Prophylactic effect of green tea and Nigella sativa extracts against fenitrothion-induced toxicity in rat parotid gland

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ABSTRACT

Objective: The aim of the present study was to compare between two antioxidant treatments in prevention of fenitrothion induced toxicity on rat parotid salivary gland.

Design: Forty adult male Wistar rats with an average weight of 120–150 g were randomised into 4 groups, control (group I), fenitrothion administration (group II), fenitrothion administration 1 h after green tea extract or Nigella sativa oil extract administration (groups III and IV respectively). The rats were then sacrificed after 28 days. The submandibular salivary glands were examined histologically, immunohistochemically and ultrastructurally.

Results: Histopathologically the fenitrothion group showed sign of acini degeneration represented by loss of normal architecture (amalgamation). The nuclei of the acinar cells revealed different sizes and shape (polymorphism). The acini relatively preserved their shape in both prophylactic groups (III and IV). Histomorphometric analysis showed significant increase in the optical density of caspase-3 cleaved activity in all experimental groups (p = 0.0001). A significant difference was observed between both prophylactic experimental groups III and IV (p = 0.0039). Ultrastructurally, the nuclei of serous acini in group II appeared pyknotic with segregation of chromatin. Condensation of the chromatin at the periphery of the nucleus was observed in the nuclei of group III, Clumping of the chromatin with darkly stained pyknotic nucleus was detected in group IV.

Conclusions: In a rat model the administration of natural antioxidants could be of beneficial effect on prevention of cytotoxicity induced by organophosphorus compounds. However, green tea showed more promising results than that of Nigella sativa.

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1. Introduction

Organophosphate insecticides (OI) represent one of the most widely used classes of pesticides with high potential for human exposure in both rural and residential environments. Individuals at greatest risk are those who most frequently handle these compounds, including formulators, applicators, farmers and home gardeners. OI are also used extensively in the home environment, and as a result, young children are at increased likelihood for exposure to these compounds.1

Organophosphates (Ops) is the term that includes all insecticides containing phosphorus. Because of the similarity of Ops chemical structures to the “nerve gases,” their modes of action are also similar.2 Ops work by inhibiting certain important enzymes of the nervous system, namely cholinesterase (ChE). This inhibition results in the accumulation of acetylcholine (Ach) at the neuromacroneurone and neurone/muscle (neuromuscular) junctions or synapses, causing rapid twitching of voluntary muscles and finally paralysis.3

Fenitrothion is a contact insecticide belonging to the organophosphate family. It is now widely used for controlling a wide range of pests, i.e. penetrating, chewing and sucking
insect pests. It also used as a fly, mosquito, and cockroach residual contact spray for farms and public health programmes.\(^4\) Unfortunately, many pesticides in common use can produce some toxic and adverse effects on liver, kidney, thyroid gland and other biological systems when tested on various types of experimental animals.\(^5,6\) These toxic effects probably occur through the generation of reactive oxygen species (ROS) causing damage to the various membranous components of cell.\(^7\)

Antioxidants have proved to be a good defence mechanism against free radical effects, which might be produced from contamination with pesticide and other toxic substances.\(^8,9\)

Our body contains its own antioxidant system, made up of enzymes like catalase, super oxide dismutase and metal-binding proteins. Cellular defence mechanism to oxidative damage is activated endogenously by glutathione and other enzymes which convert the oxidised molecules to their reduced from. The endogenous defence mechanism against oxidative damage is complemented by antioxidants like vitamin C, vitamin E, carotenoids and flavonoids (e.g. polyphenols), mainly found in vegetables, fruits and green tea.\(^10\)

Tea, a product made up from the leaf and bud of the plant Camellia sinensis, is the second most consumed beverage in the world.\(^11,12\)

Polyphenols constitute the most interesting group of green tea leaf components, and in consequence, green tea (GT) can be considered as an important dietary source of polyphenols, flavonoids.\(^13\)

Catechins (the major constituents of green tea extract) are highly potent flavonoids present in tea and serve perhaps as the best dietary source of natural antioxidants.\(^14\) Catechins act as free radical scavengers having chemo-preventative behaviour as well as protection against coronary heart disease and attenuation of high blood pressure.\(^15,16\)

Nigella sativa (NS), is an amazing herb found wild in southern Europe, northern Africa, and Asia Minor. It is described as the Melanthon of Hippocrates and Discoidres and as Gith of Fliny.\(^17\) Thymoquine, the main active ingredients of NS, has a strong antioxidant potential due to the scavenging activity towards free radicals. The effect of NS has been evaluated in animal studies. There are many reports on its biological activities including antihypertensive, anti-diabetic, anti-bacterial, anti-tumour and immunomodulator.\(^18,19\)

Based on the observations that OPI increase the prevalence of cytotoxicity, and that GT and NS are effective antioxidants in the managements of this condition. The current study was carried out to hypothesise that either GT or NS could be used as co-treatment to reduce side effects associated with use of OPI.

2. Materials and methods

2.1. Experimental procedure

Forty adult male Wistar rats with an average weight of 120–150 g were used in this study. They were randomly assigned into four groups, 10 rats each. Group I (control group) did not receive any medical treatment. Groups II, III and IV were given 1/30 LD\(_{50}\) 20 mg/kg body weight of fenitrothion\(^a\) orally once a day for 28 days.\(^20\) Group (III) was given 60 mg green tea\(^b\) extract daily 1 h before fenitrothion administration once a day for 28 days.\(^20\) Group (IV) was given 1 ml Nigella sativa\(^c\) oil extract daily 1 h before fenitrothion administration once a day for 28 days.\(^21\)

At the end of 28 days, animals were sacrificed by ketamine overdose. The parotid salivary glands were dissected. The right side of each gland was used for the light microscopic, and immunohistochemical examination, forming a total of 40 specimens (10 specimens from each group). The other 40 specimens (10 specimens from each group) of the left sides of the parotid salivary glands were used for transmission electron microscopic study.

2.2. Light microscopic examination

Specimens were immediately fixed in 10% neutral formalin for 48 h, and then rinsed in distilled water. Specimens were dehydrated in ascending grades of alcohol and embedded in paraffin. From each gland, 30–40 sections of 5 \(\mu\)m thickness were cut. The sections were subjected to haematoxylin and eosin stain according to the conventional method. Histopathologic examination was performed using light microscopy.

2.3. Immunohistochemistry (IHC)

Sections of 5 \(\mu\)m thickness were placed on positive charged slides. Briefly, the sections were deparaffinised and endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H\(_2\)O\(_2\)) in PBS for 30 min. Antigen retrieval was performed by microwaving the sections in 0.01 M sodium citrate buffer (pH 6.0). The slides were then rinsed in PBS, blocked with normal goat serum and incubated respectively with the primary antibodies PCNA (diluted 1:2000 in PBS, Sigma–Aldrich, St Louis, MO) and rabbit anti-cleaved caspase-3 monoclonal antibody (diluted 1:100; Cell Signalling Technology, Boston, MA, USA) over night at 4 °C. They were then rinsed and incubated with secondary antibodies, biotinylated goat anti-mouse and goat anti-rabbit (diluted 1:150 in PBS; Zymed, San Francisco, CA, USA), for 3 h at room temperature. Sections were washed in PBS and incubated with streptavidin-labelled peroxidase complex (diluted 1:150 in PBS; Zymed) for 3 h at room temperature, and the antibody was then visualised with 0.6 mg/ml 3,3’-diaminobenzidine tetrachloride (DAB, Sigma–Aldrich) dissolved in PBS, to which 0.03% H\(_2\)O\(_2\) was added (brown staining); the sections were subsequently counterstained, blued dehydrated and sealed with Mayer’s haematoxylin.

Caspase-3-labelled cells were identified by brown nuclear, cytoplasmic staining, as caspases translocate from the cytoplasm to the nucleus after activation.\(^22\)

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\(^a\) Sumithion KZ contains 50% fenitrothion, Produced by Kafer El-Zyat Co. for insecticide industries—Egypt.

\(^b\) Film coated tablets contains 30% polyphenol, produced by Arab Co. for pharmaceuticals and Medicinal plants—Egypt.

\(^c\) Black seed oil (cold press extract), produced by ISIS Co. for food industries—Egypt.
2.4. Histomorphometric analyses

The data were obtained using Leica Qwin 500 image analyzer computer system (England). The image analyser consisted of a coloured video camera, coloured monitor, hard disc of IBM personal computer connected to the microscope, and controlled by Leica Qwin 500 software. The Optical density (OD) of caspase-3 was measured using an objective lens of magnification 40×, i.e. of a total magnification of 400. Ten fields were measured for each specimen. After grey calibration, the image was transformed into a grey delineated image to choose areas exhibiting positive reactivity with accumulation of all grades of reactivity (minimum, maximum and median grey). Areas of positive reaction were then masked by a blue binary colour. Mean values were obtained for each specimen (Fig. 1).

Data obtained from histomorphometrical analysis were statistically described in terms of range, mean ± standard deviation (±SD), and median. Comparison between the studied groups was done using Kruskal–Wallis analysis of variance (ANOVA) test with Conover–Iman test for independent samples as post hoc multiple 2-group comparisons. A probability value (p value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programmes Microsoft Excel 2003 (Microsoft Corporation, NY, USA) and Stats Direct statistical software version 2.7.2 for MS Windows, StatsDirect Ltd., Cheshire, UK.

2.5. Transmission electron microscopy (TEM)

The left sides of the parotid glands were cut into small parts of one cubic mm and fixed in 3% glutaraldehyde in phosphate buffer (pH 7.2) for 6 h. The specimens were then washed in three changes of phosphate buffer. Secondary fixation was achieved in 1% osmium tetra-oxide at 4 °C, for 1.5 h and then rinsed in phosphate buffer. Specimens were dehydrates in graded series of ethanol (50%, 70%, 85%, 95%, and 100%) for 10 min at each concentration. The specimens were then cleared in propylene oxide and embedded in epoxy resin. Semi thin sections of 1–2 μm were cut and stained with 1% toluidine blue and examined by light microscope for detection of the site to be studied by TEM. Ultra thin sections were then cut using the ultra microtome (Leica, Germany), mounted on copper grids and stained with uranyl acetate and lead citrate. The grids were examined by Joel 1200 EXII electron microscope (Joel, Peabody, MA, USA).

3. Results

3.1. Histological results

3.1.1. The control group (I)

The parotid salivary glands of Wistar albino rats were formed of typical serous acini. The serous cells had rounded basally located nuclei. The cells were roughly pyramidal in shape and were arranged around a central narrow lumen. The serous units contained granular basophilic cytoplasm (Fig. 2).

3.1.2. The fenitrothion group (II)

Serous acini of the parotid gland appeared smaller in size with loss of normal architecture (amalgamation). The nuclei of the acinar cells revealed different sizes, shape (polymorphism) and position. The cytoplasm showed vacuolisation (Fig. 3).

3.1.3. The GT group (III)

The serous acini showed a relatively their normal appearance. The acinar cells exhibited small deeply basophilic nuclei. The cytoplasm showed less vacuolisation than that revealed in group II (Fig. 4).
An increase in the OD of caspases-3 was evident in the parotid salivary gland of the experimental groups II, III and IV. The increase was statistically evaluated and was found to be highly significant \( p = 0.0001 \). Moreover, significant difference was also observed between both prophylactic experimental groups III and IV \( p = 0.0039 \).

3.4. Transmission electron microscopy (TEM)

Ultrastructural examination of the nuclei of the parotid serous acini of control group I showed double nuclear membrane as well as prominent nucleoli. The chromatin was arranged in aggregates adjacent to the inner nuclear membrane and the rest of chromatin was dispersed in the nuclear matrix forming an open-faced nucleus (inset of Fig. 6a). The nuclei of the serous acini in group II appeared pyknotic, shrunk with segregated chromatin. Wide nuclear pore could be detected (inset of Fig. 6b). Condensation of the chromatin at the periphery of the nucleus was observed in the serous acini of group III (inset of Fig. 6c). Clumping of the chromatin with darkly stained pyknotic nucleus was detected in group IV. Wide nuclear pore could be also observed (inset of Fig. 6d).

3.1.4. The NS group (IV)

Some of the serous acini lacked their normal architecture (amalgamation). The nuclei of the acinar cells showed variability in size and density (Fig. 5).

3.2. Immunohistochemical results

We evaluated the effect of fenitrothion as well as the two prophylactic antioxidant treatments on cell apoptosis using caspases – 3 IHC. A negative caspase-3 staining was observed in either nuclei or cytoplasm of serous acini of parotid gland group I (Fig. 6a). However, group II revealed strong nuclear and granular cytoplasmic reaction. The reaction involved almost all the nuclei of the serous acini (Fig. 6b). Immuno-reactivity for caspases-3 was observed scattered in the cytoplasm or in perinuclear region of some serous cells of group III. However, most of the nuclei escaped this positive reaction (Fig. 6c). Group IV showed a relatively positive reaction in all nuclei as well as the cytoplasm of the serous acini (Fig. 6d).

3.3. Histomorphometric analyses

The OD of areas occupied by active caspases-3 in the parotid gland of the control and experimental groups is summarised by means, standard deviation and median in Table 1.

**Fig. 3** – A photomicrograph of group II showing loss of acinar architecture (amalgamation), hyperchromatic and polymorphic nuclei. Note, the presence of numerous cytoplasmic vacuoles (asterisks) (H&E, 400×).

**Fig. 4** – A photomicrograph of Group III showing a relatively normal appearance of serous acini, small deep basophilic nuclei and few cytoplasmic vacuolisation (asterisk) (H&E, 400×).
Saliva is the first digestive fluid secreted into the alimentary canal. The functions of saliva are quite diverse and include moistening and facilitate bolus formation and swallowing, providing the necessary pH buffering capacity in oral cavity, homeostasis of calcium phosphate levels and protection of oral mucosa. Salivary gland hypofunction can occur as a result of chronic prescription of drug treatment, therapeutic X-ray irradiation of head and neck regions, during cancer chemotherapy and in certain systemic diseases.

Aberrant salivary gland apoptosis may be one of the underlying pathogenic causes for the loss of salivary gland function. The absence of an adequate amount of saliva has pronounced effect on general health. For example, increased respiratory infections, loss of adequate nutrition, severe oral and fungal infections, increase rate of dental caries and periodontal disease.

In the present study, systemic administration of fenitrothion led to signs of degeneration of glandular epithelium cells that was demonstrated in serous acini of rat parotid salivary gland Histopathologically, immunohistochemically, histomorphometrically and ultrastructurally.

In the present study, histopathologically the fenitrothion group showed loss of normal architecture (amalgamation). The nuclei of the acinar cells revealed different sizes, shape (polymorphism) and position. The cytoplasm showed vacuolisation. On the other hand, the cytotoxic effect was reduced in the other prophylactic experimental groups III and IV. Our findings were in agreement with Afshar et al., who reported that fenitrothion treated rats was accompanied by similar histopathological alterations in liver and kidneys which led to nephrotoxicity that resulted in systemic toxicity. Moreover, it was demonstrated that Sumithion insecticides induced remarkable inhibition of cytochrome P-450 activity in the liver decreasing drug metabolism and detoxication. This inhibitory effect subjected the body organs to more accumulated toxin in the blood. The principle mechanism by which fenitrothion induces acute toxicity is probably through the generation of reactive oxygen species (ROS) causing oxidative damage to the various membranous components of cell.

The cause of oxidative damage has been reported to be due to the shift in the balance of pro-oxidant (free radicals) and the anti-oxidants (scavenging) mediators.

Antioxidants are compounds that help to inhibit many oxidation reaction caused by free radicals thereby preventing or delaying damage to the cells and tissues.

Recently, green tea polyphenols proved to be protect the liver from hepatotoxicity associated with oxidative damage through antioxidant, anti-inflammatory and antiapoptotic mechanisms.

Interestingly, the immunohistochemical results and histomorphometric data of the ongoing study corroborated with the histopathological results. Comparing the OD between the four studied groups, an increase in the OD of the areas occupied by active caspases-3 was evident in all experimental groups II, III and IV. The increase was statistically highly significant (p = 0.0001). Moreover, significant difference was also observed between both prophylactic experimental groups III and IV (p = 0.0039).

Feng et al. (2005) demonstrated the traffic of caspases during the apoptotic process. Activation of caspase-3 was first observed close to the inside surface of the cellular

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<th>Table 1 - Mean values and standard deviation of the optical density of caspases-3 activity in the parotid salivary gland of the control and experimental groups.</th>
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membrane, then transferred to the cytoplasm, and finally translocate to the nuclear region by active transport.\textsuperscript{37}

Recent reports proved that NS extracts contain potent components which have the ability to induce apoptosis through caspase and P53 pathway. Moreover, thymoquinone, the major constituents of NS oil extract promotes apoptosis through increasing the Bax-Bcl-2 ratio, activating caspase 3 and inhibiting histone deacetylase activity and transcription.\textsuperscript{38,39}

TEM results confirmed both histopathological and immunohistochemical results of the present research. The nuclei of the acini group II exhibited either pyknotic, shrunken and/or segregated chromatin associated with wide nuclear pores. The nuclei in group III showed only peripheral condensation of the chromatin whilst that of group IV showed dark pyknotic nuclei associated also with wide nuclear pores. The ultrastructural findings is in agreement with Rush et al., who demonstrated chromatin condensation characteristic of apoptosis in primary cortical culture exposed to diazinon OPI.\textsuperscript{40}

During apoptosis, activation of caspase-9 increases permeability of the nuclear pores, which allows cytoplasmic caspases to reach their nuclear substrates and lets soluble proteins that are normally restricted to the nucleus or cytoplasm to distribute throughout the cell.\textsuperscript{37}

Increasing the number of antioxidant’s treatments are in development and on the market. A range of differences and similarities are found between these treatment options, and

Fig. 6 – A photomicrograph showing: (a) Group I with no labelling of anti-cleaved caspases-3 antibody. (Inset): Normal ultrastructure distribution of chromatin in control nucleus (3000×). (b) Group II displayed strong nuclear and granular cytoplasmic reaction. (Inset): Pyknotic and segregated chromatin (3000×). (c) Immuno-reactivity for caspases-3 was observed scattered in the cytoplasm or in perinuclear region of some serous cells of GT group III, most of the nuclei escaped this positive reaction. (Inset): Condensation of the chromatin at the periphery of the nucleus (3000×). (d) NS group IV displayed positive reaction to all nuclei as well as the cytoplasm of the serous acini. (Inset): Clumping of the chromatin with darkly stained pyknotic nucleus with wide nuclear pore (arrows) (3000×) (IHC, 400×).
these need to be carefully evaluated before the initiation of treatment. Thus it is speculated that GT extract supplements will lead to a continuously +ve effect on salivary gland acini in contrast with NS extract antioxidants, resulting in improved quality of saliva.

The overall results of this study clearly demonstrated that oral administration of fenitrothion leads some histopathological changes in salivary glands in rats. We suggest that similar studies should be done on different body tissues of rat. It is also recommended that the use of OPs must be controlled to avoid any hazards to the living especially humans.

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Competing interests: None declared.

Ethical approval: I state that the use of experimental animals was strictly humane and followed the guidelines of the ethics committee of Kasr-El-Eini medical school. All efforts have been done to reduce animals’ suffering.

REFERENCES