Menoufia, Egypt
(drhanaanooh@gmail.com)

Abstract

Metiram is a fungicide used against fungal diseases of field crops. *Nigella sativa* L. (Black seed) is widely distributed herb with wide therapeutic uses. This work aims to study the effect of *N. sativa* oil on ovarian toxicity induced by metiram fungicide in albino rats. Treating animals with metiram caused many histopathological alterations. The number of ovarian follicles decreased and most of them degenerated which accompanied by increase of atretic follicles and increase of collagen fibers. Histochemical results revealed a decrease in carbohydrates content. Metiram significantly decreased the levels of both LH, FSH and estradiol. It also caused an increase in lipid peroxidation and decreased the ovarian antioxidant enzymes, SOD, CAT. This study revealed an ameliorative effect of *N.sativa* oil against metiram-induced ovarian toxicity through significant increase in number of healthy follicles with a decrease of the atretic follicles and collagen fibers. Moreover, *N.sativa* treatment led to a significant increase in FSH, LH and estradiol levels. A decrease in lipid peroxidation and increase in antioxidant enzymes were recorded. These results give new insight on beneficial effect *N. sativa* oil against female gonadal toxicity and this effect may be attributed to its antioxidant activity.

Keywords: Antioxidant enzymes, *N. sativa* oil, Ovarian tissue; Rat.

1 Introduction

Fungicides are a group of pesticides applied against wide range of fungal diseases of field crops, fruits, nuts and ornamentals. Although fungicides, proved useful, it does not excluded from expressing its hazardous actions in mammalian animals and human. Intoxication with these chemicals constitutes a major problem, especially because this could be happened by ingestion of contaminated crops like fruits and vegetables. Metiram (polyram) is a non-systemically acting fungicide of dithiocarbamate group. It is used to prevent crop damage in the field and to protect harvested crops from deterioration in storage or transport (Charls et al., 2000). Exposure to metiram was accompanied by adverse effects. Sortwell et al., (1977) reported follicular hyperplasia in thyroid of female rhesus monkeys treated with metiram. Kornuta et al., (1996) reported that metiram is one of the pesticides which showed genotoxic effect. Treating rabbits with high-dose level of metiram was resulted in minimal to moderate exfoliation and ulcerative dermatitis in the skin (Ullmann et al., 1987). Whalen et al., (2003) showed that the fungicides (maneb, metiram, and ziram) have cytotoxic effects on both T and NK lymphocyte function. Metiram was found to induce histopathological as well as biochemical alterations in the liver of albino mice (Sákr et al., 2009). Sakr and Badawy (2011) reported that metiram-inhibited spermatogenesis and induced apoptosis in albino mice.

Many traditional plants products are in use today due to their therapeutic potential. *Nigella sativa* L. is an annual herb belongs to Family Ranunculaceae. It grows in Mediterranean countries and it is one of the native plants that are widely distributed in Egypt. Black seeds are ascribed to have many medicinal properties in traditional medicine. It is effective against cough, bronchitis, asthma, chronic headache, migraine, dizziness, chest congestion, dysmenorrheal, obesity, diabetes, paralysis, hemiplegia, back pain, infection, inflammation, rheumatism, hypertension, and gastrointestinal problems such as dyspepsia, flatulence, dysentery, and diarrhea (Datta et al.
Animals in this group (15 rats) were given ethylenethiuram disulfide and 20% inert ingredients. Genotoxicity and ultrastructural changes induced by CCl4 ammoniate ethylenebis (dithiocarbamate)-poly (ethylenethiuram disulfide) and anti-hepatotoxicity (Farrag et al. 2007) in experimental animals. Tasawar et al. (2011) reported that black seed (tested on 80 subjects) is effective to change the lipid profile significantly in a way which is beneficial to heart. Treatment of the animals with N. sativa improved both genotoxicity and ultrastructural changes induced by CCl4 (Abou-Gabal et al. 2007). Parhizkar et al. (2011) reported that N. sativa possesses estrogenic function which can be helpful in managing menopausal symptoms as an alternative for hormone replacement therapy. Although there are many investigations on the impact of N. sativa on various tissues, its effect in metiram-induced ovarian toxicity has not been examined yet. In the present work we evaluated the effects of N. sativa oil on serum antioxidant enzyme activities, lipid peroxidation and histological changes in ovarian tissue exposed to metiram in a rat model. Also we evaluated the changes in FSH, LH and estradiol levels following the treatment of N. sativa oil.

2 Materials and Methods

Fifty five Female Wistar rats weighting 155 ± 10 g were used in this work. They were purchased from the breeding center of experimental animals at Helwan University, Helwan, Egypt. The animals were kept in the laboratory under constant temperature (24±2 °C) throughout the experimental work. They were maintained on a standard rodent pellets and water was available ad libitum. Maintenance of animals and experimental procedures was approved by the animal ethical committee in accordance with the guide for care and use of laboratory animals of Menoufi University, Egypt. Animals were divided into 4 groups:

Group I. These animals (10 rats) served as controls.

Group II. Animals in this group (15 rats) were orally given 0.2ml/100 g b.w N. sativa oil 3 days/week for 4 weeks (Kamarzaman et al. 2012). N. sativa oil was obtained from Cairo Pharmaceutical and Chemical Industries Co., Egypt.

Group III. Animals of this group (15 rats) were orally given 1/10LD50 (284 mg/kg b.w) of metiram3 days/week for 4 weeks dissolved in distilled water (Sakr and Badawy, 2011). This fungicide was obtained from SHOURA Chemical Company, Cairo, Egypt. It consists of 80% active ingredients [zinc ammoniate ethylenebis (dithiocarbamate)-poly (ethylenethiuram disulfide) and 20% inert ingredients.

Group VI. Animals in this group (15 rats) were given the same dose of metiram given to animals of group III followed by N. sativa oil (0.2ml/100 g) 3 days weekly for 4 weeks.

2.1 Hormonal study

For hormonal determination, blood sample was obtained from the inferior vena cava and then centrifuged. Sera were stored at -20 OC until assayed for the biochemical parameters. FSH, LH and estradiol were quantitatively determined in sera by enzyme immunoassay kit (Medix Biotech Inc., USA).

2.2 Ovarian oxidative stress

For determination of antioxidant enzymes, ovaries were removed and homogenized in potassium phosphate buffer solution (50 mM, pH 7.5) using a Potter-Elvehjem homogenizer to give a 10% homogenate. Homogenates were centrifuged at 1500 g for 10 min at 4°C; supernatant was recovered, placed on ice, and immediately used for the determination of MDA, CAT and SOD. The activity of superoxide dismutase (SOD) was determined according to the method of Nishikimi et al. (1972). The principal of this method depends on the ability of
SOD to inhibit the power of phenazine methosulphate-mediated to reduce the nitroblue tetrazolium. The catalase (CAT) activity was assayed according to the method of Aebi (1984). Catalase activity was determined from the rate of decomposition of H 2O2. Malondialdehyde MDA, as a marker for lipid peroxidation was measured colorimetrically in ovarian homogenate according to the method of Ohkawa et al. (1979).

**Statistical analysis**

The results were expressed as mean ± SD of different groups. The differences between the mean values were evaluated by ANOVA followed by Student’s “t” test using Minitab 12 computer program (Minitab Inc., State Collage, P.A).

3 Results

**Change in body and ovary weights**

Exposure of albino rats to metriam led to a significant decrease in the body weight of animals compared to the control (P<0.0001). A significant increase in body weight was observed in animals treated with metriam + *N. sativa*. Animals given *N. sativa* alone did not show differences in body weight compared to the controls. Similarly, ovary weights decreased in animals treated with metriam and increased in those treated with metriam + *N. sativa* (Table 1).

**Histological observations**

Examination of ovarian sections of the control group (group I) showed the normal histological structure of the ovary. The surface of the ovary was covered by a single layer of flat to cuboidal cells (covering epithelium). Follicles in different stages of development were detected. Primary oocyte was surrounded by a single layer of squamous epithelial cells and multilaminar primary follicles were surrounded by a few layers of granulosa cells, were detected. Secondary follicles were also observed and each was formed of several layers of granulosa cells with multiple fluid filled spaces. Several corpora lutea were formed of large pale-staining vacuolated granulosa-lutein and theca lutein cells, enclosing many capillaries in between. Atretic follicles and mature graafian follicle were observed (Fig. 1).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body weight(g)</th>
<th>Ovary weight(mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>155.3±3.2</td>
<td>31±7.6</td>
</tr>
<tr>
<td><em>N. sativa</em></td>
<td>156±2.05</td>
<td>31.3±5.2</td>
</tr>
<tr>
<td>Metriam</td>
<td>135.5</td>
<td>21.3±17.1</td>
</tr>
<tr>
<td>Metriam+<em>N. sativa</em></td>
<td>153.5</td>
<td>30.6±9.19</td>
</tr>
</tbody>
</table>

(*) Significant at P<0.05 compared with control group
Ovary of animals treated with metiram and *N. sativa* showed an improvement in the histological appearance. Several normal follicles and corpora lutea with few of congested blood vessels was observed (Fig.5). Stroma of control rats and those given *N. sativa* showed a few collagen fibers (Fig.6), while animals treated with metiram showed an increase in collagen fibers in the stroma and around the blood vessels (Fig.7). Animals given metiram and *N. sativa* showed a reduction in collagen fibers compared with metiram-treated animals (Fig.8).

**Quantitative results**

Data in table (2) showed that treating rats with metiram for 4 weeks caused significant decrease (P<0.05) in the number of medium, large and small follicles compared with control groups. The number of follicles was significantly increased after treating with metiram and *N. sativa*. On the other hand, the number of small and large atretic follicles increased significantly in rats treated with metiram (table 3). The number of atretic follicles decreased in animals treated with metiram and *N. sativa*. Data in table (4) showed the percentage of collagen content in different animals group. An increase in collagen content was recorded in rats treated with metiram compared with control and *N. sativa*-treated groups. On the other hand, the percentage of collagen content reduced in animals given metiram and *N. sativa*.
### Table 2. Effect of different treatments on the number of ovarian follicles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Number of follicles</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>small</td>
<td>medium</td>
<td>large</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>250±11</td>
<td>51±4</td>
<td>9±1</td>
<td></td>
</tr>
<tr>
<td>N.sativa</td>
<td>5</td>
<td>248±9</td>
<td>49±5</td>
<td>8±2</td>
<td></td>
</tr>
<tr>
<td>Metiram</td>
<td>5</td>
<td>171±5*</td>
<td>22±3*</td>
<td>3±2*</td>
<td></td>
</tr>
<tr>
<td>Metiram+N. sativa</td>
<td>5</td>
<td>222±8</td>
<td>42±3</td>
<td>7±1</td>
<td></td>
</tr>
</tbody>
</table>

(*). Significant at P<0.05 compared with control group

### Table 3. Effect of different treatments on the number of atretic follicles

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Number of atretic follicles</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>small</td>
<td>medium</td>
<td>Total number</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>9±2</td>
<td>3±1</td>
<td>12±1.4</td>
<td></td>
</tr>
<tr>
<td>N.sativa</td>
<td>5</td>
<td>10±1</td>
<td>4±1</td>
<td>14±1.5</td>
<td></td>
</tr>
<tr>
<td>Metiram</td>
<td>5</td>
<td>18±2*</td>
<td>7±2*</td>
<td>25±2.1*</td>
<td></td>
</tr>
<tr>
<td>Metiram+N. sativa</td>
<td>5</td>
<td>11±2</td>
<td>4±1</td>
<td>15±1.5</td>
<td></td>
</tr>
</tbody>
</table>

(*). Significant at P<0.05 compared with control group

### Table 4. Mean area% of collagen fiber content in different animal groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>N.sativa</td>
<td>5.8±0.6</td>
</tr>
<tr>
<td>Metiram</td>
<td>19.6±1.3*</td>
</tr>
<tr>
<td>Metiram+N. sativa</td>
<td>8.6±2.2</td>
</tr>
</tbody>
</table>

(*). Significant at P<0.05 compared with control group

### Histochemical Observations

Examination of ovary of control rats or rats treated with N.sativa revealed that the germinal epithelial cells and the stromal cells showed a strong PAS-positive reaction. The ovum of primary, secondary and Graafian follicles also showed a strong reactivity (Fig.9). Sections in ovaries of animals treated with metiram showed a marked decrease of PAS positive materials in the different parts of the ovary as compared with ovary of both control and N.sativa treated rats (Fig.10). On the other hand, rats treated with metiram and N. sativa showed an increase in the total carbohydrate contents of the stromal cells, the ovum of primary, secondary and Graafian follicles compared with those of metiram treated animals (Fig.11).

### Hormonal results

Treating animals with metiram revealed significant decrease in LH level compared with control group. Animals treated with metiram and N.sativa showed significant decrease in LH level when compared with metiram treated rats (Fig. 12). Similarly, a significant decrease in FSH was recorded in sera of animals treated with metiram while animals treated with metiram and N.sativa showed significant increase when compared with metiram treated rats (Fig.13). A significant decrease in estradiol level was observed in sera of animals treated with metiram and significant increase was recorded in sera of animals treated with metiram and N.sativa (Fig.14). Treating animals with N.sativa showed non-significant difference in levels of FSH, LH and estradiol when compared with control group.
Fig. 13. Change in FSH in different animal groups, (*). Significant at P<0.05 compared with control group.

Fig. 14. Change in estradiol in different animal groups. (*). Significant at P<0.05 compared with control group.

**Lipid peroxidation and antioxidant enzymes**

Data in table (5) showed that metiram treatment exhibited a significant increase in the level of MDA (the marker of lipid peroxidation). Significant decrease in the activities of the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), was observed in female rats administered with metiram. On the other hand, rats treated with metiram and *N. sativa* showed a decrease in MDA and an increase in the antioxidant status (SOD, CAT).

Table 5. Effect of different treatments on MDA, SOD and CAT in ovarian tissue of rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/mg protein)</th>
<th>SOD (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.5±1.5</td>
<td>220±18.6</td>
<td>530±20.4</td>
</tr>
<tr>
<td>N. sativa</td>
<td>16.3±1.8</td>
<td>222±20.5</td>
<td>541±19.5</td>
</tr>
<tr>
<td>Metiram</td>
<td>29.4±2.2*</td>
<td>165±10.6</td>
<td>258±21.5*</td>
</tr>
<tr>
<td>Metiram + N. sativa</td>
<td>20.8±1.3</td>
<td>200±12.5</td>
<td>410±16.5</td>
</tr>
</tbody>
</table>

(*) Significant at P<0.05 compared with control group.

**4 Discussion**

In the present study, many histopathological changes were seen in the ovary of albino rats after treatment with metiram. The number of ovarian follicles decreased and most of them degenerated which accompanied by increased of atretic follicles. In agreement with these results, Sakr et al. (2011) reported that topsin fungicide caused histopathological alterations in the ovary and decreased the number of follicles with an increase of the atretic follicles. They added that topsin significantly decreased serum levels of both LH and FSH and increased estradiol. Treating rats with mancozeb fungicide was found to induce a significant decrease in the number of healthy follicles with concomitant increase in the number of atretic follicles. The histologic observation of the ovary revealed the presence of less number of corpora lutea and the size of the ovary was also reduced (Bolivar and Kaliwal, 2001). Administration of edifenphos, an organophosphate fungicide, to hemicastrated albino rats caused a decrease of ovarian weight and decreased the number and duration of different phases of the estrous cycle (Nanda and Kaliwal, 2003).

Shibayama et al. (2009) reported that atrazine herbicide led to histopathological findings such as decrease in the numbers of corpora lutea, increase in large-sized atretic follicles and swelling of the luteal cells. The authors suggested that atrazine had an ovulatory effect through suppression of the luteinizing hormone surge. Goldman et al. (1997) reported that the fungicide sodium dimethyldithiocarbamate caused a dose-related suppression of oocyte release in rats and this involves two separate mechanisms, one attributable to an alteration in ovarian hormonal feedback to the brain (or pituitary), inhibiting the LH surge, and the other associated with a direct, as yet undetermined, effect on local preovulatory events within the ovary. Armenti et al. (2008) recorded that exposure to organochlorine pesticide, methoxychlor, resulted in reduced ovulation and fertility and premature aging, possibly by altering ovarian gene expression and folliculogenesis. The authors added that methoxychlor reduced serum progesterone, increased luteinizing hormone and down-regulated Cytochrome P450 side-chain cleavage.

A decrease in amount of carbohydrates was recorded in ovary of rats treated with metiram. Similarly, Sakr and Badawy (2011) observed depletion of glycogen in testicular tissue of rats given metiram. El-Sherbiny et al. (2010) reported an alteration in some enzymatic
activity that had led to metabolic degradation and
inhibited the carbohydrate synthesis in the ovarian
follicles of rats exposed to some insecticides. An
increase in lipid peroxidation marker, MDA, and a
decrease in the antioxidant enzymes, SOD and CAT,
was recorded in ovarian tissue of rats treated with
metiram. These results strongly suggested that
metiram has the capability to induce oxidative
damage. These results are consistent with the previous
study of Sakr et al. (2011) who observed an increase in
lipid peroxidation and decrease of antioxidant
enzymes in rats treated with the fungicide, topsin.

In the present study, the decrease in healthy follicles
with concomitant increase in atretic follicles in rats
exposed to metiram was interrupted by some authors.
Sarkar et al. (2000) reported that this effect may due
to inhibition of acetyl cholinesterase which alters the
pituitary as it was observed in the rats treated with
pesticide quinolphos. Another possibility may be due
to affecting catecholamine neurotransmitter
metabolism by inhibiting the GnRH release through
the inhibition of DβH (Przewlocka et al. 1975).
Pasquale et al. (1990) reported that the reproductive
toxicity of 12-dimethyl (a) anthranene may be due
to the direct effect on gonads that may impair
reproductive function by direct insult to the cell
population within the gonads resulting in the
impairment of the feedback mechanism to the
hypothalamus and pituitary. In the present work, the
ovarian toxicity may be due to the oxidative stress
generated by metiram.

The significant decrease in hormonal level (LH, FSH
and estradiol) observed in metiram treated rat may
explained by two explanations. The first one is
hypovascularity associated with congested blood
vessels. In this concern, Löseke and Spanel-Borowski
(1996) correlated hypoovulation to insufficiently
developed microvascular bed in over stimulated
ovaries. The second one is stimulation of
progesterone antagonists production that reduced
preovulatory P4-production in the ovary and down PR
expression through pituitary - hypothalamus axis
(Donath et al., 2000).

Results obtained in this work revealed the potential
protective role of N.sativa against ovarian toxicity of
metiram. Animals given N.sativa and metiram
showed an increase in the number of follicles with
decrease of atretic ones together with increase in FSH,
LH and estradiol. Similar to these results,
Kamarzaman et al. (2014) reported that
cyclophosphamide-treatment caused a significant
reduction in the mean number of normal primary and
secondary follicles, mean ovarian diameters and
increased vacuolation with irregular distribution of
granulosa cells. They added that the numbers of
normal and ovarian diameters were significantly
increased in mice given N. sativa and
cyclophosphamide as opposed to effects seen in
cyclophosphamide-alone. Bayir et al. (2012) reported
that the administration of N. sativa was effective in
reversing tissue damage induced by ischemia and/or
ischemia/reperfusion in rat ovaries. Arak and Assi
(2011) reported that adult rats exposed to lead acetate
caued a significant reduction in FSH and LH and the
treatment of N. sativa seed extract caused significant
inversion in these hormones. They added that this
elevation may be due to the active constituents of N.
sativa which stimulate hypothalamus or pituitary
glands to release and secrete gonadotropin hormones.

Attentions have been paid to the protective effects of
natural antioxidant against xenobiotics - induced
toxicities, especially when free radical generations are
involved. The present results showed that N.sativa
caused decrease of lipid peroxidation and improved
the antioxidant status in ovary of rats treated with
metiram. The antioxidant activity of N.sativa was
studied by many investigators. Burits and Bucer
(2000) tested the possible antioxidant activity of the
essential oil seeds, N.sativa. They have showed that
thymoquinone and the components carvacrol, t-
anethole and/or-terpineol demonstrated respectable
radical scavenging property. Thymoquinone is the
active compound of the essential oil with anti-
oxidative effect that works as a scavenger of various
radical oxygen species including superoxide radical
anion and hydroxyl radicals through different
mechanisms (Badary et al., 2002).

In vitro studies have shown that extracts of N. sativa
seeds protect erythrocytes against lipid peroxidation,
degradation, loss of deformability and increased
osmotic fragility caused by hydrogen peroxide H2O2
(Suboh et al. 2004). Meziti et al. (2012) reported that
N. sativa seeds have a considerable antioxidant
activity in vitro and in vivo. The antioxidant activities
of the methanolic extract and the fixed oil are
confirmed by an in vivo assay in mice, the daily oral
administration of methanolic extract and fixed oil
during 21 days, resulted in a significant enhancement
of the blood total antioxidant capacity. Kushawa
(2014) reported that N. sativa oil is effective in
Protecting rats against acetaminophen induced hepatotoxicity via increased resistance to oxidative stress and by reverse cellular damage. Awadalla (2012) reported that co-administration of *N. sativa* oil prevented cisplatin induced reproductive toxicity in male rats and decreased testicular lipid peroxidation. *N. sativa* fixed and essential oils significantly ameliorate free radicals and improve antioxidant capacity in diabetic rats (Sultan et al.2014). The ameliorative effect of *N. sativa* oil against metiram-induced ovarian toxicity observed in this study, seems to be due to the richness of this oil with antioxidant compounds.

5 References


