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Teratogenicity and neurotoxicity effects induced by methomyl insecticide on the developmental stages of *Bufo arabicus*

Amin A. Seleem*

Zoology Department, Faculty of Science, Sohag University, Sohag, Egypt
Biology Depart., Faculty of Science and Arts, Al-ula, Taibah University, Kingdom of Saudi Arabia

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ABSTRACT

Methomyl (MET) is a carbamate insecticide that has been widely used to protect the crop against insects as an alternative for organophosphorus insecticide. Thus the present study aims to evaluate the potential toxic effects of MET on the developmental stages of *Bufo arabicus*. Tadpoles were classified into three stages (25, 37, 40). Every stage was divided into two groups, control and MET-treated group (10 ppm for two weeks) after LC50 determination in acute toxicity test for 96 h. Control and MET-treated larvae were examined at the level of morphological, histological, skeleton deformities and immunohistochemical labeling of alpha-synuclein in the spinal cord and dorsal root ganglion. MET-exposed larvae showed hyperactivity, extreme agitation, abnormal swimming and kinking tail as compared to control. Alizarin Red S-Alcian blue staining showed scoliosis in MET-treated tadpoles at 25 and 37 stages; kyphosis, retarded tail regression and reduced ossification of the phalanges of digits for both fore-and hind limbs were noted in MET-exposed tadpoles at 40 stage as compared to control. Histopathological changes in myotomes, notochord and spinal cord were shown in MET-exposed tadpoles as compared to control. Immunohistochemical examination showed an over expression of alpha-synuclein either in the neurons of the spinal cord or in the dorsal root ganglion of MET-exposed tadpoles at stage 40 as compared to control. The present study concluded that MET insecticide induces malformation and teratogenicity effects which were accompanied by neurodegenerative effects for the neurons either in the spinal cord or in the dorsal root ganglion.

1. Introduction

Methomyl (MET) (C5H10N2O2S), S-methyl-1-N-[(methylcarbamoyl)-oxy]-thioacetimidate, is a carbamate insecticide that has been widely used for agriculture to protect the crop against diverse species of insects, ticks, mites, and spiders through direct contact and systemic poisoning of insects (Reiser et al., 1997; Tomlin, 2001). Carbamates occupy the third major group of insecticides and used as an alternative for organophosphorus because of their fast action on the target pests, high efficiency, low persistence in the environment, and a short lifespan (Ribera et al., 2001; Kaur and Sandhir, 2006). MET has commercial names like Lannate and has ecotoxicological effects because of the high solubility in water and contaminates groundwater (Tsatsakis et al., 1996; Kegley et al., 2014). The using of MET was restricted by Environmental Protection Agency because of its residue which was detected in soil, agricultural products, and aquatic system (EPA, 1998; El-Fakharany et al., 2011; Caetano et al., 2013). The residues of MET were detected in the brain, blood, liver, and kidney in human and experimental animals causing poisoning and mortality (William et al., 1991; Hoizey et al., 2008; Lee et al., 2011; Mohamed and Sayed, 2013). Exposure to MET insecticides represents a major public health risk and causes toxicity to diverse aquatic species such as common carp (*Cyprinus carpio*), *Daphnia magna*, ostracoda, algae, daphnids, and fishes (Rao et al., 1984; Todd and Leeuwen, 2002; Boran et al., 2007; Wang et al., 2015; Toumi et al., 2016a, 2016b). MET has serious toxic effects on non-target organisms including amphibian which are important in many ecosystems of vertebrate biomass and considered as a bioindicator for the agrochemical contamination such as insecticides (Lau et al., 2015; Trachantong et al., 2017). MET intoxication is a major health problem for farmers, workers, and pilots during aerial spraying or occupational handling (William et al., 1991; Radwan et al., 2008). MET induces disturbance in fetal development and histological changes in female rats (Alharbi, 2017). Carbamate insecticides have inhibitory effects on Acetylcholinesterase (AChE) activity which is a vital enzyme for the normal neurotransmission function and exerts its pesticidal action by inhibition of cholinesterase activity in diverse organisms

* Zoology Department, Faculty of Science, Sohag University, Sohag, Egypt.
E-mail address: amin_seleem@science.sohag.edu.eg.

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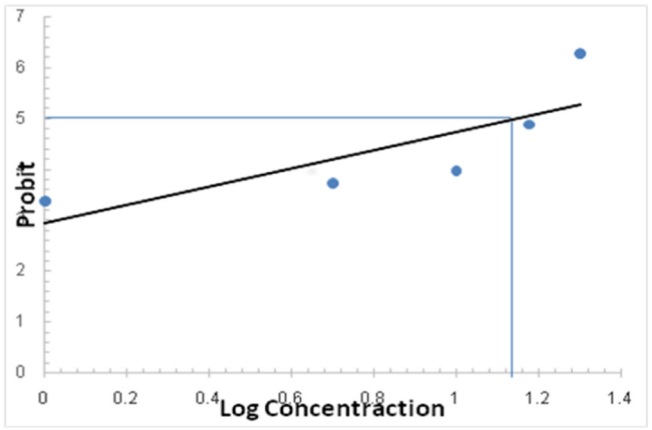

Fig. 1. Determination of median lethal concentration (LC_{50} = 14.12 ppm) after 96 h of MET exposure for *Bufo arabicus* tadpoles.

leading to neurotoxic symptoms (Silva Filho et al., 2004; Yi et al., 2006). Neurotoxicity of pesticides includes continuous nerve stimulation as a result of the accumulation of acetylcholine at peripheral and central nervous systems (Fikes, 1990; Giesy et al., 1999). The aim of this study is to evaluate the teratogenicity and neurotoxicity effects induced by MET insecticide on the non-target organism as amphibian *Bufo arabicus*.

2. Material and methods

2.1. Determination of lethal dose and mortality

Freshly fertilized eggs of *Bufo arabicus* were collected from farms of Al-Ula governorate, Al-Madinah Al-Munawarah, Kingdom of Saudi Arabia in October 2017. Fertilized eggs were transferred to the laboratory and kept in aquaria containing well-aerated dechlorinated water that was changed daily. A constant 12:12-h light/dark photoperiod was maintained throughout the exposure. The hatched tadpoles were fed on lettuce during the time of experimentation. The determination of selected stages for the study such as 25 (18.8 mm), 37 (40.8 mm), and 40 (44.0 mm) was based on the length measurements, the duration of development, external morphology and physiological characteristics reported by Ba-Omar et al., 2004. MET-based insecticide (Lannate 90% SP, made in Taiwan) was purchased from Agriculture market for pesticides in Al-Ula governorate, KSA. Preliminary trial was

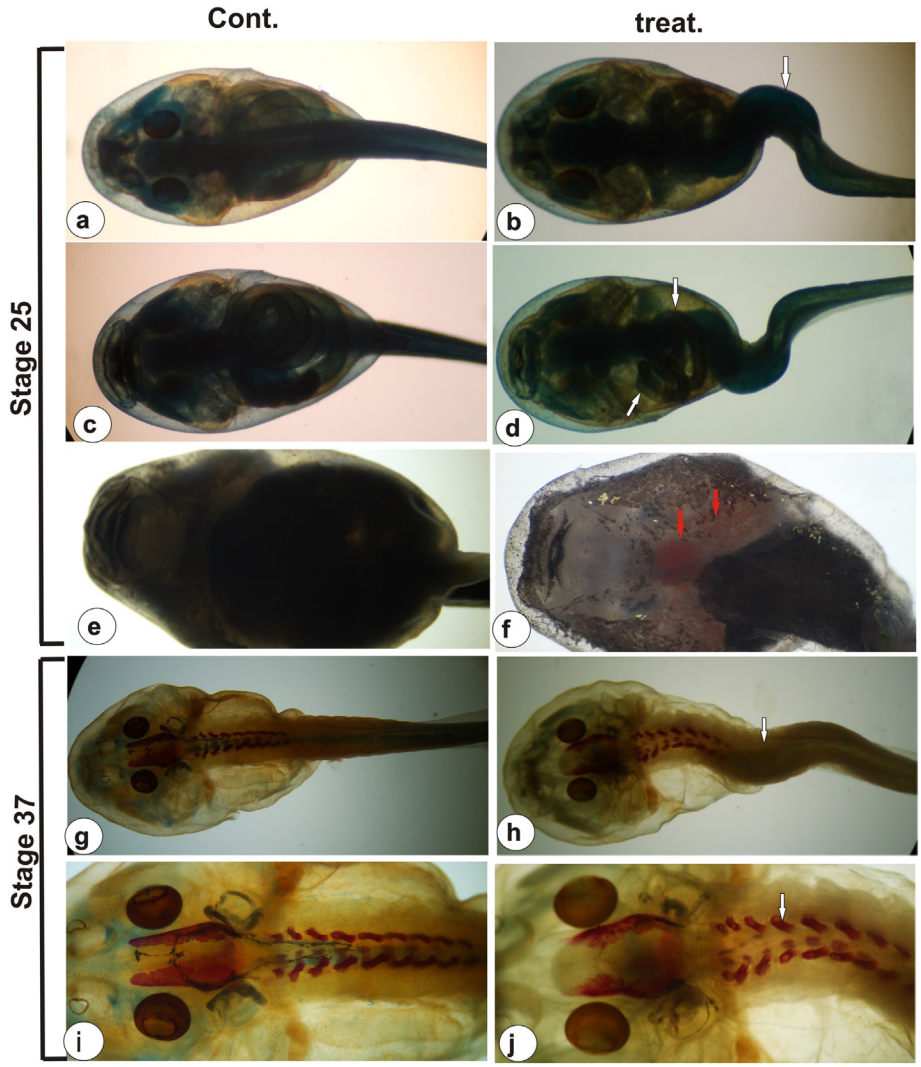

Fig. 2. Photographs of control larvae with normal morphology at stages 25 (a, c, e) and 37 (g, i). MET-exposed tadpoles showed scoliosis (vertebral and spinal curvature) at stage 25 (arrow; b) and 37 (arrows; h, j). Abnormal gut coiling (arrows; d) and redness with edema (red arrows; f) in MET-intoxicated tadpoles at stage 25 as compared to control (c, e). (a, b, g, h, I, j dorsal view) (c, d, e, f ventral view). Alizarine Red S-Alcian blue staining. a–d 30×; e–f 35×; g, h 8×; i, j 20×.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

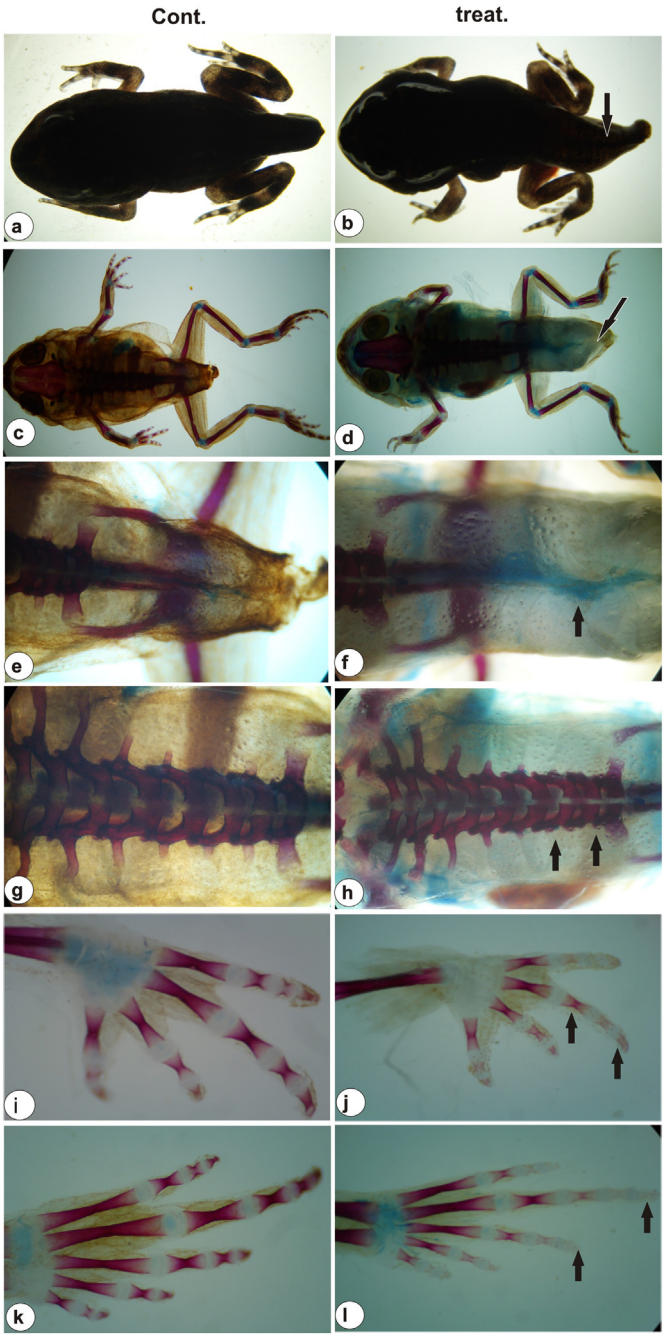

Fig. 3. Photographs of control larvae with normal morphology at stage 40 (a, c, e, g, i, k). MET-exposed tadpoles showed kyphosis (hunch back) (arrow; b), retardation of the tail regression (arrows; d, f), losing vertebral apophyses (arrows; h), and incomplete and reduced ossification of the phalanges of digits for forelimbs (arrows; j) and hindlimbs (arrows; i). Alizarine Red S-Alcian blue staining. a–d 8×; e–h 30×; i–l 35×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conducted to estimate LC50 using standardized methods (ASTM, 1997). Tadpoles at stage 25 were exposed to high concentrations of MET (20, 50, 100, 250, 500, 1000 ppm) in acute toxicity test for 96 h for LC50 determination by Probit analysis according to Finney, 1971.

2.2. Experimental design and processing

The study included three different stages (25, 37, 40) to determine which one will be more tolerant for the toxicity of MET insecticide. Every stage was divided into two groups (20 tadpoles in each group); the control group and MET-exposed (10 ppm/L) based on the acute toxicity test for LC50 determination at 96 h. The experiment was conducted for two weeks of continuous exposure with MET. Test solutions were refreshed every 24 h to maintain the pesticide concentration. At the end of the experiment, tadpoles were anesthetized and fixed in 10% formaldehyde. Whole mount of larvae at studied stages was stained with Alizarin Red S-Alcian blue stain according to Wassersug, 1976. The stained larvae were photographed by a stereo binocular microscope (Leica EZ4HD, Microsystem, Singapore). For general histology and carbohydrate detection, fixed tadpoles were rinsed in phosphate buffer, dehydrated in ascending series of absolute alcohol, cleared in xylene and embedded in paraffin wax. Serial sections of the developing larvae in studied groups were cut at 7 µ thick by rotary microtome (RM 2125RTS microtome, Leica Biosystems, Shanghai, China). Selected regions (Notochord, spinal cord, muscle, dorsal root ganglion) of studied groups were stained for general histology and carbohydrate contents with Haematoxylin and Eosin and with Periodic acid Schiff's reaction (PAS) (Drury and Wallington, 1976).

Selected tissues of the studied stages were mounted on positive slides (Superfrost/Plus) for immunohistochemical staining (Buchlowalow and Bocker, 2010). According to the manufacture protocol of Millipore company (Cat. No. Det-HP1000), the slides were deparaffinized by xylene, rehydrated by alcohol, recovered of the epitope by citrate buffer pH = 6, blocked for endogenous tissue peroxidase by 3% hydrogen peroxide, and applied drops of blocking reagent (Cat. No. 20773). The spinal cord and dorsal root nerve ganglion regions of studied groups were incubated with primary antibody alpha-synuclein (Rabbit anti-Synuclein-alpha polyclonal antibody, Cat. No. E2681, Spring Bioscience, Pleasanton, USA) for three hours at room temperature. Sections were incubated with secondary antibody, biotinylated goat anti-rabbit IgG (Cat. No. 20775) for 30 min, then incubated with Streptavidin-horseradish Peroxidase for 30 min (Cat. No. 20774). Sections were covered with freshly prepared chromogen reagent (30 µl of DAB liquid chromogen solution to 1 ml of DAB liquid buffer solution, product No. D3939, Sigma, USA). The stained sections were dehydrated by ascending grades series of ethanol, cleared by xylene, mounted by DPX with coverslip and photographed by a light microscope (Axio Lab. A1) equipped with an AxioCam ERc5s camera (Carl Zeiss, Berlin, Germany).

3. Results

3.1. Toxicological observation

The median lethal concentration (LC50) after 96 h of MET exposure is 14.12 ppm for *Bufo arabicus* tadpoles (Fig. 1). Mortality of tadpoles based on concentration and time-dependent of exposure. 100% mortality was observed after MET exposure for 10 min. at concentration 500 and 1000 ppm, 12 h at 250 ppm, 24 h at 100 ppm, 2 days at 50 ppm and 4 days at concentration 20 ppm. MET-exposed tadpoles (10 ppm) for two weeks at studied stages (25, 37, 40) showed signs of toxicity such as increasing the excretory rate of feces at the beginning of the exposure, hyperactivity, and an increase in the heart rate beats with edema and redness (Fig. 2f, red arrows) as compared to control (Fig. 2e). Abnormal swimming behavior was observed such as swimming in a spiral orbit, laying/swimming on their sides or backs, and swimming erratically for many seconds. At the end of the exposure, MET-treated tadpoles suffered from tail deformations, abnormal gut coiling (Fig. 2d, arrows), loss of appetite, loss of balance, and motionlessness as compared to control (Fig. 2a, c, e).

3.2. Morphological and skeletal abnormalities

Exposure to MET insecticides causes some of the deformities such as kinking tail which was accompanied with asymmetrical tail and

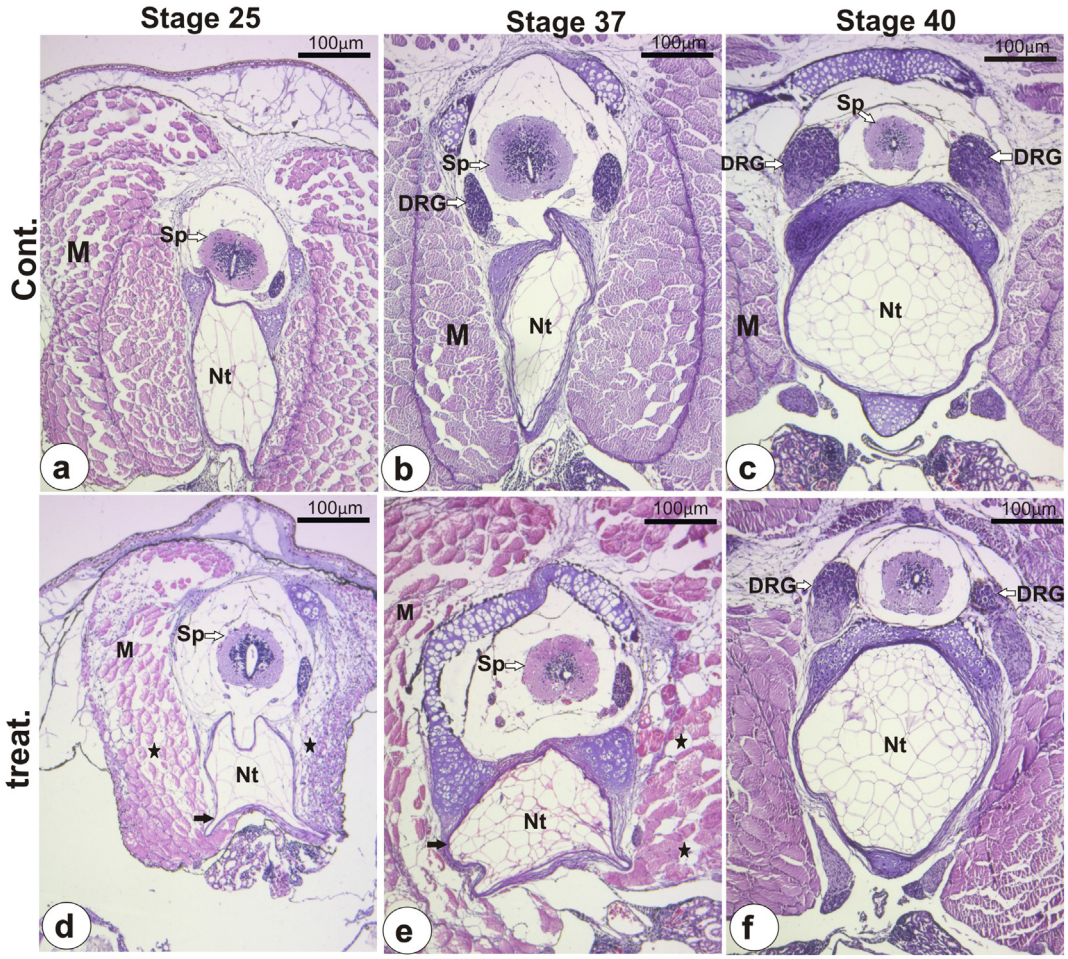

Fig. 4. Photomicrographs of H&E staining of control larvae showing the normal features of myotomes and notochord at stages 25, 37, and 40 (a, b, c). MET-exposed tadpoles showed distortion in the architecture of notochord and myotomes at stages 25, 37 (black arrows; d, e). MET-exposed tadpoles at stage 40 (f) showed less damage than that of stages 25 and 37. Spinal cord (Sp), notochord (Nt), muscle (M), dorsal root ganglion (DRG). H&E stain Scale bar 100 µm.

curvature of the spinal cord. MET-exposed tadpoles at stages 25 and 37 elucidated scoliosis (spinal curvature), tail torsion, and incomplete ossification of the vertebral column (Fig. 2b, h, j, arrows) as compared to control (Fig. 2a, g, i). It was apparent that there was more deformation observed for stages 25 and 37 compared to stage 40. MET-treated tadpoles at stage 40 showed kyphosis (hunch back) (Fig. 3b, d arrows), retardation of tail regression (Fig. 3b, d, f, arrows), missing formation of vertebral apophyses (Fig. 3h), incomplete and reduction ossification of the phalanges of digits for forelimbs (Fig. 3j), and hindlimbs (Fig. 3i) as compared to control (Fig. 3a, c, e, g, i, k).

3.3. Histopathological studies

In the control tadpoles at studied stages (25, 37, 40), myotomes were oriented in an orderly manner parallel to the notochord and attached at regular intersomatie boundaries (Fig. 4a, b, c). Exposure to MET caused many dramatic alterations like distortion and disintegration in the architecture of notochord at the stages 25 and 37 (Fig. 4d, e, black arrows) as compared to control (Fig. 4a, b). Myotomes of MET-exposed tadpoles at stages 25 and 37 revealed an uncorrected orientation, reduction in the number and volume, increase of extracellular spaces between myocytes, and vacuolated regions (Fig. 4d, e) as compared to control (Fig. 4a, b). It's obvious that notochord and muscles of MET-exposed tadpoles at stage 40 were less affected than those of stages 25 and 37 (Fig. 4f) as compared to control (Fig. 4c). The normal distribution of stored carbohydrates in muscles of tadpoles was shown in the control of studied stages (Fig. 5a, b, c arrows). The muscles of MET-exposed tadpoles showed a decrease in the carbohydrate content (Fig. 5d, e, f arrows) as compared to control group.

In the control larval stages (25, 37 and 40), neurons of the gray matter in spinal cord possessed prominent central nuclei and thin basophilic cytoplasm (Fig. 6a, b, c). Histopathological lesions were detected in the spinal cord of MET-intoxicated tadpoles such as necrosis of neurons, pyknotic nuclei, vacuolated regions, degeneration of neurons, fragility and spongy of white matter (Fig. 6d, e, f stars, arrows). Alpha-synuclein expression in the normal spinal cord of control larvae at studied stages was shown in the lining cells of the ependymal canal and in the neurons (Fig. 7a, b, c). Abnormal increase of alpha-synuclein expression was detected in the lining cells of the ependyme and in the neurons of the spinal cord of MET-exposed tadpoles at studied stages (Fig. 7d, e, f arrows). An increase of melanin was detected in the spinal cord of exposed larvae at stage 25 (Fig. 7d, blue arrow). Light microscope examination of dorsal root ganglion in control larvae at stage 40 showed the typical morphology of normal ganglion cells with round nuclei, centrally located, and distinct nucleoli (Fig. 8a). Dorsal root ganglion in MET-intoxicated tadpoles at stage 40 displayed inflammatory cells (Fig. 8b, red arrow), degeneration of the neurons, some of the chromatolytic nuclei were shrunken or replaced by vacuoles (Fig. 8b, black arrow). Intracellular localization of alpha-synuclein around the nucleus was shown in the normal dorsal root ganglion at stage 40 (Fig. 8c). Abnormal accumulation of alpha-synuclein and peripheral localization were observed in dorsal root ganglion cells of MET-intoxicated tadpoles at stage 40 as compared to control (Fig. 8d, red arrows).

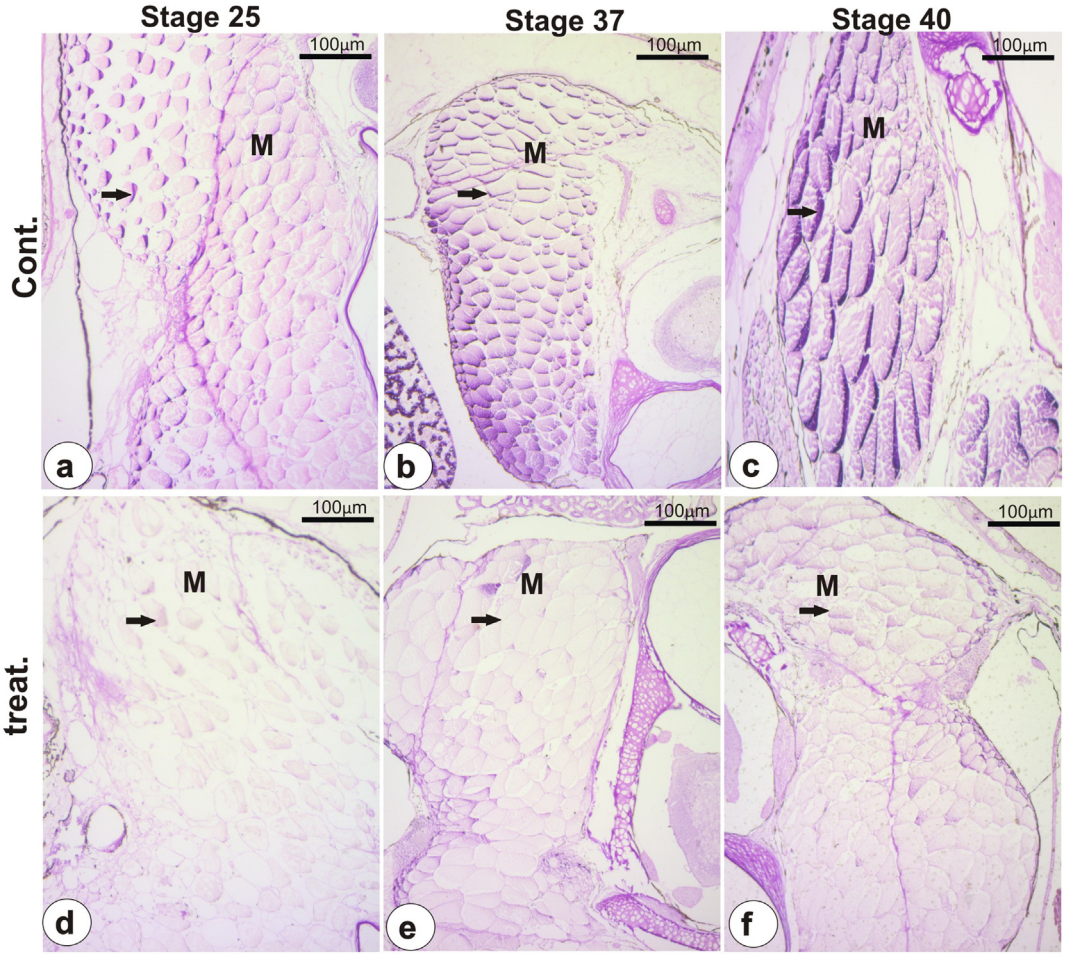

Fig. 5. Photomicrographs of PAS staining of control larvae showing the normal distribution of stored carbohydrates in muscles at studied stages (a, b, c). The MET-intoxicated tadpoles showed a decrease in the carbohydrates content of muscle (d, e, f). arrows indicate the carbohydrates stored in muscle (M). PAS stain Scale bar 100 µm.

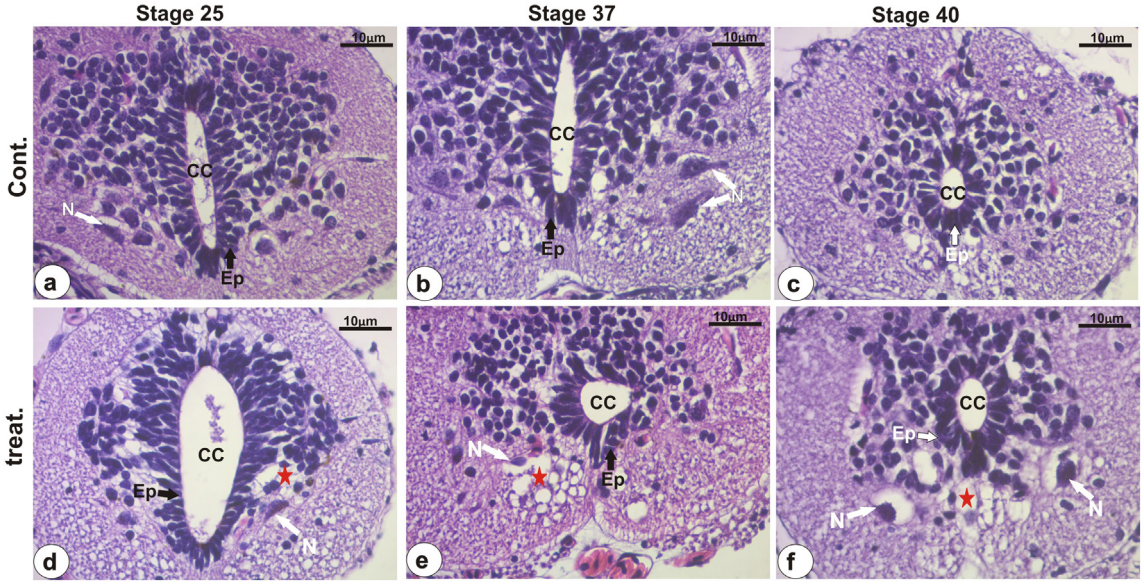

Fig. 6. Photomicrographs of H&E staining showing the normal structure of the spinal cord in the control group at stages 25, 37, and 40 (a, b, c). MET-intoxicated tadpoles showed vacuolated regions and degeneration of neuronal cells (stars, white arrows; d, e, f). Central canal (CC); ependyme (Ep); neuron (N). H&E stain Scale bar 10 µm.

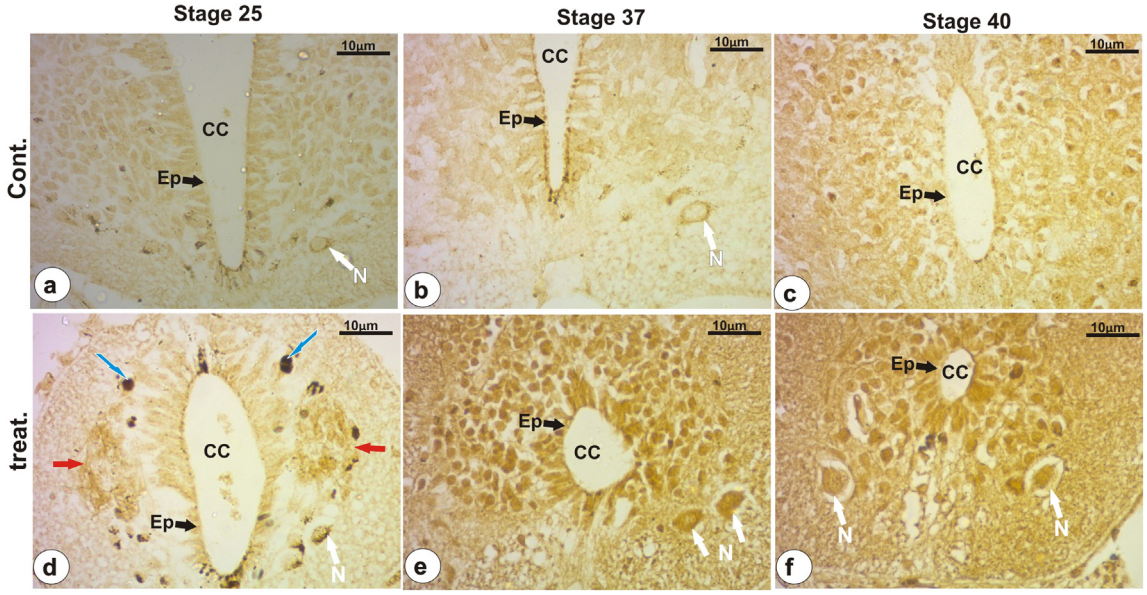

Fig. 7. Photomicrograph of alpha-synuclein immunostaining showing the normal expression in the lining ependyme cells and in the neurons of the spinal cord at studied stages (25, 37 and 40) (a, b, c). Abnormal an increase localization of alpha-synuclein in the lining cells of the ependyme and neurons of the spinal cord in MET-exposed tadpoles at studied stages was noted (red arrows d, black and white arrows e, f). Blue arrows indicate the abnormal accumulation of melanin in MET-exposed larvae at stage 25. Alpha-synuclein immunostained. Scale bar 10 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

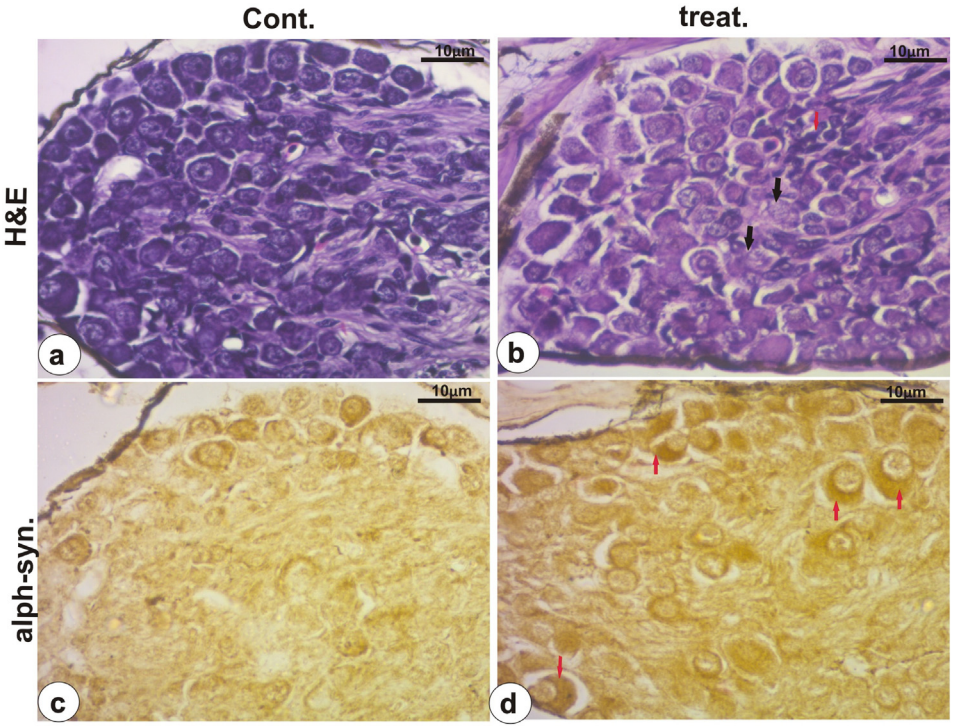

Fig. 8. Photomicrographs of H&E staining of control larvae showed typical morphology of dorsal root ganglion cells (a). MET-intoxicated dorsal root ganglion at stage 40 displayed degeneration of the neurons (black arrow; b) and inflammatory cells (red arrow; b). The normal localization of alpha-synuclein in the normal dorsal root ganglion was detected in the intracellular around the nucleus (c). An increase and an accumulation of alpha-synuclein expression were noted in dorsal root ganglion of MET-intoxicated tadpoles (red arrows; d). a, b: H&E stain Scale bar 10 µm.; c, d: Alpha-synuclein immunostained. Scale bar 10 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Signs of toxicity which are observed in the behavior of *Bufo arabicus* tadpoles such as extreme agitation, tremors, and seizures after MET exposure were close similar to exposure of amphibian tadpoles to organophosphorus insecticides such as carbaryl, carbofuran, malathion, chlorpyrifos, endosulfan, and dimethoate (Sayim and Akyurtlakl, 1999; Bonfantia et al., 2004; Sayim and Kaya, 2006; Sayim, 2008; Bernabò et al., 2011; Bandara et al., 2012). These behavioral effects might be attributed to neurotoxicity of such pesticides which are linked to inhibition of acetylcholinesterase (Fulton and Key, 2001) and lead to the induction of morphological alteration such as axial-muscular abnormalities, uncontrolled and continuous contractions of the tail musculature, and body shaking (Karalliedde and Henry, 1993; Behra et al., 2002; John et al., 2003). Also, insecticides disrupt and alter the concentrations of melatonin, acetylcholine, serotonin, dopamine, noradrenaline, and norepinephrine in cholinergic neurotransmitter systems (Berrill et al., 1998; Harris et al., 2000; Park et al., 2001; Rohr et al., 2003; Goulet and Hontela, 2003). Malformed tadpoles increase the susceptibility to the risk of predation, decrease the foraging efficiency,

and reduce their probability to complete metamorphosis development (Bishop et al., 1992; Bridges, 2000). The present study stated that the median lethal concentration (LC_{50}) of MET for *Bufo arabicus* is 14.12 ppm for 96 h. Previous study reported LC_{50} for other species of amphibian such as *Bufo vulgaris formosus* which is 23 ppm (Nishiuchi, 1980), *Rana limnocharis* which is 27.16 ppm (EPA, 2001), *Bufo bufo japonicas* which is 40 ppm (Hashimoto and Nishiuchi, 1981), and *Hoplobatrachus rugulosus* is 8.69 ppm (Trachantong et al., 2017). The difference in the values of LC_{50} might be attributed to the acute toxicity of pesticides which depends on species, developmental stage, and testing protocols (Berrill et al., 1998; Bridges and Semlitsch, 2000; Ezemonye and Tongo, 2009; Jones et al., 2009; Lau et al., 2015). MET application includes the treatment of different crop types to control various insect and nematode pests by spraying or soil applications (Desaeger et al., 2011; Van Scoy et al., 2013). MET is classified as a restricted-use insecticide; thus ecological consequences of MET application represents highly toxic effects to multiple non-targeted organisms such as mammals, birds, amphibians, fishes, and aquatic invertebrates (Farre et al., 2002; Van Scoy et al., 2013). The previous study showed contaminated corn kernels with MET causes death of hundreds of pigeons because the exceeded median lethal dose (Villar et al., 2010). The perspective of the concentrations used in this study is relative to those that would typically be used in agricultural applications that might happen by repeated spraying of MET and accumulation of MET in tissues during the development of *Bufo arabicus*. Also, the developing tadpoles might be exposed to the multiple doses of MET during the course of development from fertilized egg to the mature stage that starts from the breeding season in October (Ba-Omar et al., 2004).

The muscle and notochord in the tail region of MET-exposed tadpoles showed histopathological alterations which comply with the effects of organophosphorus insecticides such as abnormal notochord curvature, disorganized myotomes, tail flexure, abnormal gut coiling, and teratogenic effect (Bonfantia et al., 2004; Sayim, 2008; Bandara et al., 2012). The similar kinking tail was observed in insecticides-exposed tadpoles such as pyrethroid, cypermethrin, esphenvalerate, and carbaryl (Materna et al., 1995; Bridges, 2000; Greulich and Pflugmacher, 2003). The abnormal tail flexure observed in MET might be due to the consequence of the cholinergic reaction, where acetylcholinesterase inhibition is the primary mode of toxicity of neurotoxicant as organophosphorus or carbamate pesticides (Fulton and Key, 2001; Gill et al., 2011). Thus, signs of anti-cholinesterase toxicity cause neurological synapses as repeated firing, continuous contraction of tail muscles, complex posturing movements, uncontrollably, and body shaking; leading to the axis tail folding, paralysis, muscular damage, and finally to death usually by asphyxiation (Lien et al., 1997; John et al., 2003; Scholz et al., 2006; Sparling and Fellers, 2007). Acetylcholinesterase (AChE) is required for muscular and neuronal development, therefore gene mutation causes tail twitching and severe myopathy (Behra et al., 2002). AChE inhibition causes overstimulation of the postsynaptic membrane and excessive Ca2 + influx which leads to myopathy at the neuromuscular junction, cytotoxicity, and cell death (Leonard and Salpeter, 1979; Orrenius et al., 1989; Karalliedde and Henry, 1993; De Bleecker et al., 1994).

MET causes a marked damage of neuronal cells of gray matter and dorsal root ganglion that might be due to the role of insecticide in the disruption of the cytoskeleton, hypoxia, calcium overload, diminishing mitochondrial energy metabolism, induction of oxidative stress, production of toxic metabolites, increase levels of lipid peroxidation, formation of reactive oxygen species, hydroxyl radical, and hydroperoxide (Lemercier et al., 1983; Fikes, 1990; Giesy et al., 1999; Zhang et al., 2003; Costa et al., 2008; Mansour et al., 2009, 2017; Akhgari et al., 2013). The present study demonstrated the normal intracellular localization of alpha-synuclein in the control tadpoles at neurons of the gray matter of spinal cord and dorsal root ganglion. Similar localization of alpha-synuclein was reported in species-specific differences (Giasson et al., 2001; Ahn et al., 2012). The neurons of the spinal cord and dorsal root ganglion in the MET-exposed tadpoles showed overexpression of the alpha-synuclein immunoreactivity that may be attributed to the role of alpha-Synuclein in chaperone-like activity (Souza et al., 2000; Takenouchi et al., 2001). Some pesticides such as paraquat and dithiocarbamates exhibit dopamine cell loss and aggregate cytosolic alpha-synuclein (Brooks et al., 1999; McCormack et al., 2002; Norris et al., 2007). Methamphetamine drug induces neurotoxicity and overexpression of alpha-synuclein (Francesco, 2008). Aggregation of neuronal alpha-synuclein indicates the neurodegeneration and impairment of motor function in transgenic mice and Drosophila (Feany and Bender, 2000; Masliah et al., 2000; Van der Putten et al., 2000). Overexpression of mutant alpha-synuclein reduces tyrosine hydroxylase activity and dopamine storage synthesis, leading to impaired motor function in transgenic mice (Perez et al., 2002; Yavich et al., 2005). Mice lacking alpha-synuclein displayed functional deficits in the nigrostriatal dopamine system (Abeliovich et al., 2000). Pesticides can accelerate the rate of alpha-synuclein fibril formation which is considered the main factor in Parkinson's disease (Uversky et al., 2001). Also, pesticides inhibit the ubiquitin-proteasome system (UPS) which normally degrade alpha-synuclein and reduce the UPS activity, leading to the accumulation of alpha-synuclein which is associated with neurodegenerative diseases (Wang et al., 2006; Chou et al., 2008).

5. Conclusion

The present study concluded that MET insecticide induces teratogenicity and neurotoxicity. MET induces neurodegenerative effects that were accompanied by overexpression of alpha-synuclein in the neurons of the spinal cord and dorsal root ganglion during developmental stages of *Bufo arabicus*. The study recommends not to use MET during the course of *Bufo arabicus* development.

Conflict of interest

The author declares that there is no conflict in this paper with any other work.

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