Research Article

Tracking of Apoptosis Induced by Emamectin Benzoate in Fetuses of Hypothyroid Rats

Alaa Azoz[1*], Khairy A. Ibrahim[2], Ibrahim Y. Abdel Kader[1], Abdel Rahman Tawfik[1]

1. Zoology Department, Faculty of Science, Cairo University, Egypt.

2. Mammalian Toxicology Department, Central Agricultural Pesticides Laboratory, Agricultural Research Center, Dokki, Giza, Egypt.

***Corresponding author's E-mail:** alaa.azoz.lotfy@gmail.com

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ABSTRACT

The objective of the present study is to investigate if daily administration of 0.479 mg/kg (equivalent to 1/200 LD50) of the insecticide emamectin benzoate (EMB) in hypothyroid pregnant rats may induce apoptosis in brain and liver of the fetuses during the phase of organogenesis (from day 6 to day 20 of pregnancy). A total of forty pregnant rats were divided into four groups, ten animals for each. They are control group received 1 ml of corn oil/kg, carbimazole (CBZ) group (to induce a case of hypothyroidism), emamectin benzoate group, and the fourth group received mixture of emamectin benzoate and carbimazole. The results showed that each of CBZ, EMB, and their mixture induced a case of hypothyroidism in pregnant dams as indicated by the decrease in the concentrations of their plasma T3 and T4. Detection on emamectin benzoate-induced apoptosis observed that the gene expressions of caspase-3 mRNA, caspase-8 mRNA, and caspase-9 mRNA were significantly increased in the brain and liver of the fetuses after pregnant dams were orally administrated with CBZ, EMB, and their mixture. In brain and liver of the fetuses, the comet assay showed significant decreases in head DNA and increases in both tail DNA and tail moment after oral administrated of CBZ, EMB, and their mixture. Depends on the previous results, EMB considered as a teratogen due to its embryo toxic effects on the fetus of hypothyroid rats and pregnant females especially with a case of hypothyroidism should avoid the exposure to EMB during the gestation period.

Keywords: Hypothyroidism, Emamectin benzoate, Carbimazole, Caspases, Comet assay.

INTRODUCTION

Thyroid gland is a butterfly-shaped endocrine gland usually located in the lower front of the neck below the larynx (the voice box). The main hormone made by the thyroid is thyroxin, also called T_4 because it contains four iodine molecules. Small amounts of another and more potent thyroid hormone containing three iodine molecules, triiodothyronine (T_3) are also made by the thyroid gland. However, most of the T_3 in the blood is made from T_4 in other tissues[1, 2].The gland is time accelerating body growth [3].Hypothyroidism is a condition in which the thyroid gland is not able to produce enough thyroid hormone[4].

Thyroid hormones are affected by pollutant like pesticides[5].Emamectin benzoate (EMB) is the salt of the naturally occurring avermectins[6].Avermectins are used against parasitic nematodes causing onchocerciasis and lymphatic filariasis[7].Many studies suggest an association between environmental exposure of pregnant women to certain agricultural pesticides and malformation in their fetuses[8].

Aim of the Work

The objective of the present study is to investigate if administration of the medium lethal dose (LD_{50}) of the insecticide emamectin benzoate in hypothyroid pregnant rats may induce apoptosis in some organs of the fetuses during the phase of organogenesis. The present proposal evaluates the toxicity of emamectin benzoate administration through the effects on DNA, cytomorphological alterations, and the ability to induce apoptosis in brain and liver fetuses.

MATERIALS AND METHODS

Pesticides and thyroid drug

The pesticide EMB was dissolved in corn oil in final volume of 1 ml/kg body weight before use. Carbimazole (CBZ) from Sigma was prepared daily by dissolving in water and animals were received 6 mg/Kg body weight.

Animals

The handling of the laboratory animals during the experimental work was performed according to the guidance for care and use of IACUC (Institutional Animal Care and Use Committee) with approval number CU/I//S/43/16.

Male and female albino rats (*Rattus norvegicus*) aged 12 – 14 weeks, were obtained from the breeding colony of the Mammalian Toxicology Department - Central Agricultural Pesticides Lab, Giza, Egypt. Females (2-3) had been transferred into each male cage for mating. The pregnant rats were isolated from male rats and housed in separate cages.

Experimental design

A total of forty pregnant rats were divided into four groups, ten animals for each as the following:

Control group

Rats were daily received one ml of corn oil/kg from day 6 to day 20 of pregnancy.

Carbimazole group (positive control for hypothyroidism)

Rats were daily received 6 mg/kg of carbimazole from day 6 to day 20 of pregnancy.

Emamectin Benzoate (EMB) group

Rats were daily received 0.479 mg/kg (equivalent to 1/200 LD_{50}) emamectin benzoate from day 6 to day 20 of pregnancy. The acute oral median lethal dose (LD_{50}) of EMB for female rats was undertaken according to the guideline of EPA (Environmental Protection Agency) for acute oral toxicity. LD_{50} values were derived by the moving average method using special tables given by[9].

Emamectin benzoate and carbimazole group

Rats were daily received 0.479 mg/kg of emamectin benzoate and 6 mg/kg of carbimazole from day 6 to day 20 of pregnancy.

Blood sample was withdrawn from retro-orbital plexus[10]. The tubes were immediately centrifuged using Sigma Laboratory Centrifuge (3K30, Germany) at 3600 r.p.m for 15 min. Plasma samples were separated and kept at −80 C^o until used for thyroid hormones determination. Rats were sacrificed and brain and liver from their fetuses were quickly frozen at – 80 ^0C and saved for the biochemical parameters assay.

Methods

Thyroid hormones assay

Immunoassays for the in vitro quantitative determination of total triiodothyronine (T_3) and thyroxine (T_4) in blood were obtained from Atlas Medical, UK.

Detection of apoptotic genes expression

Caspase-3, caspase-8 and caspase-9 genes expression were determined by Real-Time Polymerase Chain Reaction (*RT-PCR*). All PCR experiments were performed in the Eppendorf, Realplex instrument, Germany for the signal detection and analysis.

Table 1: Oligonucleotide Primers used in SYBR Green real time PCR

Gene	Primer sequence (5'-3')
ß-actin [11]	*F*-TCCTCCTGAGCGCAAGTACTCT *R*-GCTCAGTAACAGTCCGCCTAGAA
Caspase-3 [12]	*F*-AGTTGGACCCACCTTGTGAG *R*-AGTCTGCAGCTCCTCCACAT
Caspase-8 [12]	*F*-GCGACAGGTTACAGCTCTCC *R*-GCAGCCTCTGAAATAGCACC
Caspase-9 [12]	*F*-CTGGCCCAGTGTGAATACCT *R*-CTCAGTCAACTCCTGGGCTC

Gene-specific primers were designed based on the gene sequences of *Rattus norvegicus* present on the NCBI homepage (*http://www.ncbi.nlm.nih.gov*). Expression was normalized to ß-actin gene, which was used as an internal housekeeping control. Highly purified salt-free primers were obtained from Metabion, Germany and the sequences of these primers obtainable in table (1)[11, 12].

Single-cell gel electrophoresis (Comet assay)

The comet assay used in the present study was applied under alkaline conditions using ordinary microscope slides[13]. For visualization of DNA damage, observations were made of EtBr-stained DNA using a 40x objective on a fluorescent microscope. The degree of DNA damage was performed on coded slides by one reader to avoid variability by comet 5 image analysis software (Liverpool, UK) linked to a CCD camera.

Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics version 22 software package (SPSS, IBM, Chicago, IL, USA). Data are presented as means ± standard errors of mean (S.E.M). To determine differences between groups, analysis of variance (ANOVA) followed by Equal Variances Assumed LSD post hoc analysis for multiple comparisons between different groups. The level of statistical significance was set at probability $P \leq 0.05$.

RESULTS

Thyroid hormones assay

Total Tri-iodothyronine (T_3)

The concentrations level of T_3 of pregnant dams were significantly decreased as after oral gavages with CBZ ($P<0.05$), EMB ($P<0.05$), and their mixture ($P<0.01$) by percentage differences of 17.19, 14.54, and 12.94, respectively compared with the control. Pregnant dams orally administrated mixture of CBZ and EMB showed non significant change in the concentration of T_3 as compared to pregnant dams orally administrated with CBZ or EMB by percentage differences of 4.88 and 1.84, respectively (Table 2).

Table 2: Concentrations (means ± S.E.) of T_3 and T_4 in plasma (µlU /ml) of pregnant dams orally gavages with CBZ, EMB, and their mixture

	T_3	T_4
Control	55.05±2.67	6.18±0.76
CBZ	45.58±0.68[a]	2.70±0.23[a]
EMB	47.04±1.80[a]	4.60±0.23[a]
Mixture	47.92±1.36[a]	3.18±0.36[a]

Significant decrease as compared to control.

Total Tetra-iodothyronine (T_4)

The oral gavages of pregnant dams with CBZ, EMB, and their mixture showed significant decrease ($P<0.05$) in the

concentrations level of plasma T_4 as compared to control by percentage differences of 56.31, 25.56, and 48.54, respectively. Pregnant dams orally administrated mixture of CBZ and EMB showed non significant change as compared to pregnant dams orally administrated with CBZ by percentage difference 15.09 and significant decrease (P<0.05) in the concentration of T_4 as compared to the pregnant dams orally administrated with EMB by percentage difference of 44.65 (Table 2).

Caspase-3 gene expressions using Real-Time PCR at the 20th day of gestation

Brain

It was observed that the gene expressions of caspase-3 mRNA were significantly increased after orally administrated with CBZ (P<0.01), EMB (P<0.05), and their mixture (P<0.01) by percentage differences of 757.3, 481.1, and 1129.7, respectively as compared to the control. Pregnant dams orally administrated mixture of CBZ and EMB showed significant increases (P<0.01) in the gene expressions of caspase-3 mRNA as compared to pregnant dams received CBZ or EMB by percentage differences of 44.22, 57.85, and, respectively (Table 3).

Liver

It was observed that the gene expressions of caspase-3 mRNA were significantly increased after orally administrated with CBZ (P<0.01), EMB (P<0.05), and their mixture (P<0.01) by percentage differences of 490.32, 271.24, and 741.92, respectively as compared to the control. Pregnant dams orally administrated mixture of CBZ and EMB showed significant increases (P<0.01) in the gene expressions of caspase-3 mRNA as compared to pregnant dams received CBZ or EMB by percentage differences of 29.88 and 54.72, respectively (Table 3).

Table 3: Caspase-3, 8 and 9 mRNA gene expressions (means ± S.E.) in brain and liver of fetuses at 20th day of gestation

	Caspase-3 mRNA gene expressions		Caspase-8 mRNA gene expressions		Caspase-9 mRNA gene expressions	
	Brain	Liver	Brain	Liver	Brain	Liver
Control	1.02±0.01	1.01±0.05	1.02±0.05	1.00±0.04	1.02±0.02	1.00±0.04
CBZ	8.71±0.57 [a]	5.92±0.32 [a]	5.21±0.44 [a]	4.01±0.30 [a]	9.147±0.62 [a]	10.05±0.36 [a]
EMB	5.90±0.21 [a]	3.82±0.47 [a]	2.94±0.20 [a]	2.72±0.42 [a]	6.91±0.28 [a]	5.24±0.36 [a]
Mixture	12.49±0.85 [a,b,c]	8.44±0.41 [a,b,c]	7.88±0.44 [a,b,c]	6.17±0.38 [a,b,c]	16.40±0.62 [a,b,c]	12.68±0.72[a,b,c]

(a) Significant increase as compared to the control; (b) Significant increase as compared to CBZ group; (c) Significant increase as compared to EMB group.

Caspase-8 gene expressions using Real-Time PCR at the 20th day of gestation

Brain

It was observed that the gene expressions of caspase-8 mRNA were significantly increased after orally administrated with CBZ (P<0.01), EMB (P<0.05), and their mixture (P<0.01) by percentage differences of 408.99, 187.59, and 669.99, respectively as compared to the control. Pregnant dams orally administrated mixture of EMB and CBZ showed significant increases (P<0.01) in the gene expressions of caspase-8 mRNA as compared to pregnant dams received CBZ or EMB by percentage differences of 33.97, 62.65 and, respectively (Table 3).

Liver

It was observed that the gene expressions of caspase-8 mRNA were significantly increased after orally administrated with CBZ (P<0.01), EMB (P<0.05), and their mixture (P<0.01) by percentage differences of 300.09, 171.77, and 516.38, respectively as compared to the control.Pregnant dams orally administrated mixture of CBZ and EMB showed significant increases (P<0.01) in the gene expressions of caspase-8 mRNA as compared to pregnant dams received CBZ or EMB by percentage differences of 35.09, 55.98 and, respectively (Table 3).

Caspase-9 gene expressions using Real-Time PCR at the 20th day of gestation

Brain

It was observed that the gene expressions of caspase-8 mRNA were significantly increased after orally administrated with CBZ (P<0.01), EMB (P<0.05), and their mixture (P<0.01) by percentage differences of 795.88, 576.88, and 1506.07, respectively as compared to the control. Pregnant dams orally administrated mixture of CBZ and EMB showed significant increases (P<0.01) in the gene expressions of caspase-9 mRNA as compared to pregnant dams received CBZ or EMB by percentage differences of 44.22, 57.85 and, respectively (Table 3).

Liver

It was observed that the gene expressions of caspase-8 mRNA were significantly increased after orally administrated with CBZ (P<0.01), EMB (P<0.05), and their mixture (P<0.01) by percentage differences of 903.59, 423.78, and 1166.83, respectively as compared to the control. Pregnant dams orally administrated mixture of CBZ and EMB showed significant increases (P<0.01) in the gene expressions of caspase-8 mRNA as compared to pregnant dams received CBZ or EMB by percentage differences of 20.78, 58.65 and, respectively (Table 3).

Comet assay

Head DNA

Brain

There were significant decreases (P<0.05) in head DNA after oral administrated of CBZ, EMB, and their mixture (P<0.01) by percentage differences of 17.15, 12.03, and 30.49, respectively as compared to the control. Pregnant dams orally administrated mixture of CBZ and EMB showed significant decreases (P<0.01) in head DNA as compared to CBZ or EMB by percentage differences of 19.19 and 26.54, respectively (Table 4).

Liver

There were significant decreases (P<0.05) in head DNA after oral administrated of CBZ, EMB, and their mixture (P<0.01) by percentage difference of 18.27, 10.51, and 26.55, respectively as compared to the control. Pregnant dams orally administrated of mixture of CBZ and EMB showed significant decreases (P<0.01) in head DNA as compared to CBZ or EMB by percentage differences of 11.27, 21.83, respectively (Table 4).

Tail DNA

Brain

There were significant increases (P<0.05) in tail DNA after orally administrated of CBZ, EMB, and their mixture as compared to the control by percentage differences of 188.21, 132.08 , and 334.64, respectively (Table 4). Pregnant dams orally administrated mixture of CBZ and EMB showed significant increases in tail DNA (P<0.01) as compared to CBZ or EMB by percentage differences of 33.69 and 46.60, respectively (Table 4).

Table 4: Comet parameters (means ± S.E.) in brain and liver of fetuses at 20th day of gestation

	Head DNA		Tail DNA		Tail Moment	
	Brain	Liver	Brain	Liver	Brain	Liver
Control	91.65±1.47	92.52±2.40	7.48 ±2.40	8.35±1.15	0.12±0.04	0.10 ±0.01
CBZ	75.94 ±2.88[a]	75.62±2.55[a]	24.38±2.55[a]	24.06±2.88[a]	2.06±0.19[a]	2.65±0.62[a]
EMB	82.79±2.53[a]	80.62±1.78[a]	17.21±2.53[a]	19.38 ±1.78 [a]	1.51±0.34[a]	1.24 ±0.20[a]
Mixture	67.96±3.95[a,b,c]	63.71 ±2.36[a,b,c]	32.04±3.95[a,b,c]	36.29±2.36 [a,b,c]	4.72±1.32[a,b,c]	4.43 ±0.44[a,b,c]

(a) Significant increase as compared to the control; (b) Significant increase as compared to CBZ group; (c) Significant increase as compared to EMB group.

Liver

There were significant increases (P<0.05) in tail DNA after orally administrated of CBZ, EMB, and their mixture as compared to the control by percentage differences of 225.94, 130.03 , and 328.36, respectively. Pregnant dams orally administrated mixture of CBZ and EMB showed significant increases in tail DNA (P<0.01) as compared to CBZ or EMB by percentage differences of 23.91 and 46.29, respectively (Table 4).

Tail moment

Brain

Significant increases in tail moment as compared to the control were observed when pregnant dams orally administrated with CBZ (P<0.05), EMB (P<0.05), and their mixture (P<0.01) by percentage differences of 2442.89, 1084.84, and 4153.45, respectively. Pregnant dams received mixture of CBZ and EMB showed significant increases in tail moment (P<0.01) as compared to CBZ or EMB by percentage differences of 40.22 and 72.14, respectively (Table 4).

Liver

Significant increases in tail moment as compared to the control were observed when pregnant dams orally administrated with CBZ (P<0.05), EMB (P<0.05), and their mixture (P<0.01) by percentage differences of 1634.57, 1174.79, and 3881.62, respectively.Pregnant dams orally administrated mixture of CBZ and EMB showed significant increases in tail moment (P<0.01) as compared to CBZ or EMB by percentage differences of 56.44 and 67.98, respectively (Table 4).

DISCUSSION

Contamination of human food by insecticides mostly occurs to farmers and agriculture workers[14]. Studies suggest an association between environmental exposure of pregnant women to certain agricultural pesticides and malformation in their fetuses[8]. The most susceptible period for fetal anomalies to take place is the period of organogenesis (6-15 days of gestation). Drugs and chemicals given during this period are more likely to cause fetal defects[15].

Emamectin benzoate (EMB) is an important macro cyclic lactone insecticide that belongs to the avermectin family[16].Emamectin benzoate (EMB), an avermectin derivative, is found to be effective against variety of pests and now widely used[17]. This function of EMB has been ascribed to its neurotoxicity via stimulating γ-amino butyric acid (GABA) receptor and glutamate-gated chloride channels[18]. Initially, EMB is considered to be safe and less toxic because GABA-reactive neurons are limited in central nervous system in human beings[19]. However, EMB is lipophilic and easily pass through cell membrane into cytoplasm[20].

Hypothyroidism is the most common pathological deficiency of thyroid hormones that accounts approximately 2% of women and 0.1-0.2% of men[21]. It is

common in pregnancy with a predictable prevalence of 2-3% and 0.3-0.5% for subclinical and overt hypothyroidism respectively [22].

Given the high frequency of hypothyroidism in the human population[23], it is essential to understand the cellular and molecular mechanisms that are altered by this disorder especially due to the exposure to the insecticide EMB. In the present work, the investigation of cytotoxicity of EMB administration in hypothyroid pregnant rats was examined. The examination was done through the detections of apoptosis and DNA damage in brain and liver in their fetuses.

In the present work carbimazole (CBZ) is used to produce a case of hypothyroidism in pregnant rats. Thionamide, an antithyroid drug of the same group as that of carbimazole, used during pregnancy and lactation is known to suppress the thyroid status of both mother and fetus suggesting that even the therapeutic dose taken during pregnancy can induce hypothyroidism[24].

The results showed that each of CBZ, EMB, and their mixture induced a case of hypothyroidism in pregnant dams as indicated by the decrease in the concentrations of their plasma T_3 and T_4.

Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents[25].

Apoptosis is a coordinated and often energy-dependent process that involves the activation of a group of cysteine proteases called caspases[26].Caspases (cysteine-aspartic proteases) are a family of protease enzymes playing essential roles in programmed cell death including apoptosis, pyroptosis, and necroptosis[27].

Ten major caspases were identified [28]that broadly categorized into initiators (caspase-2,-8,-9,-10), effectors (caspase-3,-6,-7), and inflammatory (caspase-1,-4,-5).Caspases are sub classified into three types[29]: Initiator, Executioner and Inflammatory. Initiator caspases include caspases 2, 8, 9, and 10. Executioner caspases include caspases 3, 6, and 7. Inflammatory caspases include 1, 4, 5, 11, and 12. Caspase-3 is a caspase protein that interacts with caspase-8 and caspase-9. So the present work dealt with the examination of caspases 3, 8, and 9.

Detection on emamectin benzoate-induced apoptosis observed that the gene expressions of caspase-3 mRNA, caspase-8 mRNA, and caspase-9 mRNA were significantly increased in the brain and liver of the fetuses after pregnant dams were orally administrated with CBZ, EMB, and their mixture as compared to the control. Pregnant dams orally administrated mixture of CBZ and EMB showed significant increases in the gene expressions of caspase-3 mRNA, caspase-8 mRNA, and caspase-9 mRNA in brain and liver of the fetuses as compared to pregnant dams received only CBZ or EMB.

Consistent with this finding is the report showing an increase in the expression of apoptotic signaling molecules such as Bax/Bcl2 and cell damage in the CA3 hippocampal region during hypothyroidism[30].

The data of [23] that analyzed the cellular and molecular changes in the adult brain occurring with the development of experimental hypothyroidism suggested that adult hypothyroidism affects the hippocampus by a mechanism that alters the composition of postsynaptic density (PSD), reduces neuronal and astrocyte survival, and alters the content of the signaling neurotrophic factors, such as brain-derived neurotrophic factor (BDNF). These observations support the notion that hypothyroidism is harmful for the hippocampus and neocortex. Because BDNF is a neurotrophic factor involved in neuronal protection, cell death, and synaptic plasticity, they also analyzed the brain BDNF content of hypothyroid rats. They detected an increase in BDNF in hypothyroid rats. Specifically, BDNF mRNA increased in all hippocampus regions and at the layers II, III, and V of the neocortex.

In adult rats, hypothyroidism causes altered expression of mRNA encoding for the subunits comprising the tyrosine kinase receptor, TrkB[31].

The quantified the cytotoxicity of EMB through the detections on cell viability, DNA damage, and cell apoptosis in the fall armyworm *Spodoptera frugiperda* Sf-9 cells in vitro [32]showed that EMB caused a concentration- and time-dependent reduction on the viability of Sf-9 cells, and the median inhibitory concentrations (IC50) were 3.34µM at 72h of exposure. The dual acridine orange/ethidium bromide staining showed that exposure to EMB induced a significant time- and concentration-dependent increase on cell apoptosis.

The identified genotoxicity and cytotoxicity of EMB to human normal liver cells (QSG7701 cell line) in vitro [33]declared that EMB-enhanced apoptosis of QSG7701 cells concurrent with generated ROS, a loss of mitochondrial membrane potential, cytochrome-c release; up regulate the Bax/Bcl-2 and the activation of caspase-9/-3.

Caspase-3 is the major effector protein of apoptosis that is activated by an initiator caspase as caspase-9[33]. They indicated that EMB-induced QSG7701 cell apoptosis via activation of both caspase-3 and caspase-9 to degrade intracellular proteins to carry out cell death program.

Caspase-9 induces death signals by triggering other types of caspase activation[34], and its expression greatly influences the onset of apoptosis. In this study, they demonstrated that the transcription factor AP-4 directly bound to the promoter region of Casp-9 and regulated the expression of Casp-9.

Many apoptosis-inducing stimuli induce the apoptotic mechanism by causing the release of cytochrome *c* from the mitochondria and by inducing the oligomerization of

Apaf-1, followed by the recruitment of procaspase- 9 into a large complex apoptosome[35, 36]. After activation by Apaf-1, caspase (Casp)-9 initiates a proteolytic cascade by cleaving and activating both Casp-3 and the downstream caspases and thereby inducing cell death[37].

Casp-9 is an initiator of a cascade of cleavage and activation of other caspases, and the deletion of Casp-9 from cell extracts abrogates the cytochrome *c*-inducible activation of Casp-2, -3, -6, -7, -8, and -10 [38] .

The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events[39].

These stimuli cause changes in the inner mitochondrial membrane that result in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial trans membrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol [40]. The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi [41, 42]. These proteins activate the caspase dependent mitochondrial pathway. Cytochrome c binds and activates Apaf-1 as well as procaspase-9, forming an apoptosome [43]. The clustering of procaspase-9 in this manner leads to caspase-9 activation [44].

Upon activation, caspase-9 cleaves executioner caspases including caspase-3 at specific sites, leading to the activation of the downstream caspases and the ultimate disassembly of the committed cell[45].

Studies of caspase-8 have focused on the ability of this caspases to interact with receptors of the tumor necrosis factor (TNF)/nerve growth factor (NGF) family and to signal for their cell death-inducing effect [46]. In mediating cell death induction by receptors of the TNF/ NGF family, caspase-8 helps to eliminate injured and infected cells and maintain leukocyte homeostasis[47]. Under certain situations, ligation of death receptors leads to the activation of caspase-8, which in turn cleaves and activates BID, a pro-apoptotic Bcl-2-family protein [48]. Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor mediated apoptosis can be inhibited by a protein called c-FLIP (a master anti-apoptotic regulator) which will bind to Fas-associated protein with death domain (FADD) and caspase-8, rendering them ineffective[49].

An overview of methods often being used for detecting DNA fragmentation as one of the most specific findings in apoptosis is provide by [50]. They declared that three routine assays have been developed for detecting DNA fragmentation: DNA ladder assay, TUNEL assay, and comet assay. Comet assay can be used for detecting nucleus breakdown producing single/double-strand DNA breaks.

DNA in cells can be damaged in a number of ways. Phenazine derivatives can intercalate DNA due to their plannar structure and hydrophobicity, causing DNAdamage[51, 52]. The exposure to organophosphorus insecticides such as chlorpyrifos and methyl parathion that can produce oxidative stress to alter the activity of antioxidant enzymes, to trigger excessive nitric oxide production, and/or to increase lipid peroxidation in humans and animals, is an important cause of DNA damage and the impairments in the DNA repair protein system[53, 54].

DNA damage is an important index in genotoxicity assessment of environmental poison to organism. It could be caused by DNA single-strand breaks, DNA double-strand break, DNA adducts formations, DNA–DNA and DNA-protein cross-links resulting from the interaction of the pesticide or its metabolites and DNA[55].

In the present work the single-cell gel electrophoresis (Comet assay) is used as a simple method for measuring deoxyribonucleic acid (DNA) strand breaks. The lengths of head DNA, tail DNA (the distance of DNA migration from the body of the nuclear core), and tail moment (the distance between the center of the head DNA and the center of the tail DNA, were measured. The tail moment incorporates a measure of the smallest detectable size of migrating DNA in the comet tail and the number of broken pieces from the nuclear core.

In brain and liver of the fetusus, the present work showed significant decreases in head DNA and increases in both tail DNA and tail moment after oral administrated of CBZ, EMB, and their mixture. Fetuus of the pregnant dams orally administrated mixture of CBZ and EMB showed significant decreases in brain and liver in head DNA and increases in both tail DNA and tail moment as compared to CBZ or EMB.

Exposure to insecticides such as methomyl and avermectin is reported to induce DNA damage that is involved in the genotoxic process in cells[56,57]. The levels of DNA strand breakage have been proposed as a sensitive indicator of genotoxicity.

The quantified cytotoxicity of EMB through the detections on cell viability, DNA damage, and cell apoptosis in arthropod insect *Spodoptera frugiperda* ovary Sf-9 cells (a clonal isolate of *Spodoptera frugiperda* Sf-21 cells) in vitro was done by [32]. The dual acridine orange/ethidium bromide staining showed that exposure to EMB induced a significant time- and concentration-dependent increase on cell apoptosis. The alkaline comet assay revealed that EMB induced significant increases on single-strand DNA breaks, and the percentage of γH2AX-positive cells (γH2AX is an important phosphorylated form of H2AX histone family member X which forms when double-strand breaks appear) represented a time- and concentration-dependent formation of DNA double-strand breaks in Sf-9 cells.

The chromatin condensation and nuclei fragmentation in Sf-9 cells were also observed in response to EMB exposure by using DAPI staining[58]. These data indicate that EMB induced the nuclear aberrations and DNA damages that may be related with cytogenetic toxicity. We initially speculate that the cause of EMB poisoning the Sf-9 cells is somewhat associated with oxidative stress during the exposed cell culturing process.

DNA damage caused by low dosage of avermectin was evaluated in hemocytes of silkworm (*Bombyx mori*) using the alkaline single-cell gel electrophoresis (SCGE). Results showed that there was positive correlation between avermectin concentration and DNA damage in three tested treatments. In this study, significant increase of comet appearance of hemocytes was observed in 24 h after silkworms were treated by avermectin, which suggested that the effect of avermectin on DNA damage was rapid and direct[59].

The exposure to insecticides such as methomyl and avermectin is reported to induce DNA damage that is involved in the genotoxic process in cells[33]. The levels of DNA strand breakage have been proposed as a sensitive indicator of genotoxicity[60, 61].

CONCLUSION

EMB considered as a teratogen due to its embryo toxic effects on the fetus of hypothyroid rats. The teratogenicity of EMB may be mediated by its ability to elevate apoptosis and DNA damage in brain and liver during organogenesis of the fetuses. Depends on the previous comments pregnant females especially with a case of hypothyroidism should avoid the exposure to EMB during the gestation period. Further researches should be focused on the mechanisms by which EMB causes DNA damage and cell apoptosis in order to completely understand the teratogenicity induced by EMB.

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