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REVIEW ARTICLE

Glutathione S-transferase is a good biomarker in acrylamide induced neurotoxicity and genotoxicity

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ABSTRACT

Glutathione S-transferases (GSTs) are major defence enzymes of the antioxidant enzymatic system. Cytosolic GSTs are more involved in the detoxification than mitochondrial and microsomal GSTs. GSTs are localized in the cerebellum and hippocampus of the rat brain. Acrylamide (AC) is a well assessed neurotoxin of both animals and humans and it produces skeletal muscle weakness and ataxia. AC is extensively used in several industries such as cosmetic, paper, textile, in ore processing, as soil conditioners, flocculants for waste water treatment and it is present in daily consumed food products, like potato chips, French fries, bread, breakfast cereals and beverages like coffee; it is detected on tobacco smoking. GST acts as a biomarker in response to acrylamide. AC can interact with DNA and therefore generate mutations. In rats, low level expression of glutathione S-trasferase (GST) decreases both memory and life span. The major aim of this review is to provide better information on the antioxidant role of GST against AC induced neurotoxicity and genotoxicity.

KEY WORDS: glutathione S-transferases; biomarker; acrylamide; neurotoxicity; genotoxicity

Introduction

Glutathione S-transferases (GSTs) (EC 2.5.1.18) are multifunctional phase II versatile detoxification and xenobiotic metabolizing enzymes (Hayes *et al.,* 2005; Frova, 2006). There are seven classes of cytosolic glutathione S-transferases identified in mammals, which are classified on the basis of amino acid sequence similarities, named as alpha (α), mu (μ), pi (π), sigma (σ), theta (θ), omega (ω) and zeta (ξ) (Hayes & McLellan, 1999; Sheehan *et al.,* 2001).

Detoxification role of GST

The cytosolic GST isoenzymes belong to the same class sharing greater than 40% identity. Between different classes the identity is less than 25% (Hayes *et al.,* 2005). GSTs are ubiquitous and inactivation of cancerous agents through metabolism makes them important in cancer therapy (certainly alpha, mu, pi and theta class GSTs) (Lo *et al.,* 2007). The tri-peptide glutathione (GSH) is an efficient scavenger of reactive oxygen species (ROS). GSTs are responsible for detoxification of oxidative stress by products of metabolism (Hayes *et al.,* 2005). Toxic chemicals, oxidation and variation in temperature can regulate the expression of GSTs (Frova, 2006). The conjugation of electrophilic compounds by GST with glutathione detoxifies harmful drugs and environmental chemicals and thus GSTs are toxicologically important enzymes (Arakawa *et al.,* 2013). Figure 1, shows general reaction catalysed by GST. GSTs are playing a key role in cellular detoxification system to protect the cell from reactive oxygen metabolites and they contribute to biotransformation of xenobiotics and carcinogens (Hayes *et al.,* 2005). GST based drugs would be the next generation therapeutics to deal with drug resistance, cancer as well as neurological and neurodegenerative diseases (Kumar *et al.,* 2017).

GST enzymes have developed many functions throughout evolution (da Fonesca *et al.,* 2010). The cellular protective role of GST superfamily was taken towards positive selection on GST duplicates and they acquired other functions including sex hormone metabolism and apoptosis regulation which are vital for the retention of duplicates. Metabolism of xenobiotics was the main cause for the expansion of the GST family (da Fonesca *et al.,* 2010).

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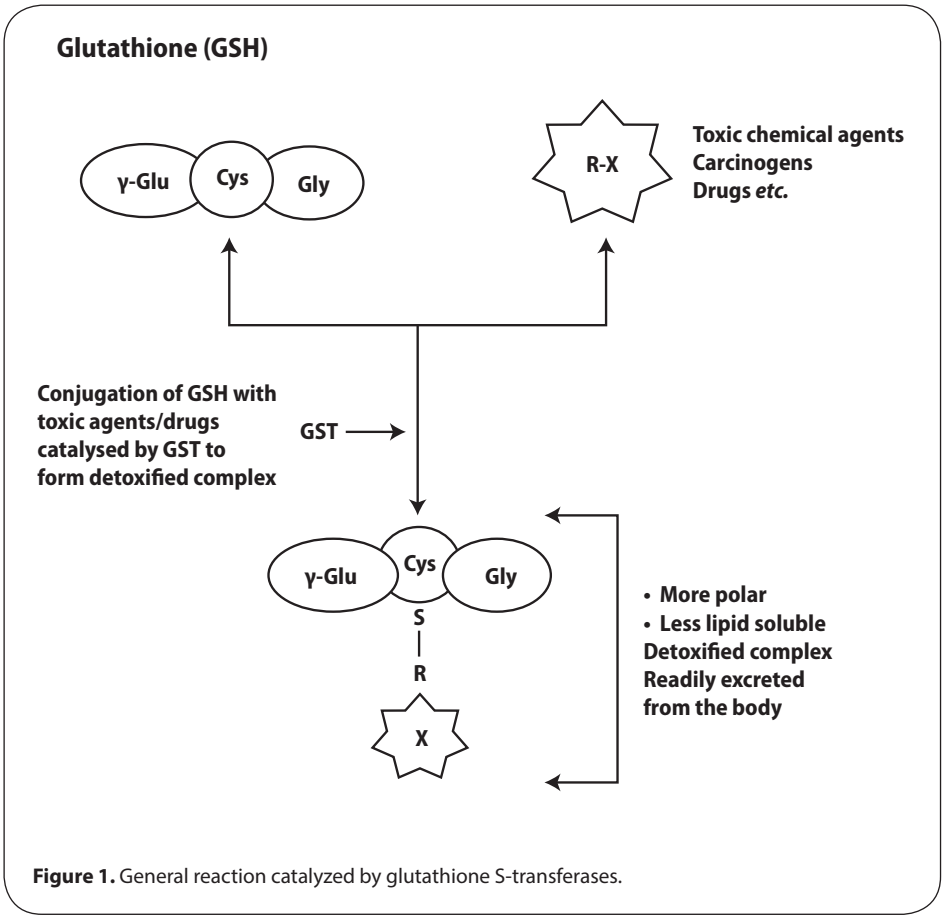

Figure 1. General reaction catalyzed by glutathione S-transferases.

GSTs exhibit an adaptive response to cellular stress (Hayes *et al.,* 2005). In addition, cytosolic GST promoter possesses the antioxidant response element (Frova, 2006). Glutathione (GSH) acts as scavenger of reactive oxygen species (ROS) and GSTs are responsible for metabolism of oxidative stress by-products (Hayes *et al.,* 2005). It was identified that soluble GSTs in mitochondria may work against reactive oxygen species (ROS) generated by the respiratory chain (Haider *et al.,* 2002). GST binding to reactive electrophiles may be important for preventing DNA damage. Ffor other molecules GSTs act as intracellular carriers (Hayes *et al.,* 2005). GSTs are multifunctional antioxidant enzymes that exhibit selenium independent glutathione peroxidase (GPx) activity in addition to glutathione transferase (GST) activity. With these activities GST can detoxify a variety of toxic chemicals (Dasari *et al.,* 2017a)

Structural characterization of GST

Ther are two binding sites in the cytosolic glutathione S-transferases (GSTs), *i.e.* glutathione (GSH) binding site (G site) and substrate (xenobiotic) binding site (H site). Two distinct xenobiotic binding sites are there in rat alpha class GST, including certain GST isoenzymes (Ding *et al.,* 2003).

It is suggested that domain I (G site) is composed of smaller N-terminal α/β helices, which include 1–78 amino acid residues of α and θ class, 1–82 of class μ, 1–74 of class π and σ and domain II (H site) is composed of larger α helix, which includes 86–222 amino acid residues of class α, 90–217 of class μ, 81–207 of class π, 81–202 of class σ and 85–208 of class θ (Armstrong, 1994; Wilce *et al.,* 1995). GST specific activity is decided by amino acid residues in the H-site (Armstrong, 1994).

Brain GSTs

Table 1 shows localization and importance of GST. Several studies reported that alpha (α), mu (μ) and pi (π) class GSTs were purified from rat brain; Yc of α class, Yb and Yβ of μ class, Yδ of π class GSTs with relevant molecular weight 27.5 KD, 26.3 KD and 26 KD, and 24.8 KD respectively, are expressed in response to toxic chemicals. Increased levels of total GST activity and relative density of that enzyme was studied in two regions of the brain, *i.e.* cerebellum and hippocampus (Struzynska *et al.,* 2002). The π class GST has been associated with myelin forming cell, probably to protect the myelin structure (Tansey & Cammer, 1991). Several studies reported that Yb and Yβ subunits of π class GST are more expressed in the rat brain, which may play a key role in detoxification.

Table 1. Rat brain glutathione S-transferases localization and their role.

Yb3 subunit of μ class GST is specifically expressed in rat brain	Abramovitz & Listowsky, 1987
GST isoenzymes are localized in glial cells of rat brain	Cammer *et al.,* 1989
π class GST may protect myelin structure	Tansey & Cammer, 1991
GSTA4 of alpha (α) class is localized in ependymal cells of the chorioid plexus, endothelial cells and perivascular end-feet of astrocytes	Johnson *et al.,* 1993
Ya subunit in nuclei and Yc subunit in nucleoli of α class GST is localized in rat brain neuron	
Both α and μ class GSTs protect neurons from toxic compounds	
GST activity is identified in detectable level in both cerebellum and hippocampus	Struzynska *et al.,* 2002
GSTA4 and GSTM1 of α and μ class GSTs inhibit oxidative damage to lipids and proteins in rat brain	Shao *et al.,* 2005
GSTA4 isoenzyme may protect microenvironment of brain cell	Abbott *et al.,* 2006
Absence of GSTM1 of π class GST is increase the risk of schizophrenia and tardive dyskinesia	De Leon *et al.,* 2005
Low level expression of GST decrease life span and memory	Bjork *et al.,* 2006

According to brain immunohistochemical studies, GSTA4 is predominantly distributed in ependymal cells of the chorioid plexus, endothelial cells and perivascular endfeet of astrocytes (Johnson *et al.,* 1993). The GSTA4 isoenzyme in the blood-brain barrier was strategically positioned to defend the microenvironment of brain cells (Abbott *et al.,* 2006). The GSTA4 expression levels were increased and nearly doubled in the cerebral cortex of old rats compared to young adult rats (Martinez-Lara *et al.,* 2003). The absence of GSTM1 increases the risk of schizophrenia and tardive dyskinesia (De Leon *et al.,* 2005). The mood stabilizers, like lithium or valproate, induce expression of both GSTM1 and GSTA4 and they inhibit oxidative damage to lipids and proteins and thus protect the brain from exotoxicity (Shao *et al.,* 2005). A low level of GST expression is associated with a decrease of life span memory performances in rats and it may model assessment of brain aging and neurodegeneration (Bjork *et al.,* 2006).

Immunohistochemical studies reported μ class GST to be localized in astrocytes, subventricular zone cells and ependymal cells (Cammer *et al.,* 1989), π class GST is localized in oligodendrocytes and also in association with myelin (Cammer *et al.,* 1989) of the rat brain central nervous system. Localization of GST Ya subunit was identified in nuclei and Yc subunit in nucleoli of the rat brain neuron (Johnson *et al.,* 1993). There are at least two GST isoenzymes localized in glial cells of the rat brain (Cammer *et al.,* 1989). Yb3 subunit of μ class GST was found to be specifically expressed in the rat brain (Abramovitz & Listowsky, 1987).

Cytosolic GST activity and total concentration of GST protein as well as the concentration of μ class GST are almost equal in the cerebellar cortex of the rat (Johnson *et al.,* 1993). In rats, the cytoplasmic localization of microsomal GST and the nuclear localization of α class GSTs in neurons, the relationship between the concentration of Yb2 subunit of μ class GST and also the resistance of neurons to toxic compounds in the cerebellar cortex indicate that the GSTs may protect against exogenous as well as endogenous neurotoxic metabolites (Johnson *et al.,* 1993).

Role of biomarkers

The environment is continuously loaded by foreign chemicals as well as metals due to urbanization and industrialization. From the beginning of the 20th century several thousands of organic pollutants have been by several ways released into the environment (Helm *et al.,* 2011). Most of these chemicals are undegradable, extremely toxic and accumulated both in territorial and aquatic ecosystems, transported to different environments by air, water and migratory species from their production place (Choi & Wania, 2011). Biochemical markers are useful in examining the effects of toxicants in various tissues (Van der Oost *et al.,* 2003). Figure 2, shows the expression of GST in response to AC. Biochemical markers can provide basic warning signals of particular stress and also give information on the health status of the organism (Korte *et al.,* 2000).

By ecotoxicological studies the interaction between chemicals and the organism can be assessed at different levels by using various biomarkers such as biotransformation enzymes, antioxidative compounds, oxidative

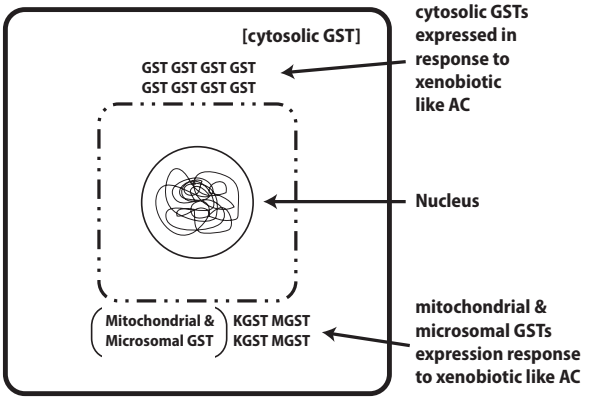

Figure 2. Xenobiotic versus GST in cell.

stress parameters, biotransformation products, stress proteins, both hematological and histological parameters, immunological, reproductive and endocrine parameters, genotoxic, neuromuscular, physiological and morphological and many other parameters (Van der Oost *et al.,* 2003). Most of the xenobiotics are metabolized by conjugation with glutathione (GSH) catalyzed by glutathione-S-transferase (GST) (Zimniak, 2008). A variety of organic xenibiotics, drugs and toxic compounds are matabolized by glutathione S-transferases (Halliwell & Gutterdige, 2015). In response to xenobiotics, the expression of GST as biomarker can be measured with the substrate 1-chloro-2, 4-dinitrobenzene by a using spectrophotometer (Habig *et al.,* 1974).

Acrylamide (AC)

Acrylamide (AC) is a very reactive and easily soluble substance in water and it is commonly used in industries as well as laboratories (Nordin *et al.,* 2003). As shown in Figure 3, AC is used in cosmetic, paper and textile industries as well as in ore processing, as soil conditioners and flocculants for waste water treatment (Friedman, 2003). AC is carcinogenic to experimental animals; it was discovered in various food products which are routinely consumed by humans, a situation raised public health concerns (Weiss, 2002). Generally, individuals can be victimized to AC at the work place (Dearfield *et al.,* 1995). AC enters the human diet through carbohydrate and amino acid rich food products prepared at high temperature (during food processing) (Stadler *et al.,* 2002; Mottram *et al.,* 2002); heat treated food products contain AC (Konings *et al.,* 2003). Lower level of AC is formed when cooking at lower temperature (Rydberg *et al.,* 2003). As shown in Figure 3, commonly consumed foods such as breakfast cereals, French fries and potato chips, as well as beverages (e.g., coffee) contain a significant quantity of AC (Tareke *et al.,* 2002).

It is well established that AC once entered into the biological system is quickly passed via cell membranes and widely distributed to all tissues (LoPachin & DeCaprio, 2005). AC shows neurotoxic, mutagenic and carcinogenic effects (Zhang *et al,* 2011; Maier *et al.,* 2012). AC forms glutathione S-conjugate by interacting with vital cellular nucleophiles having -SH, $-NH_2$ and -OH groups, which is an initial step of biotransformation of electrophiles to mercapturic acid (Awad *et al.,* 1998). AC interacts with glutathione S-trsansferases (GSTs) (Das *et al.,* 1982). AC induced oxidative stress is more effective at high doses (Yousef & El-Demerdash, 2006).

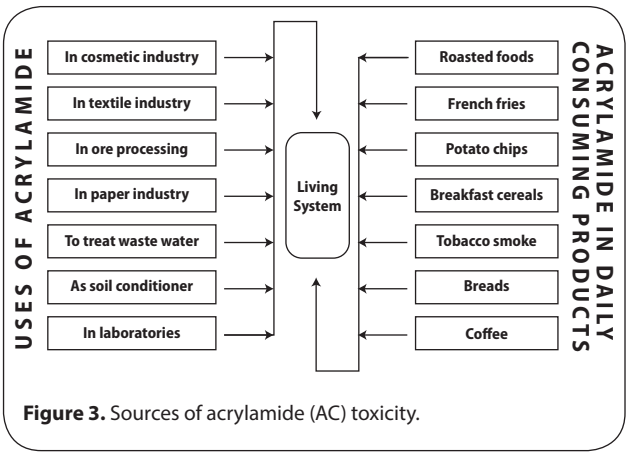

Figure 3. Sources of acrylamide (AC) toxicity.

Influence of AC on GST

A significant decrease in glutathione (GSH) content and GST activity was observed in AC administered rat brain (Shukla *et al.,* 2002). Depletion of GSH content as well as inhibition of GST activity was found both *in vitro* and *in vivo* (Srivastava *et al.,* 1986). A high level of GST and GST associated peroxidase (GPx) can protect the brain from AC toxicity up to certain level (Dasari *et al.,* 2017b).

Neurotoxicity of AC

The occurrence of adverse changes at the structural and functional level in the nervous system induced by a toxic compound is considered neurotoxicity and the substance responsible for the pathological condition of the nervous system is a neurotoxin (Bull, 2007). AC induced neurotoxic symptoms are characterized as ataxia, skeletal muscle weakness, and cognitive impairment including numbness of the extremities (Deng *et al.,* 1993). AC neurotoxicity in both human and experimental models was associated with cerebellar Purkinje cell death, degeneration of distal axons and nerve terminals in both the peripheral and central nervous systems (PNS and CNS) (LoPachin *et al.,* 2003).

Several rat studies suggest that axon degeneration might not be a primary neurotoxic effect of AC (Lehning *et al.,* 2003). Long-term treatment and low-dose administration of AC lead to degeneration of peripheral nerve tissue such as sciatic, tibial and sural nerves. Silver stain study of rat cerebellum revealed that AC can induce progressive degeneration of Purkinje cell axons (Lehning *et al.,* 2003). Central-peripheral neuropathy was observed in rats, monkeys and humans exposed to AC (Seale *et al.,* 2012). Allam *et al.* (2013) identified AC toxicity in the cerebral cortex as pyknosis and neurocyte chromatolysis in all stages of their investigation.

When the adduct formation exceeds and intoxication continues up to a disproportional increase of dysfunctional proteins, the related presynaptic processes are progressively disabled, which leads to the characteristic cumulative neurotoxicity of AC (LoPachin *et al.,* 2006). AC was found to suppress both metabolism and axonal transport in neurons, which leads to deficiency of nutritional factors (Honing & Rosenberg, 2000). Both prenatal and perinatal exposure of rodent pups to AC causes developmental neurotoxicity (Garey & Paule, 2010). The

accumulation of weak type-2 alkene electrophiles such as AC, methyl acrylate, ethyl methacrylate accelerate the progressive nerve terminal demise associated with Alzheimer's disease (LoPachin & Gavin, 2012). Both cerebellar dysfunctions and proprioceptive sensations are causes for abnormal performances after AC administration (Allam *et al.,* 2011).

Genotoxicity of AC

Toxicogenomic studies are based on gene expression evaluation to detect toxicity signals and identify new sensitive markers (Ellinger-Ziegelbauer *et al.,* 2008). Yet the specificity of thousands of genomic biomarkers are sometimes confuse and thereby not significant enough (Zhang *et al.,* 2011). When gametes are subjected to artificial reactive oxygen species (ROS) DNA damage may occur, such as modification of all bases, generation of base-free sites, deletions, frameshifts, DNA cross links, including chromosomal rearrangements (Duru *et al.,* 2000). Figure 2 shows the expression GST in response to AC.

DNA adduct formation is completely non-dose dependent and mutations can form at lower concentrations of AC, indicating the generation of promutagenic DNA adducts (Besaratinia & Pfeifer, 2003). Both AC and glycidamide (GC) (epoxide metabolite of AC) can damage DNA and glycidamide is mainly responsible for the mutagenicity of AC (Besaratinia & Pfeifer, 2004). Even micromolar doses of AC can effectively induce promutagenic DNA adducts and this calls for a reconsideration of AC presence in human diet as well as in the environment (Besaratinia & Pfeifer, 2004).

Adler *et al.* (1993) suggested that AC can generate chromosomal aberrations, sister chromatid exchanges, and mitotic disturbances. Both chromosomal aberration and micronucleus assays proved that AC might have genotoxic potency (Yang *et al.,* 2005). In AC treated rats, feulgen stain (specific for DNA) color intensity is decreased in the medulla neurons when compared to control, due to a marked loss of DNA in the medulla neurons (Allam *et al.,* 2013). During the process of apoptosis, serious and irreversible DNA damage occurs. Glutathione S-transferases and its peroxidase activities are destabilized by the excess accumulation of AC, leading to interaction with DNA (Sreenivasulu & Balaji, 2016).

Conclusion

This review summarizes the neuro and geno-toxicity of AC and the important role of GSTs in detoxification of toxic chemical agents like AC, and their localization in the brain, *i.e.* the cerebellum, hippocampus, neurons and glial cells. AC is a neurotoxin with symptoms like ataxia, skeletal muscle weakness, cognitive impairment and numbness. AC is a potent genotoxic agent forming chromosomal aberrations and micronuclei. Glutathione conjugation with toxic agents, catalyzed by GSTs, is the most important phase of detoxification. High expression of GST in cytotoxic conditions reveals that it is an efficient biomarker and enhances more tolerance of biological systems to toxic chemicals.

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REVIEW ARTICLE

Antidotal effects of thymoquinone against neurotoxic agents

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ABSTRACT
Several plants which contain the active component thymoquinone (TQ) have been traditionally used in herbal medicine to treat various diseases. Several studies indicated the protective effects of TQ against neurotoxic agents. The present study was aimed to highlight the protective effects of TQ against neurotoxic agents. For this reason, the literature from 1998 to 2017 regarding the protective effects of TQ against neurotoxic agents and their involvement mechanisms has been studied. The present review suggests the protective effects of TQ against neurotoxic agents in experimental models. More clinical trial studies are however needed to confirm the antidotal effects of TQ in human intoxication.

KEY WORDS: thymoquinone; neurotoxic agents; antioxidant; antidote

ABBREVIATION
ACR: Acrylamide; **ALT:** Alanine transaminase; **CAT:** Catalase; **DM:** Diabetes mellitus; **FAS:** Fetal alcohol syndrome; **GSH:** Glutathione; **GSH-Px:** Glutathione peroxidase; **GST:** Glutathione S-transferase; **LDH:** Lactate dehydrogenase; **LPS:** Lipopolysaccharide; **MDA:** Malondialdehyde; **Met:** Metformin; **mRNA:** Messenger ribonucleic acid; **NE:** Norepinephrine; **NMDA:** N-methyl-D-aspartate; **NO:** Nitric oxide; **PA:** Passive avoidance; **PTZ:** Pentylenetetrazole; **ROS:** Reactive oxygen species; **SOD:** Superoxide dismutase; **STZ:** Streptozotocin; **TQ:** Thymoquinone

Introduction

Plants as an important source of active compounds have been used in traditional medicines (Samarghandian *et al.*, 2017; Samarghandian *et al.*, 2017). *Nigella sativa* (of the family ranunculaceae) and its seeds are commonly called black cumin, fennel flower, or nutmeg flower (Ahmad *et al.*, 2013). It is considered a medicinal herb with some religious usage, called the 'remedy for all diseases except death' (Prophetic hadith) and Habatul Baraka "the Blessed Seed" (Mohammad *et al.*, 2013). The black cumin oil consists of active components such as ocopherols, phytosterols, polyunsaturated fatty acids, thymoquinone, ρ-cymene, carvacrol, t-anethole and 4-terpineol. Thymoquinone (2-isopropyl-5-methylbenzo-1, 4-quinone) (TQ) has been found in many medicinal plants, as *e.g.* in several genera of the Lamiaceae family (Monarda) and the Cupressaceae family (Juniperus) (Farkhondeh *et al.*, 2017). TQ is the main ingredient of the *Nigella sativa*, which is effective for the treatment of various diseases, such as neurodegenerative disorders, coronary artery diseases, respiratory failures, and urinary system failures (Ahmad *et al.*, 2013). TQ has been indicated to possess antioxidant, anti-inflammation, anticancer, antimicrobial, anti-mutagenic and anti-genotoxic activities (Asaduzzaman Khan *et al.*, 2017). TQ may be considered a therapeutic agent for prevention of neurodegenerative diseases. However, the therapeutic effects of TQ against toxic agents remains nascent in the literature. The present review aimed to critically review studies from 1998 to 2017 regarding the protective effects of TQ against neurotoxic agents.

Safety study

The LD50 value of TQ was found to be 10 mg/kg intraperitoneally (i.p.) in the rat. I.p. injection at doses of 4, 8, 12.5, 25 and 50 mg/kg TQ in mice has no effect on biochemical

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indices, such as serum alanine transaminase (ALT) and lactate dehydrogenase (LDH) (Mansour *et al.*, 2001). However, i.p. injection of TQ higher than 50 mg/kg was lethal in mice (Mansour *et al.*, 2001). Several toxicological studies indicated that oral administration of TQ in the range of 10–100 mg/kg has no toxic or lethal effects in mice (Kanter, 2008; Kanter, 2011a). The maximum tolerated dose of TQ was 22.5 mg/kg in male and 15 mg/kg in female rats when injected i.p., whereas in both male and female rats it was 250 mg/kg after oral administration (Kanter, 2011b). The difference in toxicity response between i.p. injection and oral ingestion of TQ can be related to the complete absorption of TQ into the systemic circulation after i.p. injection, whereas with oral administration, TQ is biotransformed in the gastrointestinal tract or metabolized in the liver.

Methods

Online literature resources were checked using different search engines such as Medline, PubMed, Iran Medex, Scopus, and Google Scholar from 1998 to 2017 to identify articles, editorials, and reviews about antidotal effects of TQ against neurotoxic agents. TQ, neurotoxicity, and neurotoxic agents were key words used to search the literature.

Protective effects of TQ against neurotoxic agents (Table 1)

Lead

Lead (Pb^{2+}) is one of the most hazardous heavy metals that threatens human health (Samarghandian *et al.*, 2013). Lead causes damage to the brain by disrupting ionic balance in neuronal cells, modifying normal brain function, interrupting neural signal transmission between neurons and inducing neurodegeneration and progressive neuronal cell death (Lidsky & Schneider, 2003). Chronic occupational exposure to low levels of lead may be a risk factor for some neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (Coon *et al.*, 2006; Wu *et al.*, 2008). Chelation therapy is the most effective treatment for lead poisoning (Flora *et al.*, 2012). Natural antioxidants have been used to improve lead toxicity in experimental studies (Reckziegel *et al.*, 2011). Lead acetate exposure [0.5 g/l (500 ppm)] was found to cause degeneration of hippocampal and cerebellar neurons, endothelial lining of brain blood vessels with perivascular cuffing of mononuclear cells consistent to lymphocytes and chromatolysis and neuronal and also congestion of choroid plexus blood vessels, ischemic brain infarction, microglial reaction, neuronophagia, and axonal demyelination. TQ treatment (20 mg/kg in corn oil (0.5 ml/rat)) improved the lead-induced brain lesions in rats due to its antioxidant properties (Radad *et al.*, 2014), suggesting that TQ attenuated brain oxidative stress induced by lead. However more studies are needed to determine the underlying mechanisms of such protection of TQ against lead neurotoxicity.

Morphine

Morphine was indicated to induce oxidative stress in brain (Guzmán *et al.*, 2006; Özmen *et al.*, 2007; Ibi *et al.*, 2011). Reactive oxygen species (ROS), glutamate release, and nitric oxide (NO) have important roles in morphine tolerance, dependence and withdrawal symptoms (Sepulveda *et al.*, 1998; Özek *et al.*, 2003; Wen *et al.*, 2004; Mori *et al.*, 2007; 2011). Activation of the ionotropic N-methyl-D-aspartate (NMDA) subtype of glutamate receptors plays a crucial role in the development of morphine analgesic tolerance and dependence (Bajo *et al.*, 2006; Murray *et al.*, 2007; Wang *et al.*, 2007). Over-activation of the glutamatergic system increases ROS production (Alekseenko *et al.*, 2012). The protective effects of TQ against morphine induced tolerance and dependence have been indicated (Abdel-Zaher *et al.*, 2013). Repeated administration of TQ prevented the development of morphine tolerance and dependence in mice, decreased brain malondialdehyde (MDA) and NO levels, increased brain intracellular glutathione (GSH) level and glutathione peroxidase (GSH-Px) activity. TQ had no effect on the increased glutamate level in the brain induced by repeated administration of morphine. However, TQ inhibited morphine tolerance and dependence-induced increase in inducible NO synthase but not in neuronal NO synthase mRNA expression in mouse brain. It was indicated that TQ ameliorated the development of morphine tolerance and dependence via decreasing the brain glutamate level, oxidative stress, inducible NO synthase expression, and NO overproduction. The study suggested that the protective effect of TQ is very likely due to its strong antioxidant activities.

Ethanol

Ethanol exposure during brain development might cause neurodevelopmental defects referred to as fetal alcohol syndrome (FAS) (Jones *et al.*, 1973). Ethanol disturbs brain development by the dysregulation of neurogenesis, cell migration and cell survival (Miller, 1986; 1996; Naseer *et al.*, 2010). Ullah *et al.* (2012) suggested that TQ and metformin (Met) have neuroprotective effects against ethanol-induced apoptosis via regulating calcium (Ca^{2+}) homeostasis, mitochondrial function, cytochrome-c release, caspase activation and the Bcl-2 family of proteins. ROS generation is an important mediator of ethanol-induced apoptotic cell death (Ramachandran *et al.*, 2003; Young *et al.*, 2005; Antonio *et al.*, 2008). Exposure to ethanol accompanied with Met (10 mM), TQ (10, 15, 25 and 35 μM) or Met plus TQ inhibited ROS generation, which triggers apoptotic cell death pathways during early development of rat cortical and hippocampal neurons. TQ plus Met decreased the levels of Ca^{2+} induced by ethanol in the brain. Administration of TQ plus Met to rats reduced ethanol-induced apoptosis in cortical and hippocampal neurons by decreasing Bax/Bcl-2 ratio. Met plus TQ prevented cell death in primary rat cortical neurons induced by ethanol due to its antioxidant effect that

Table 1. Antidotal effects of TQ against neurotoxic agents.

Dose of TQ	Toxic agents	Experimental study	Mechanism	Ref
20 mg/kg	Lead acetate	Rat	Prevented neurotoxicity by modulating oxidative stress	Radad *et al.*, 2014
10 mg/kg	Morphine	Mice	Ameliorated the development of morphine tolerance and dependence via decreasing the brain glutamate level, oxidative stress, inducible NO synthase expression, and NO overproduction	Abdel-Zaher *et al.*, 2013
10, 15, 25 and 35 µM	Ethanol	Primary rat cortical neurons	Prevented neurotoxicity by decreasing elevated $[Ca^{2+}]c$ levels, decreasing Bax/Bcl-2 ratio in the mitochondrial of neurons, inhibition of ROS generation	Ullah *et al.*, 2012
50 mg/kg	Toluene	Rat	Prevented neurotoxicity by decreasing the immunoreactivity of degenerating neurons and apoptosis	Kanter, 2008 and 2011
0–100 µM	Glutamate	Human SH-SY5Y neuroblastoma cells	Prevented neurotoxicity by decreasing the ROS generation, mitochondrial dysfunction and apoptotic cascade	Al Mamun *et al.*, 2015
2.5, 5, 10 mg/kg	Acrylamide	Rat	Prevented neurotoxicity by inhibiting lipid peroxidation in cerebral cortex resulted in improved severe gait abnormalities	Mehri *et al.*, 2014
10 mg/kg	Streptozotocin	Rat	Prevented neuropathy diabetes via decreasing NO and MDA , increasing GSH, CAT and GST, and also decreasing norepinephrine	Hamdy & Taha, 2009
10 mg/kg	Lipopolysaccharide	Rat	Prevented neurotoxicity by decreasing hippocampal IL-6 and TNF-α level and increasing SOD and CAT activities.	Bargi *et al.*, 2017
5, 10 and 20 mg/kg	Pentylenetetrazole	Mice	Prevented neurotoxicity by decreasing glutamate, oxidative stress and NO production	Abdel-Zaher *et al.*, 2017

maintains mitochondrial integrity. The mechanism of TQ neuroprotection was similar to Met including stabilization of mitochondrial membrane potential, reducing Ca^{2+} overload and inhibition of apoptotic cascades.

Toluene

Toluene is an industrial aromatic solvent usually found in gasoline, paints, resins, cosmetic products, lacquers, inks, nail polish, paint thinners, and adhesives (Kurtzman *et al.*, 2001). Acute intoxication with toluene causes euphoria and disinhibition followed by hallucinations, tinnitus, ataxia, confusion, nausea and vomiting, an increased tendency to sleep, frequent headaches, and eye irritation in humans (Flanagan *et al.*, 1989; Echeverria *et al.*, 1991; Evans & Balster, 1991). Brain magnetic resonance imaging indicated cerebral and hippocampal atrophy with a loss in brain volume in toluene/solvent abusers (Flanagan *et al.*, 1989; Echeverria *et al.*, 1991; Evans & Balster, 1991; Yamanouch *et al.*, 1995; Kamran& Bakshi, 1998; Deleu & Hanssens, 2000). Toluene causes CNS depressant effects such as psychomotor impairment (Hester *et al.*, 2011), excitation, inhibition of locomotor activity (Shiotsuka *et al.*, 2000), and loss of righting reflex and sedation (Conti *et al.*, 2012). Furthermore, peripheral nerve dysfunction has been observed after toluene exposure (Hester *et al.*, 2011). I.p. injection of toluene disturbed the oxidant-antioxidant balance in the brain (Greenberg *et al.*, 1997; Zabedah *et al.*, 2001). It is suggested that increased lipid peroxidation and apoptosis with reduced antioxidant content in the brain are the important mechanisms involved in the neurotoxicity of toluene. The protective effect of TQ against neurodegeneration in the hippocampus after chronic toluene (3,000 ppm inhalation) exposure in rats has been shown (Kanter, 2008). TQ (50 mg/kg, p.o) treatment was found to decrease the immunoreactivity of degenerating neurons after chronic exposure to toluene. TQ treatment decreased the number of apoptotic neurons and prevented the deterioration of the hippocampal neuron, as well as memory and learning disabilities in animal models. TQ also improved morphological alteration in the hippocampus of rats after chronic exposure to toluene by ameliorating apoptosis. (Kanter, 2011) evaluated the protective effects of TQ on the neuronal injury in the frontal cortex of rats after chronic exposure to toluene (3,000 ppm inhalation). Chronic exposure to toluene caused severe degenerative changes, shrunken cytoplasm, slightly dilated cisternae of endoplasmic reticulum, and swollen mitochondria with degenerated cristae and nuclear membrane breakdown with chromatin disorganization in neurons of the frontal cortex. TQ treatment (50mg/kg p.o) was reported to ameliorate the severity of degenerative changes in the cytoplasm and especially in the cell nucleus of rats exposed to toluene. TQ treatment significantly decreased the immunoreactivity of degenerating neurons and the number of the apoptotic neurons (TUNEL positive neurons). The study also confirmed that TQ treatment improved morphologic neurodegeneration in frontal cortex tissues of rats exposed to toluene by modulating apoptotic pathways.

Glutamate

(Al Mamun *et al.*, 2015) investigated the protective effects of TQ against glutamate-induced (8 mM) cell death in SH-SY5Y neuronal cells. The findings indicated that TQ (0–100 µM) treatment had protective effects against glutamate induced viability loss, ROS generation, mitochondrial dysfunction and increased the apoptotic cascade via decreasing Bax/Bcl-2 ratio as well as caspase-9 expression. The study suggested that TQ protected against glutamate-induced cell death in SH-SY5Y neurons

by inhibiting ROS production, mitochondrial dysfunction and intrinsic apoptotic cascade.

Acrylamide

Acrylamide (ACR) is a neurotoxic agent that target both the central and peripheral nervous system. ACR can lead to neurotoxicity characterized by ataxia, skeletal muscle weakness and body weight loss (Lopachin, 2005; Zhu *et al.*, 2008). Various mechanisms are responsible for neurotoxicity induced by ACR including disruption of presynaptic nitric oxide (NO) signaling, nerve-terminal degeneration, axonal degeneration, increment of lipid peroxidation, reduction of antioxidant capacity of the nervous system and induction of apoptosis signaling. The effects of TQ on ACR-induced (50 mg/kg/day i.p.) neurotoxicity on rats have been investigated (Mehri *et al.*, 2014). TQ (2.5, 5, 10 mg/kg i.p.) inhibited lipid peroxidation in cerebral cortex and improved the severe gait abnormalities in animals. A significant decrease in the number of apoptotic neurons was observed after TQ treatment in rats exposed to ACR. TQ treatment also ameliorated morphologic changes in the frontal cortex of rats exposed to ACR. The protective effects of TQ against ACR-induced toxicity may be due to its antioxidant effects (Mehri *et al.*, 2014).

Streptozotocin

Neuropathy is one of the main complications of diabetes mellitus (DM) with ROS involved in its pathogenesis (Gaweł *et al.*, 2003). The effects of TQ on brain function in streptozotocin (STZ)-induced (60 mg/kg i.p.i) diabetes model have been investigated (Hamdy & Taha, 2009). It was suggested that TQ prevented the development of diabetes-mediated complications via decreasing the levels of NO and MDA with increasing the levels of GSH, catalase (CAT) and glutathione S-transferase (GST) enzymes. The levels of norepinephrine (NE) in the brain of diabetic rats decreased after TQ treatment (10 mg/kg p.o). Correlation analysis indicated that correction of the monoamine levels in the TQ treated diabetic rats was related to the increase in the levels of GST in these animals. The findings suggested that TQ protected the brain against STZ-induced diabetes by modulating oxidative stress.

Lipopolysaccharide

Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria that is used in research for the evaluation of LPS structure, metabolism, immunology, toxicity, physiology, and biosynthesis (Wang & Quinn, 2010). It has also been used to induce inflammation in animal models. Inflammation has been considered a major mechanism involved in the disruption of learning and memory (Chesnokova *et al.*, 2016; Wolf *et al.*, 2016). Inflammation induced by LPS resulted in releasing pro-inflammatory cytokines and inducing ROS production (Valero *et al.*, 2014; Song *et al.*, 2016). It has been suggested that natural antioxidants with anti-inflammatory properties may be effective against memory impairment. It has been reported that TQ improved learning and memory impairments induced by LPS (1 mg/kg i.p.) in rats (Bargi *et al.*, 2017). The protective effects of TQ on memory function has been evaluated using water maze test.

The findings indicated that TQ treatment (2, 5, 10 mg/kg i.p.) decreased the time to find the platform and improved remembered location of the platform in rats. The protective effects of TQ on learning and memory task was also indicated in passive avoidance (PA) test, which was presented by a longer delay for entering to the dark room after the shock. TQ also increased the time spent in the light, while decreased the time spent in the dark compartment where they have previously received a shock. TQ decreased the levels of IL-6 and TNF-α and increased superoxide dismutase (SOD) and CAT activities in the hippocampus of rats. The study suggested antioxidative and anti-inflammatory effects of TQ against learning and memory impairments induced by LPS.

Pentylenetetrazole

Pentylenetetrazole (PTZ) is a drug used as a circulatory and respiratory stimulant; however, side-effects such as epilepsy were difficult to avoid (Dhir, 2012). Cognitive diseases are a group of mental health disorders that affect learning, memory, perception, and problem solving, and include amnesia, dementia, and delirium. It was confirmed that overproduction of ROS and RNS in the brain are involved in the pathogenesis of cognitive impairments (Qu *et al.*, 2012; Chindo *et al.*, 2015; Jain *et al.*, 2015; Singh& Kumar, 2015). PTZ-kindling is a known animal model which simulates epilepsy (Hassanzadeh *et al.*, 2014; Kaur *et al.*, 2015). One study indicated that administration of TQ (5, 10 and 20 mg/kg i.p.) inhibited PTZ-induced (35 mg/kg i.p) kindling and cognitive impairment in mice. According to the results of the study, TQ ameliorated PTZ-induced kindling in mice via decreasing the levels of glutamate, oxidative stress and NO production in the brain (Abdel-Zaher *et al.*, 2017).

Conclusion

The term "neurotoxicity" pointed to damage to the nervous system induced by exposure to natural or chemical toxic agents (Adewale *et al.*, 2015). These toxic agents may modify the nervous system function in ways that can destroy the neurons (Adewale *et al.*, 2015).

In the present study, several studies from 1998 to 2017 have been reviewed to identify the protective effects of TQ against neurotoxic agents. Based on the present findings, TQ acts as an antidote in neurotoxicity induced by toxic agents. Lead, ethanol, toluene, glutamate, ACR, LPS and STZ are some examples of chemical agents against which TQ could protect the brain. TQ showed protective effects against some chemical drugs such as morphine and PTZ which have organ toxicities, particularly in overdose.

The above mentioned agents are risk factor for causing neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, learning and memory deficiency, epilepsy, *etc.* Inhibition of oxidative stress, inflammation

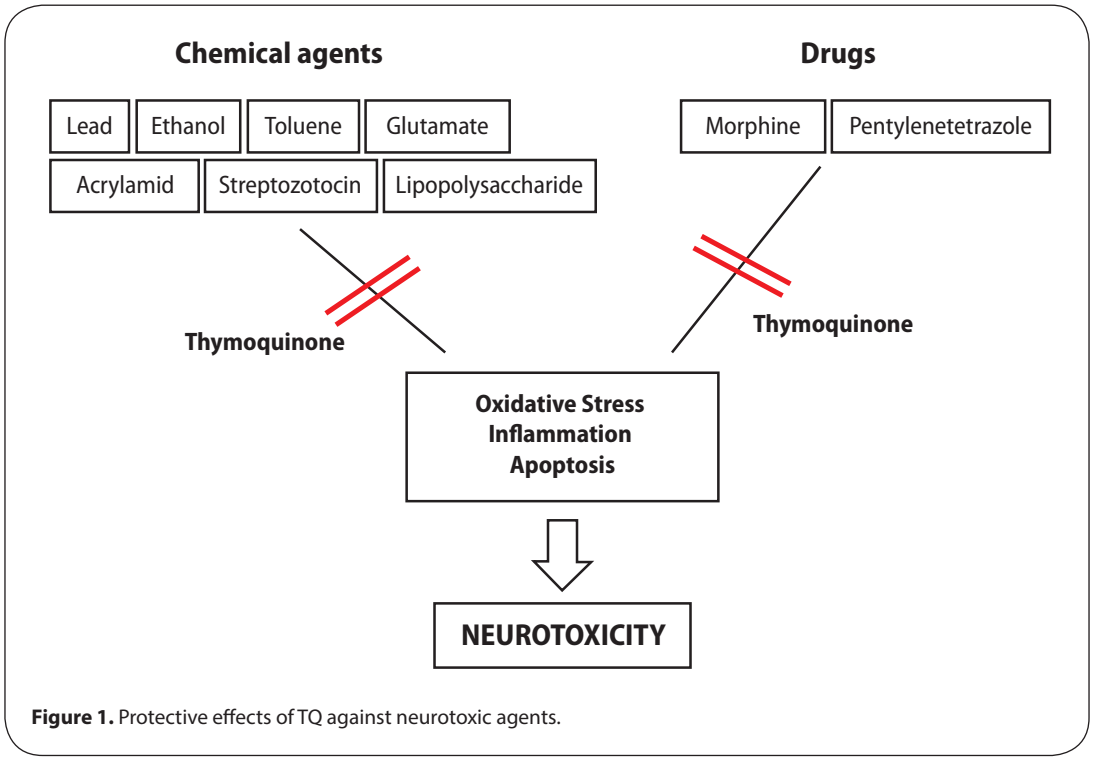

Figure 1. Protective effects of TQ against neurotoxic agents.

and apoptosis are responsible for antidotal effects of TQ (Figure 1). Oxidative stress is recognized as a main mechanism involved in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, anxiety disorders, depression, *etc.* (Salim, 2016). The present review indicated that TQ prevented CNS damage induced by lead, morphine, ethanol, glutamate, ACR, STZ, LPS and PTZ via modulating the oxidant-antioxidant system. TQ could balance between oxidant-antioxidant system via enhancing antioxidant contents and decreasing free radical production. Additionally, the strong antioxidant effects of TQ may be related to its free radical-scavenging activity (Badary *et al.*, 2003).

Neuronal apoptosis has an important role in the developing brain and also in neurodegenerative diseases (Esen *et al.*, 2017). However, there are main differences in the mechanisms by which apoptosis is initiated. Apaf-1 (apoptotic protease-activating factor 1), proteins of the Bcl-2 and caspase families are the key molecular components of apoptosis in neurons (Udhayabanu *et al.*, 2017). Neutrophils modulate neuronal apoptosis via activating main protein kinase cascades including phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase. Similarly, abnormal protein structures such as amyloid fibrils activate the apoptosis pathways in Alzheimer's disease (Yan *et al.*, 2017). The present study observed that ethanol, toluene, glutamate and ACR caused neuronal apoptosis by elevating intracellular Ca^{2+}concentration and disrupting mitochondrial membrane potential. Activation of the caspase families might be the key factor in the neurodegenerative diseases induced by toxic agents. Selective caspase inhibition might be an effective approach against neurotoxic agents. The present review also confirmed that TQ prevented neuronal injuries by modulating the activation of caspase families.

Inflammation is recognized as a major effective factor against acute and chronic CNS diseases. Inflammatory mediators such as complement and adhesion molecules, cyclooxygenase enzymes, and cytokines are elevated in neurodegenerative disease. Inflammation may have beneficial and also detrimental effects in the CNS, especially in repair and recovery. Several anti-inflammatory targets have been triggered for CNS disorders treatment. It was observed that TQ prevented learning and memory problems induced by LPS via decreasing hippocampal IL-6 and TNF-α level. In conclusion, the present review confirmed the protective effects of TQ against neurotoxic agents in experimental models, however more clinical trials should be done to confirm the antidotal effects of TQ in human.

Authors' contributions

ARS provided most of the reference and drafted the first version of the paper, designed the table in the paper, SS designed the study and did the overall editing of the paper, TF helped with the design of the paper. All authors read and approved the final manuscript.

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REVIEW ARTICLE

Dichlorvos toxicity: A public health perspective

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ABSTRACT

Pesticides are used in agriculture and in domestic pest control. Dichlorvos, an organophosphate, is a predominant pesticide used in domestic insect control in developing countries. Acute and prolonged exposure may lead to death, genotoxic, neurological, reproductive, carcinogenic, immunological, hepatic, renal, respiratory, metabolic, dermal and other systemic effects. Its toxicity is due to the ability of the compound to inhibit acetyl cholinesterase at cholinergic junction of the nervous system. This study is a review of the toxicological effects of dichlorvos in a public health perspective.

KEY WORDS: dichlorvos; organophosphate; pesticide; insecticide; acetyl cholinesterase; toxicity

Introduction

Dichlorvos, also known as DDVP (2,2-dichlorovinyl dimethyl phosphate is an orgnophosphate insecticide cum pesticide (USEPA, 2007). It is traded under names such as DDVP, Dedevap, Nogos, Nuvan, Phosvit, Vapona, Sniper and Daksh (Owoeye *et al.*, 2012; Meister 1992). Dichlorvos (Figure 1) has the molecular formulation $C_4H_7Cl_2O_3P$, molecular weight of 220.98, vapor pressure of 1.2×10^{-2} mmHg at 20 °C, and density of 1.415 g/ml at 25 °C (Budavari, 1998). It is classified by the WHO as a class 1B, "highly hazardous" chemical (WHO, 1992).

Dichlorvos is usually used as a household and agricultural pesticide. It is the most commonly used organophosphate pesticide in developing countries (Binukumar and Gill, 2010). It is also used as an antihelminthic agent on dogs, horses and swine (USEPA, 1994). Dichlorvos has been used in fish farming to eradicate crustacean ectoparasites (Varo *et al.*, 2003). It has been in use since the early 1960s and has been the subject of many toxicity studies (Durkin & Follansbee, 2004). The present study is an attempt to review the public health concerns of dichlorvos toxicity.

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Production of dichlorvos

One method of production of dichlorvos is the dehydrochlorination of trichlorfon in aqueous alkali at 40–50 °C (WHO, 1989). It is also produced commercially by a reaction of trimethyl phosphate and chloral (Sittig, 1980).

Legislations/regulations of dichlorvos

Dichlorvos present a legislative conundrum as the body of experimental result relating to its safety and non-safety continues to expand (Mennear, 1998). Different countries view both sides of the argument differently while considering the economic implications.

Dichlorvos has been reviewed under the Biological Product Directive (BPD) and the decision not to include dichlorvos in Annex 1/1A of the BDP commission approved list of active substances for use in biocidal products in Europe was reached. This implies that insecticide products containing dichlorvos shouldl no longer be placed on the market with effect from November 1, 2012 (EU, 2012). This decision was reiterated later in 2013 (EU, 2013). However, there is significant variability in its use across the European Union (EU), with some member states not using the substance at all and others still having a range of uses (EC, 2011).

Following the review of dichlorvos in the United Kingdom (UK), decision was taken on suspension of sale of all insecticide products containing dichlorvos in 2002

(EC, 2011), following recommendation of the Advisory Committee on Pesticide (HSE, 2002).

Dichlorvos status in some countries that has restrictions or ban are listed in Table 1. However, there have been non-governmental interventions in the form of conventions: the Stockholm convention on persistent organic pollutants that aimed at protecting human health and the environment from persistent organic pollutants; the Rotterdam Convention that takes the obligation of early warning about bans and restrictions of pesticides that has been banned by two countries in two regions (Imig, 2010).

Environmental degradation

The initial release of dichlorvos is mostly into air. In contact with water, hydrolysis reaction becomes the major mechanism for degradation (Faust & Suffet, 1996; Lamoteaux, 1978). In the environment, abiotic degradation via hydrolysis is the major transformation process.

Dichlorvos does not strongly absorb ultraviolet light above 240 nm (Howard, 1991; Gore *et al.*, 1971) and hence it is unlikely to be photolyzed in the atmosphere. On theoretical consideration, dichlorvos is likely to be degraded in the air from free radicals, such as hydroxyl groups or ozone.

Dichlorvos is highly soluble in water and has the tendency to remain in solution with very little tendency to sorb to sediment. In solution, dichlorvos becomes subject to both abiotic and biological degradation, with the predominant mechanism being hydrolysis. Dichorvos is hydrolyzed to dichloroethanol, dichloroacetaldehyde, dichloroacetic acid, dimethylphosphate and dimethylphosphoric acid (WHO, 1989). The degradation is more rapid in alkaline pH (Lamoteaux, 1978) and high temperature (Faust, 1996). A study by Lartiges and Garrigues (Lieberman & Alexander, 1983) investigated the degradation of dichlorvos in different types of water under different environmental conditions. The study reported that in winter temperature, dichlorvos was still present in filtered water (pH 6.1) after 180 days; residues in river water (pH 7.3) and filtered river water (pH 7.3) disappeared after 81 days; residues in sea water (pH 8.1) disappeared after 34 days. In summer, dichlorvos residues disappeared after 81, 55, 34 and 180 days for filtered water, river water, filtered river water and sea water, respectively. Microorganisms found in sewage sludges apparently can degrade dichlorvos which may require acclimation. However, the rate of degradation is slower than abiotic degradation (Lieberman & Alexander, 1983).

In soils harboring good quality of moisture, hydrolysis reaction similar to those in aqueous solutions are expected to occur (PIP, 1993). However, half-life (first-order kinetics) of 17 days in soil have been reported (Hayes, 1990), though the authors were not specific of the soil type. Hayes and Laws (1990) reported an average half-life of 16 days in silty clay soil (pH 5.5) and sandy clay soil (pH 6.9) irrespective of the soil type. More so, Lamoreaux (1978) investigated the rate of dichlorvos in Houston Black clay soil for 10 days at room temperature of 26 °C. They reported that the fate of dichlorvos degradation in the soil was directly related to the presence of bacterium *Bacillus cereus*, the pH of the soil perfusion system and the extent of dichlorvos adsorption. The study reported more rapid degradation in the presence of *B. cereus* (3.9 days half-life) as against 10 days for sterile soil. Hydrolysis and other non-biological processes accounted for 70% of total degradation of dichlorvos, while bacteria degradation accounted only for 30% in the soil perfusion system (Lamoteaux, 1978). The proposed pathway for the breakdown of dichlorvos in the soil and water/sediment system is shown in Figure 2 as adapted from APVAM (2008).

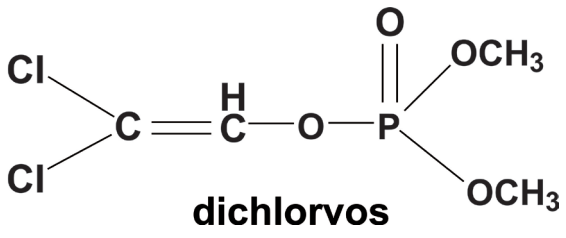

Figure 1. Structural formula of dichlorvos.

Table 1. Dichlorvos status in some countries.

Country	Status	Reference
Sri Lanka	Banned	Eddleston et al., 2003
USA	Restricted	ICAR-RC, 2015
Kuwait	Restricted	ICAR-RC, 2015
Korea	Restricted	ICAR-RC, 2015
Bangladesh	Banned	FAO-UN, 2013
Cambodia	Banned	FAO-UN, 2013
Vietnam	Restricted	FAO-UN, 2013
Australia	Restricted	APVMA, 2011
Canada	Restricted	Health Canada, 2011
Denmark	Banned	European Commission, 2011

Metabolic fate of dichlorvos in human and other mammals

Of the organophosphates, dichlorvos is distinct for its rapid metabolism and excretion by mammals. Dichlorvos was not detected in the blood of mice, rats and humans after exposure at atmospheric concentration of up to 17 times that normally reached for insect control in homes. This rapid disappearance is due to the presence of degrading enzymes in tissues and blood plasma (Hayes & Laws, 1990; Hayes, 1982). Dichlorvos does not accumulate in body tissues and has not been detected in the milk of cow or rat even at doses capable of producing symptoms of poisoning (Hayes & Laws, 1990).

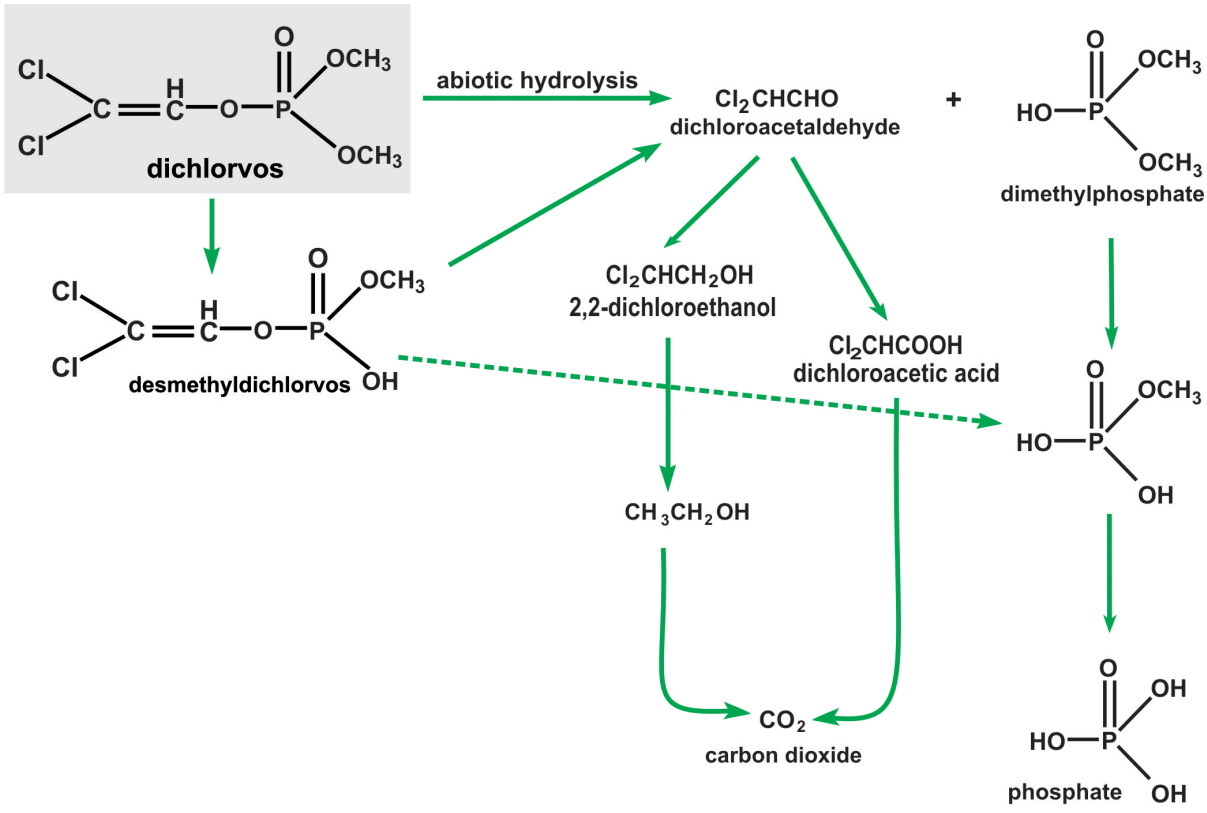

Figure 2. Proposed pathway for the breakdown of dichlorvos in soil and water/sediment system, including abiotic hydrolysis and microbial degradation steps (APVAM, 2008).

The liver is the major site of dichlorvos detoxification (Gains *et al.*, 1996; Casida, 1966). However, blood, kidney, lung, spleen metabolize dichlorvos mostly to dimethyl phosphate. Other metabolites are desmethyl dichlorvos, monomethyl phosphate, and inorganic phosphate (Loeffler *et al.*, 1976). The pathway of metabolism of dichlorvos is shown in Figure 3 as adapted from Wright *et al.* (1979).

Studies have reported the metabolism of dichlorvos in humans (Hutson & Hoadley, 1972a), rats (Casida et al 1962; Hutson & Hoadley, 1972b), mice (Hutson & Hoadley, 1972b), Syrian hamsters (Hutson & Hoadley, 1972a), pigs (Loeffler *et al.*, 1976), goats (Casida *et al.*, 1962) and cows (Casida *et al.*, 1962). The submissions of these studies showed that dichlorvos metabolism is generally similar in different species of mammals with minor difference in quantification and in relation of the rate of the metabolic pathway (ATSDR, 1997). Two enzymatic mechanisms exist for the breakdown of dichlorvos; the glutathione indepdendent mechanism catalyzed by "A" type esterases that produces dimethyl phosphate and dichloroacetaldehyde (Wright *et al.*, 1979) and the glutathione dependent mechanism that produces desmethyl dichlorvos and S-methyl glutathione. Subsequent breakdown of desmethyl dichlorvos to dichloroacetaldehyde and monomethyl phosphate is also catalyzed by "A" esterases (Wright *et al.*, 1979). S-methyl-glutathione is broken down to methylmercapturic acid and excreted in urine of the subject animal.

Mechanism of toxicity

Dichlorvos exerts its toxic effect by irreversibly inhibiting neural acetylcholinesterase (Wang *et al.*, 2004). The inhibition provokes the accumulation of acetylcholine in synapses with disruption of nerve function (Wang *et al.*, 2004). The consequences of the altered cholinergic neurotransmission in the parasympathetic autonomic nervous system includes perspiration, nausea, lacrimation, vomiting, diarrhea, excessive bronchial secretion or even death (ATSDR, 1997). Effects on motor nerve fibers in skeletal muscles can include muscle cramps, muscle fasciculation, muscle weakness and flaccidity. The cholinergic effect in the central nervous system results in drowsiness, fatigue, mental confusion, headache, convulsions, coma and even death (ATSDR, 1997).

However, the nervous system can tolerate a certain amount of acetylcholinesterase inhibition without overt toxic effects. In humans and mammals, toxic signs were not generally seen until at least 20% acetylcholinesterase was inhibited (Ecobichon, 1991).

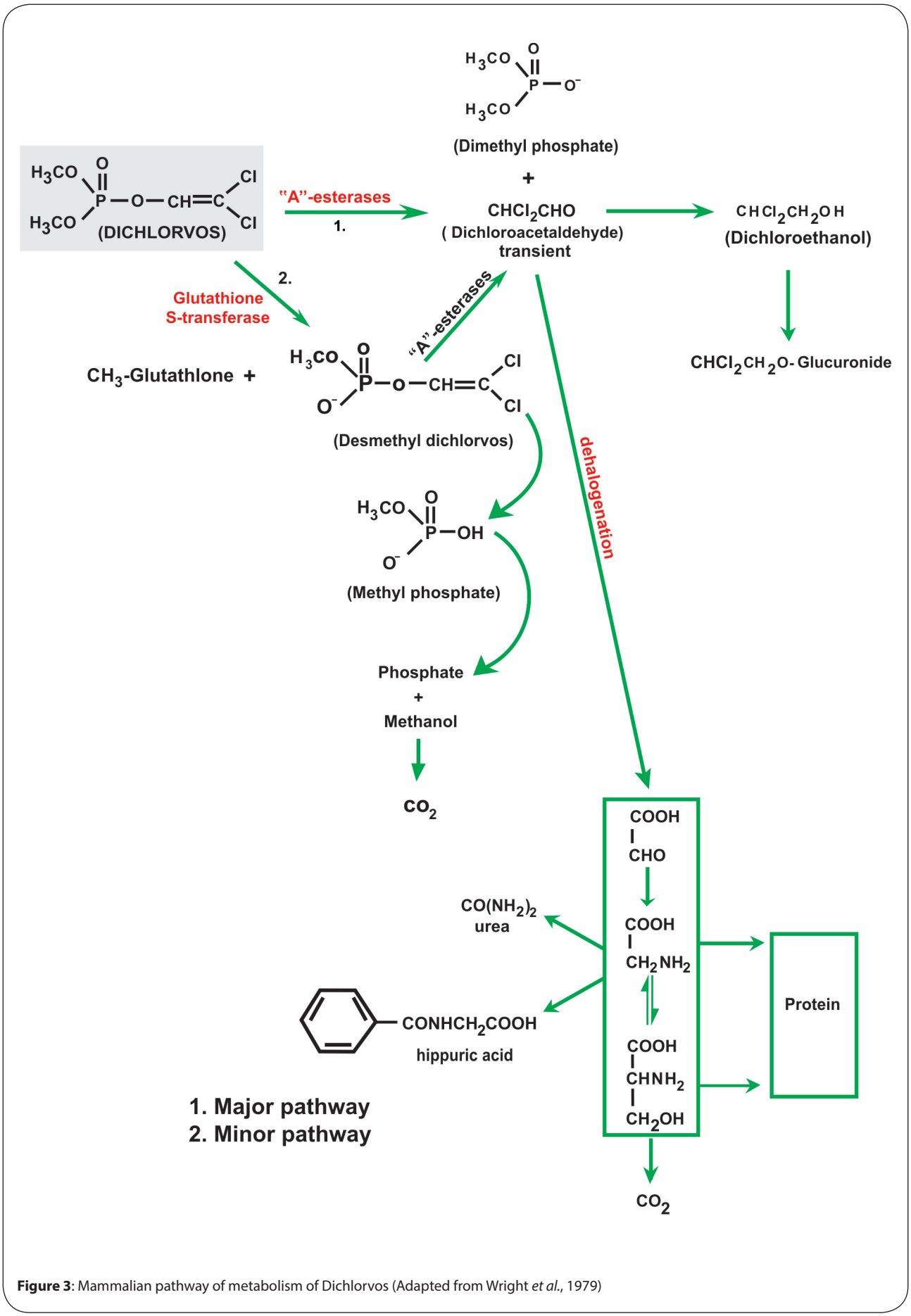

Figure 3: Mammalian pathway of metabolism of Dichlorvos (Adapted from Wright *et al.*, 1979)

Routes of exposure

One of the routes of exposure of dichlorvos is inhalation. People living near harzadous waste sites containing dichlorvos or those using it as domestic pesticide could be potentially exposed to its inhalation. Another possible route of exposure is skin contact with soil contaminated with dichlorvos or body splash. There is also possible oral exposure by ingesting food items contaminated with dichlorvos or direct ingestion (Gallo and Lawryk, 1991; USPHS, 1995).

Health effects

Exposure to dichlorvos could result in acute or chronic toxicity. Because dichlorvos is volatile, inhalation is the most common route of acute toxicity. Acute illness from dichlorvos is usually limited to cholinesterase inhibition (Casida *et al.*, 1962). Repeated or prolonged exposure to dichlorvos may result in the same effect as acute exposure including delayed symptoms. The specific toxicity effects are discussed.

Death

There was no available study on death via inhalation of dichlorvos by humans. However, Hayes (1982) documented the case of a woman who died a day after ingestion of dichlorvos. The second report was that of a 19-month-old girl that died following ingestion of a cake-like bait that contained dichlorvos (Hayes, 1982). Moreover, it was reported that two pesticide workers in Costa Rica died after spilling a dichlorvos containing insecticide on their skin without washing it off properly (Hayes, 1982).

Earlier animal studies reported death within 7 to 62 hours after exposure to air saturated with dichlorvos for rats (Durham *et al.*, 1957). Thorpe and Colleagues (1972) reported death of pregnant rabbits after inhalation of dichlorvos for 28 days at 4 mg/m^3 and 6.25 mg/m^3. Acute toxicity studies reported LD_{50} of 50 mg/kg (Durham *et al.*, 1957), 97.5 mg/kg (Keda *et al.*, 1990), 133 mg/kg (Haley *et al.*, 1975) and 139 mg/kg (Haley *et al.*, 1975) for female Sherman rats, male fisher rats, female mice, and male mice, respectively. Aquatic studies have reported LC_{50} values ranging from 0.2 to 12 mg/L for freshwater and estuarine fish (Suchismita, 2013).

Genotoxic effect

In vitro genotoxicity of dichlorvos has been reported. Dichlorvos has been reported not to be genotoxic in vivo in animal studies (Nazam & Shaikh, 2013; Dean & Thorpe, 1972). However, in an *in vitro* study, Fiore and colleagues (2013) reported disruption of mitotic division, production of mitotic arrest and chromosome aneuploidy/polyploidy in the proliferation of cell population in human cell culture by dichlorvos. The study demonstrated that the major effect of dichlorvos on mitosis spindles with monopolar microtubule arrays that are associated with hypercondensed chromosomes and pyknotic chromatin masses.

Neurological effect

Dichlorvos exerts its toxic effects in humans and animals by inhibiting neural acetylcholinesterase (ATSDR, 1997). Neurological effects have been reported in a number of animal studies following acute oral exposure with little information on humans. Luiz and Colleagues (2002) reported a case of organophosphate-induced delayed neuropathy of two weeks later in a 39-year-old lady who drank large amount of a dichlorvos based insecticide. In an animal study, Aditya *et al.* (2012) reported activation induced apoptotic cell death in primary rat microglia. Activation of the microglia cells resulted in microgliosis manifested by increased damage in the affected regions. The study reported that the microglial cells undergo cell death after 48 hours of dichlorvos treatment. In another study, Binukumar *et al.*, (2010) reported Nigrostriatal neuronal death following chronic exposure of 2.5 mg/kg/daily of dichlorvos in rat. The study submitted that chronic exposure to dichlorvos causes nigrostriatal dopaminergic degeneration accompanied by 60–70% reduction in striatal dopamine and tyrosine hydrolase levels. In an earlier study, using male Fischer 344 rats exposed to dichlorvos via olive oil gavage, an LD_{50} study showed signs of severe cholinergic stimulation including salivation, tremors, lacrimation, fasciculation, irregular respiration and prostration (Ikeda *et al.*, 1990).

Reproductive effect

There is no available literature on the reproductive effect of dichlorvos in humans. However, a study on the effects of dichlorvos on fertility of male mice via intraperitoneal injection reported significant decrease in sperm number and increase in sperm abnormalities (Faris, 2008). In another study, Ezeji and Collegues (2015) reported significant reduction in testosterone levels of adult male rats fed water contaminated with dichlorvos. The study also reported levels of distortions in the cells of the seminiferous levels as well as hypertrophy of the spermatogonia cells (Ezeji *et al.*, 2015).

Developmental effect

There is no literature on developmental effect of dichlorvos following exposure on humans. However, several animal studies on developmental effects of dichlorvos have been reported. Sisman (2010) examined the effect of varying concentrations of dichlorvos on the embryonic development of zebrafish. The study reported developmental abnormalities such as lack of blood flow, cardiac edema, delayed hatching and vertebra malformations in embryos and larvae. Another study by Thorpe and Collegues (Thorpe *et al.*, 1972) investigated developmental effects of dichlorvos via inhalation in fifteen (15) pregnant E rats within their twenty-day gestational period. The study reported stillbirths, resorption sites, skeletal defects, gastroschisis and other external malformations.

Carcinogenic effect

There is no literature on the carcinogenic effects on humans. Several animal studies, including that of Wang and Colleagues (2013), examined the risk assessment of mouse gastric tissue cancer induced by dichlorvos and dimethoate using varying doses on male Kunming mice. The study reported upregulation of p16, BCL-2 and C-myc genes in mouse gastric tissue in the orally administered 40mg/kg/day dose category. Hence the authors submitted that mouse gastric tissues exposed to high doses of dichlorvos in the long term have the potential to become cancerous (Wang *et al.*, 2013). In an earlier study (NTP, 1989) with 50 Fischer 344 rats using oral gavage in corn oil, the authors reported significant neoplasm in the pancreas and the hematopoietic system of the male rats and the mammary gland of the female rats; mononuclear cell leukemia and pancreatic adenoma increased in the male, benign mammary tumors increased in females (NTP, 1989; Ishmael *et al.*, 2006). Moreover, the same study reported significant positive trend of forestomach carcinoma and squamous cell papilloma in female mice.

Immunological effect

There is evidence from occupational exposures that dichlorvos has the potential to cause skin sensitization. Human diagnostic patch tests of occupational flower growers with a history of pesticide dermatitis have shown an allergic contact dermatitis response to dichlorvos (NIOSH, 2017; Ueda *et al.*, 1994; Fuji, 1985). In an animal study, Desi *et al.* (1980) studied the effect of daily oral administration of LD_{50} 1/40, 1/20, 110 dichlorvos in male rabbits (2.0–2.5 kg body weight) after vaccination with *Salmonella typhi*. The study reported a dose-dependent fall in the serum antibody titer of the treated animals in contrast with the control group.

Hepatic effect

Zhao *et al.*, (2015) reported a case of dichlorvos induced autoimmune hepatitis in a 49-year-old Chinese woman following chronic exposure to dichlorvos. The diagnosis was made two and a half years after initial symptoms of exposure. On initial admission, she was presented with alanine transaminase (ALT) 1558 U/L (Normal: 5–40 U/L), aspartate transaminase (AST) 1267 U/L (normal: 10–40 U/L), total bilirubin (TBIL) 133.5 µmol/L (normal: 3–20 µmol/L, alkaline phosphatase (AKP) 182 U/L (normal: 15–130 U/L). In an animal study, Romero-Navarro and Colleagues (2006) reported that acute exposure to dichlorvos (20 mg/kg/body weight) decreased the activity of hepatic glucokinase in rats. More so, histological changes in liver of rats exposed to dichlorvos has been reported (Owoeye, 2012). There was diffuse vascular degeneration of hepatocytes with necrotic hepatocytes as well as moderate peri-portal cellular infiltration by mononuclear cells in rats exposed for one week. Moderate to severe vacuolar degeneration and necrosis of hepatocytes were reported in rats exposed for two weeks while rats treated for three weeks showed loss of hepatocyte outline. Rats treated for four weeks were reported to have portal triad, completely obscured and circumscribed vessels by connective tissue, necrotic plaque, peri-portal cellular infiltrations and diffuse necrosis.

Renal effect

There is no information on dichlorvos effect on the human renal system. However, an animal study by Hou *et al.* (2014) examined the nephrotoxicity effect of dichlorvos in rats for ninety (90) days. The study reported significant increase in activities of catalase, glutathione peroxidase and superoxide dismutase; level of malondialdehyde in kidney tissues; serum level of creatinine and urea nitrogen; level of β2-microglobulin, level of retinol conjugated protein, activities of N-acetyl-β-d-glucosaminidase in urine and significant decrease in uric acid level with renal injury including tubular and glomerular filtration. However, an earlier study (NTP, 1989) reported contrasting results of no renal effect on Fisher 344 rats and B6C3Ft mice.

Respiratory effect

Respiratory irritation following dichlorvos exposure was reported in a study (Mathur *et al.*, 2000) involving children. The study reported strong correlation between acute respiratory symptoms and exposure to dichlorvos. However, the authors could not rule out irritant effects of the solvents used to disperse the dichlorvos. An animal study on the acute toxic effect of inhaled dichlorvos vapor on respiratory mechanism in guinea pigs reported significant decrease in respiratory frequency and significantly increased tidal volume in the 35 mg/mL and 75 mg/mL treated animals (Taylor *et al.*, 2008). A histological study on the lungs of rats exposed to dichlorvos reported an extension in the basal associated lymphoid tissue (BALT) in rats exposed for one, four and five weeks (Owoeye, 2012).

Metabolic/endocrine effects

An animal study (Lucic, 2002) on the effect of dichlorvos treatment on butyrylcholinesterase (BuChE) activity and lipid metabolism of rats reported significant decrease in BuChE activity in both sexes of the rats as well as significant increase in triglycerides (60–600%) and total cholesterol (35–75%). In another animal study, rats administered a single dose of dichlorvos equal to 50% of the LD50, were reported to develop hyperglycemia (Teichert-kuliszewska, 1979). Moreover, cytoplasmic vacuolation of adrenal cortical cells were reported in male Fischer 344 rats following oral administration of 4 or 8 mg/kg/day of dichlorvos for 5 days a week for two years (NTP, 1989).

Dermal and musculoskeletal effect

Vesicle cellulitis and thrombophlebitis of the extremities and bullae appearance have been reported in acute injection of dichlorvos in attempted suicide patients (Cahfer *et al.*, 2004; Sundarka *et al.*, 2000). An earlier report of dermatitis of the neck, anterior chest, dorsal hands and forearms in a 52 year old truck driver who had dermal exposure to dichlorvos was reported by Mathias (1983). In an earlier study (Snow and Watson, 1973), a tenfold increase in serum creatinine phosphokinase, suggestions

of muscle damage (ATSDR, 1997), was reported in greyhound dogs treated with 11 mg/kg dichlorvos capsule. However, contrasting studies (Laudari, *et al.*, 2014; NTP, 1989) reported no gross or histological treatment related damage to skeletal muscles in Fischer 344 rats treated with up to 8 mg/kg/day dichlorvos for 5 days a week for 2 years by oral gavage and B6C3F1 mice treated with up to 40 mg/kg/day dichlorvos for 5 days a week for 2 years.

Other systemic effects

There was no direct cardiovascular effect on dichlorvos exposure of humans except a generalized study on organophosphate exposure that reported prolonged QT and ventricular extrasystole. However, Durham and Colleagues (Durham, 1957) reported paleness of the extremities, suggestive of poor perfusion in Sherman rats within 2 hours of exposure before death. However, a contrasting report of no gross or histological effects on the cardiovascular system of rats and mice exists (NTP, 1989).

Sclera icterus has been reported following chronic dichlorvos exposure (Zhao *et al.*, 215). An earlier animal study (Ubels, 1987) reported maximal pupillary constriction (pin point pupil) with complete recovery within 4 hours following exposure to household aerosol containing 0.2–2% dichlorvos in rabbit and corneal epithelial erosion and corneal swelling in monkey.

Celic and Collegues (2009) reported lack of hematological effect except leukocytosis of sublethal dose of dichlorvos in rats following oral administration.

Diagnostic/biomakers of dichlorvos exposure

Dichlorvos at high doses will elicit classical symptoms of organophosphate toxicity such as miosis, tremor, increased salivation, lacrimation, pulmonary secretions and perspiration (ATSDR, 1997). Dichlorvos exposure can be diagnosed based on its tendency to inhibit cholinesterase activity. Hence, serum cholinesterase appears to be more sensitive to inhibition by dichlorvos and other organophosphate than erythrocyte acetylcholinesterase. However, serum cholinesterase activity recovers more rapidly than erythrocyte acetylcholinesterase because of the high turnover rate of the serum protein compared to erythrocytes (Kazemi *et al.*, 2012; ATSDR, 1997). In conditions of chronic exposure, the patient may demonstrate only reduced erythrocyte acetylcholinesterase activity and normal serum cholinesterase activity, thus giving false negative result. The true reflection of depressed cholinesterase activity is found in erythrocyte activity. Erythrocyte acetylcholinesterase recovers at the rate of 1% per day in untreated patients and takes about 6 to 12 weeks to normalize, whereas serum cholinesterase levels may recover in 4 to 6 weeks. It is pertinent to note that confirmation (aside patient history) of specific exposure to dichlorvos is difficult as the cholinesterase inhibition is similar to other organophosphate pesticides and requires elaborate analytical chemistry. The rapid metabolism of dichlorvos by liver and blood esterases makes it almost impossible to detect intact dichlorvos in humans and rarely in animals. More so, the major metabolites (dimethyl phosphate and glucuronide conjugate of dichloroethanol) are rapidly excreted into urine and will have left the body within a day or two of cessation of exposure (ATSDR, 1997). Dimethyl phosphate has been measured in the urine of pesticide applicators by extraction with an ion exchange resin, derivitization and gas chromatography (ATSDR, 1997; Das *et al.*, 1983). Dichloroethanol has been detected in the urine of a human volunteer after glucuronidase treatment and gas-liquid chromatography (Hutson and Hoadley, 1972).

Conclusion

Dichlorvos has become increasingly popular for domestic, industrial and agricultural use, however, the public health concern of dichlorvos has littered toxicological literature indicating possible toxicological implication in unregulated and unrestricted use of the pesticide. The available literature beckons on all the regulatory agencies to live up to their task. Considering the reports of dichlorvos usage and the corresponding biotic and abiotic toxicity, it has necessitated the need to make and enforce stringent rules in the use of dichlorvos containing pesticides. Unregulated and unrestricted use of dichlorvos is a "time bomb" and a matter of public health concern.

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ORIGINAL ARTICLE

Toxicity assessment of agrochemical Almix in *Heteropneustes fossilis* through histopathological alterations

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ABSTRACT

The present study was designed to assess the adverse effects of the agrochemical Almix on comparative basis in gill, liver and kidney of *Heteropneustes fossilis* through histological and ultrastructural observations under field (8 g/acre) and laboratory (66.67 mg/L) conditions. Exposure duration of both experiments was 30 days. Gill showed atrophy in secondary lamellae, hypertrophied gill epithelium, damage in chloride and pillar cells, and detachment of chloride cells from gill epithelium under laboratory condition, but hypertrophy in gill epithelium and fusion in secondary lamellae were seen under field condition. In gill, scanning electron microscopy (SEM) showed fragmentation in microridges, hyper-secretion of mucus and loss of normal array in microridges, while transmission electron microscopy (TEM) displayed dilated mitochondria and rough endoplasmic reticulum (RER), abnormal sized vacuolation in chloride cells under laboratory condition. In liver, hypertrophied and pyknotic nuclei, disarrangement of hepatic cords, and cytoplasmic vacuolation were prominent under laboratory study but in field condition the liver showed little alterations. TEM study showed severe degeneration in RER and mitochondria and cytoplasmic vacuolation under laboratory condition but dilated mitochondria were prominent in field observation. Kidney showed severe nephropathic effects including degenerative changes in proximal and distal convolute tubule, damage in glomerulus under light microscopy, while deformity in nucleus, fragmentation in RER, severe vacuolation and necrosis in kidney were prominent under TEM study. The results clearly demonstrated that responses were more prominent in laboratory than field study. Thus the responses displayed by different tissues of concerned fish species exposed to Almix could be considered as indications of herbicide toxicity in aquatic ecosystem.

KEY WORDS: Almix; scanning electron microscopy; transmission electron microscopy; *Heteropneustes fossilis*

Introduction

In modern agricultural practices, the introduction of new technology for crop production and protection has several times increased the use of herbicides. Herbicides play an important role in controlling the annual grasses, broad leaved weeds and sedges from various agricultural fields. Indiscriminate uses, careless handling, accidental spillage, or discharge of untreated effluents on herbicidal uses into natural waterways, including fish farms, can cause damage in fish population and other aquatic animals or plants (Sarikaya & Yilmaz, 2003; Fonseca *et al.,* 2008). The application of environmental toxicological studies on non-mammalian vertebrates has been rapidly expanding in recent times, and for aquatic systems, fish have become indicators for the evaluation of the toxic effects of these noxious compounds. In aquatic toxicological studies, laboratory experiments are performed to estimate the potential hazards of these chemicals to establish "safe" levels of these xenobiotics (Anto´n *et al.,* 1994).

Almix® 20 WP is the new fourth generation herbicide. It is widely used to control broad-leaf weeds and sedges both in terrestrial and aquatic systems. Almix is a selective, contact as well as systematic and both pre-emergent and post-emergent herbicide of the sulfonylurea group. It is composed of 10.1% metsulfuron methyl ($C_{14}H_{15}N_5O_6S$) [methyl 2-(4-methoxy-6-methyl-1,3,5-triazin-2-yl-

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carbamoyl-sulfamoyl) benzoate], 10.1% chlorimuron ethyl ($C_{15}H_{15}ClN_4O_6S$) [ethyl 2-(4-chloro-6-methoxy-pyrimidine-2yl-carbamoyl-sulfamoyl) benzoate] and 79.8% adjuvants (DuPont Safety Data Sheet, 2012).

Documentation of Almix herbicide toxicosis to freshwater teleostean catfish, *Heteropneustes fossilis* has been started recently (Samanta *et al.,* 2014a, b; Samanta *et al.,* 2015a, b). Low concentrations of Almix, such as those used in rice fields, might cause changes in metabolic and enzymatic parameters of catfish, *H. fossilis* (Samanta *et al.,* 2014a, b) and other fish species such as *Anabas testudineus* and *Oreochromis niloticus* concerning reduction of protein level and glutathione S-transferase (GST), and enhancement of acetylcholinesterase (AChE), lipid peroxidation (LPO) and catalase (CAT) activities in different tissues of *A. testudineus* and *O. niloticus* (Senapati *et al.,* 2012; Samanta *et al.,* 2014a, b; Samanta *et al.,* 2015a, b). However, only metabolic and physiological activities alone do not satisfy the complete understanding of pathological alterations of the tissues under toxic stress. In order to know the extent of tissue damage it is thus useful to have an insight into the analysis of cellular and subcellular orientations, although the severity of damage depends on toxic potentiality of the particular toxic compound (Tilak *et al.,* 2001; Srivastava *et al.,* 2008). The great advantages of using histopathological biomarkers in environmental monitoring is that it allows an easy examination of specific target organs including gills, liver and kidney, which are responsible for vital physiological functions, such as respiration, accumulation and biotransformation, and excretion of xenobiotics (Gernhöfer *et al.,* 2001; Camargo & Martinez, 2007). A number of studies have been reported by several authors to understand the biochemical, physiological and metabolic alterations caused by exposure to different pesticides and/or herbicides on animals and fishes (Geetha *et al.,* 1999; Sambasiva Rao, 1999; Aruna *et al.,* 2000; Sornaraj *et al.,* 2005). However, studies regarding histology and ultrastructural effects of Almix herbicide on fish tissues and other aquatic invertebrates are relatively scanty (Senapati *et al.,* 2012) and still need to be evaluated when compared with mammals and was carried out only in laboratory study. Nevertheless, field studies using histopathology and ultramicroscopic observations of fish tissues as biomarkers of aquatic contamination by Almix herbicide have not so far been reported. Thus the present study was aimed to investigate the marked changes in the histological and ultrastructural architectures in gills, liver and kidney of *H. fossilis* to Almix intoxication on comparative basis under laboratory and field conditions (*i.e.,* higher *vs* lower).

Materials and methods

Chemicals

Commercial formulation of the Almix herbicide (Almix® 20 WP, DuPont India Pvt. Ltd., Gurgaon, Haryana, India) was used in both the experiments. Delafield's hematoxylin stain, eosin yellow, xylene, DPX, amyl acetate, acetone, glutaraldehyde solution, sodium hydroxide, tricaine methanesulphonate, uranyl acetate (EM grade), ethanol, disodium hydrogen phosphate, dihydrogen sodium phosphate, lead citrate (EM grade), epoxy resin (EM grade), paraformaldehyde (EM grade) and araldite CY212 (EM grade) of analytical grade were purchased from Merck Specialities Private Limited. Osmium tetraoxide was purchased from Spectrochem Pvt. Ltd., Mumbai, India.

Fish

Freshwater teleostean fish, *Heteropneustes fossilis* (Bloch) of both the sexes with an average weight of 37.91±5.43 g and total length of 18.58±0.959 cm were procured from a local fish farm and brought to the laboratory. Fish were acclimatized under congenial conditions for 15 days in aquarium (250 L). Fish were kept in continuously aerated water with a static-renewal system and experiments were conducted under natural photoperiod (12-h light/12-h dark). During the acclimatization, the average value of water parameters were as follows: temperature, 18.61±0.808°C; pH, 7.23±0.082; electrical conductivity, 413.67±0.90 µS/cm; total dissolved solids, 295.11±1.16 mg/L; dissolved oxygen, 6.46±0.215 mg/L; total alkalinity, 260.00±16.90 mg/L as $CaCO_3$; total hardness, 177.33±5.50 mg/L as $CaCO_3$; ammoniacal-nitrogen, 2.31±0.43 mg/; and nitrate-nitrogen, 0.30±0.058 mg/L. After acclimatization, fish were divided into two groups: one group was transferred to field ponds situated at Crop Research Farm premises of the University of Burdwan and the other group was transferred to laboratory aquarium. Fish were fed once a day with commercial fish pellets (32% crude protein, Tokyu) during both acclimation and exposure periods. Therefore, the study was carried out under two different experimental conditions: field and laboratory, both for the duration of 30 days.

Field experiment

Fish were again divided into two groups as follows: control groups (triplicate cages), each cage contained 10 fish species, and Almix-exposure group with 10 fish species in three separate cages (Figure 1). The desired dose (8 g/acre) corresponds to the concentration recommended for rice culture was dissolved in water and applied once (Samanta *et al.,* 2014a; Samanta *et al.,* 2015b). Duration of the exposure period was 30 days. It was sprayed on the first day of the experiment on the surface of each Almix-treated cage. For these field experiments, special type of cage was prepared and installed separately at two different ponds of Burdwan University Crop Research Farm, University of Burdwan. Cages were prepared for the culture of experimental fish species as per Chattopadhyay *et al.* (2012) with some modifications. All the cages were square in shape having an area of 2.5×1.22 m and cage height was 1.83 m (submerged height was 0.83 m). Cages were framed by light strong bamboo. The four-sided wall, cage floor and top of the cage cover were fabricated with nylon net and embraced by two PVC nets: the inner and outer nets bearing mesh sizes of 1.0×1.0 mm^2 and 3.0×3.0 mm^2, respectively. During the experimentation

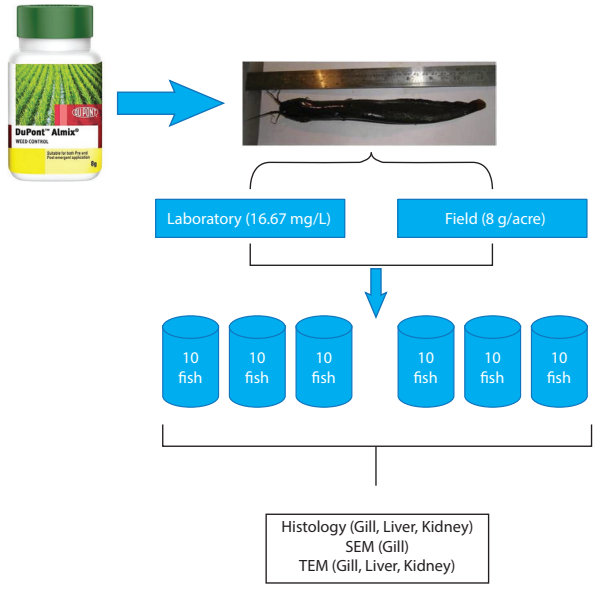

Figure 1. Experimental design of the present study.

period, pond water had the following average values: temperature 15.67±0.145 °C; pH 7.89±0.033; electrical conductivity 390.33±2.19 µS/cm; total dissolved solids 276.33±1.45 mg/L; dissolved oxygen 7.47±0.088 mg/L; total alkalinity 101.33±0.67 mg/L as $CaCO_3$; total hardness 152.00±2.31 mg/L as $CaCO_3$; ammoniacal-nitrogen 6.06±0.875 mg/L; and nitrate-nitrogen 0.58±0.016 mg/L.

Laboratory experiment

Fish were divided again into two groups (control and Almix-treated) and maintained in six aquaria (three for control and three for treatment), containing 10 fishes in each aquarium in the Ecotoxicology Lab, Department of Environmental Science, the University of Burdwan. Fish were exposed to sub-lethal dose of Almix, *i.e.,* 66.67 mg/L (40 L) for a period of 30 days (Samanta *et al.,* 2015a, b). Doses were applied every alternate day. During experimentation, Almix-treated and control were subjected to the same environmental conditions. During experimentation period, the average water parameters were as follows: temperature 19.67±0.293 °C; pH 7.48±0.052; electrical conductivity 478.33±9.70 µS/cm; total dissolved solids 341.44±6.56 mg/L; dissolved oxygen 5.82±0.394 mg/L; total alkalinity 317.30±15.60 mg/L as $CaCO_3$; total hardness 188.89±8.58 mg/L as $CaCO_3$; ammoniacal-nitrogen 6.63±1.15 mg/L, and nitrate-nitrogen 0.46±0.108 mg/L.

Sampling

During the experimentation period, water quality parameters were analyzed as per APHA (2005). After completion of the experiment, *i.e.,* 30 days, fish were collected both from aquarium and pond and were anesthetized with tricaine methanesulphonate (MS 222). After that gill, liver and kidney were taken immediately after dissection and proceeded in specific ways for histological, scanning and transmission electron microscopic study.

Histological analysis

Gill, liver and kidney from control and treatment fish were collected and fixed in aqueous Bouin's fluid solution for overnight. After fixation, tissues were dehydrated through graded series of ethanol and finally embedded in paraffin. Paraffin sections were then cut at 3–4 µ using Leica RM2125 microtome. Finally, sections were stained with hematoxylin-eosin (H&E) solution and pathological lesions were examined under Leica DM2000 light microscope. Additionally, semi-quantative analysis was also carried out by observing the frequency of pathological lesions based on Pal *et al.* (2012) with some modifications.

Ultrastructural analysis

For scanning electron microscopic study, tissues were fixed in 2.5% glutaraldehyde solution prepared in phosphate buffer (0.2 M, pH 7.4) for 24 h at 4 °C and then post-fixed with 1% osmium tetraoxide prepared in phosphate buffer (0.2 M, pH 7.4) for 2 h at 4 °C. After fixation, tissues were dehydrated through graded series of acetone, followed by amyl acetate and subjected to critical point drying with liquid carbon dioxide. Tissues were then mounted on metal stubs and sputter-coated with gold with thickness of approximately 20 nm. Finally, tissues were examined with a scanning electron microscope (Hitachi S-530) at the University Science Instrumentation Centre of the University of Burdwan.

For transmission electron microscopic study, tissues were fixed in Karnovsky fixative (mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer) for 12 h at 4 °C and then post-fixed with 1% osmium tetraoxide in phosphate buffer (0.2 M, pH 7.4) for 2 h at 4 °C. After fixation, tissues were dehydrated through graded acetone, infiltrated and embedded in epoxy resin (araldite CY212). Ultrathin sections (70 nm) were then cut using glass knife on an "Ultracut E Reichart – Jung" and collected on naked copper-meshed grids. After air-drying, grids were stained with uranyl acetate and lead citrate. Finally, tissues were examined under TECHNAI G2 high resolution transmission electron microscope at Electron Microscope Facility, Department of Anatomy, AIIMS, New Delhi.

Ethical statement

The experiment was carried out in accordance with the guidelines of the University of Burdwan and was approved by the Ethical Committee of this University.

Results

Gill

Histologically, gill is composed of primary and secondary gill lamellae. Free edges of the lamellae are extremely thin, covered with stratified epithelium and contain a vast network of capillaries supported by pilaster cells. Primary gill lamella was supported by gill rays which were bony in nature (Figure 2.1).

Semi-quantitative evaluation of frequency of pathological lesions in gill of laboratory and field condition

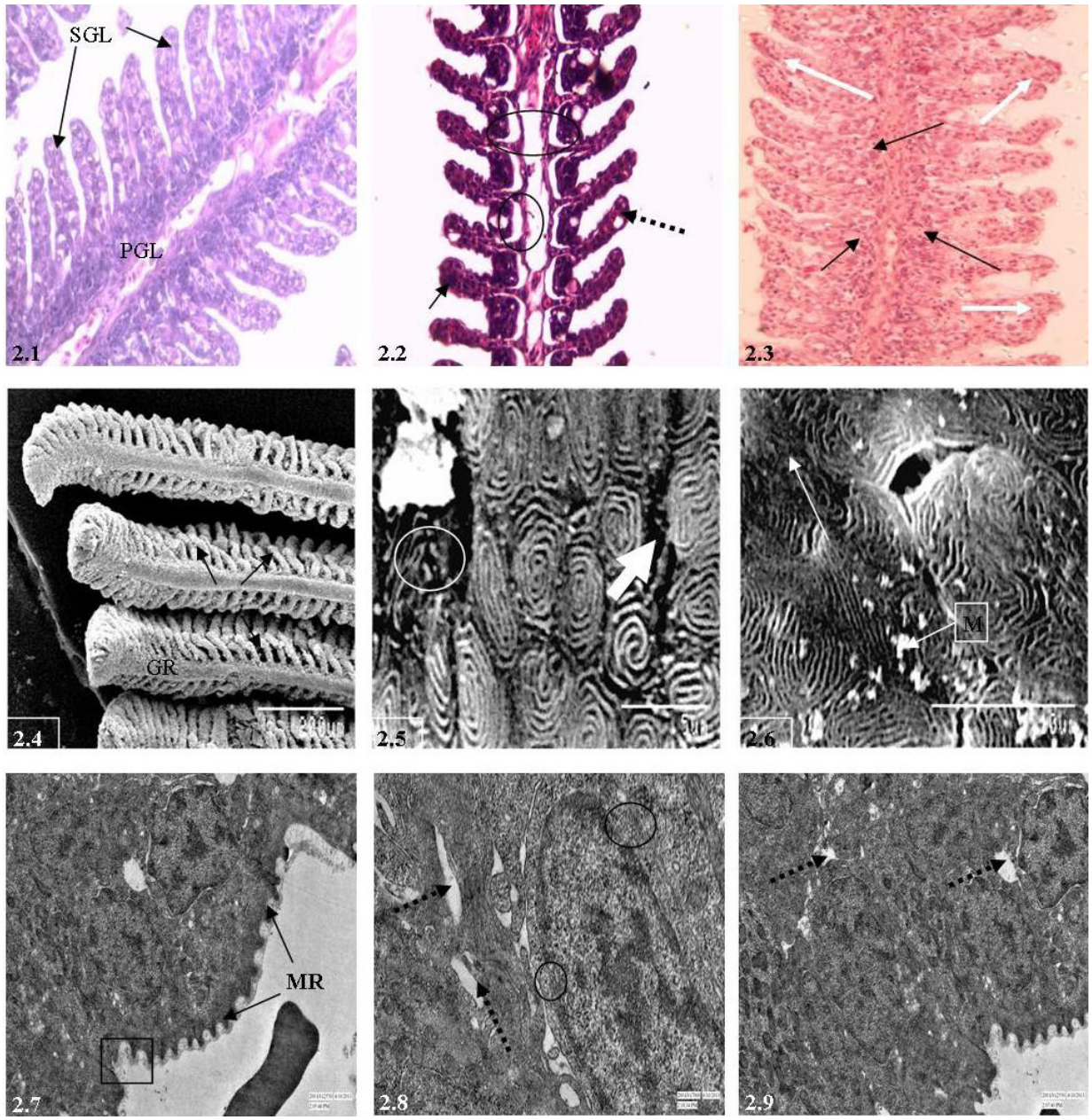

Figure 2. Histopathological photomicrographs of gill of *H. fossilis* under control condition (C), Almix treated laboratory condition (AL), Almix treated field condition (AF). **2.1** Showing normal structure of primary gill lamellae (PGL) and secondary (SGL) lamella under light microscopy (C×400). **2.2** Showing atrophy and hypertrophy of gill epithelium and SGL (arrow), damage in chloride (oval) and pillar cells (broken arrow) under light microscopy (AL×400). **2.3** Showing hyperplasia (arrow) and partial fusion of SGL (white arrow) under light microscopy (AF×1000). **2.4** Scanning electron microscopy **s**howing normal arrangement of gill rackers (GR) with primary gill lamellae (PGL) and stratified epithelial cells (SEC) on the PGL (C×150). **2.5** Gill epithelium **s**howing fragmentation of MR (oval) and loss of MR in SEC (arrow) under scanning electron microscopy (AL×6000). **2.6** Damage in MR (arrow) under SEM (AF×5000). **2.7** Gill epithelial cell under transmission electron microscopy showing normal chloride cell (CC), pavement cells (PC) with prominent mitochondria (M) (C×2550). **2.8** A double-layered nucleus (oval) and vacuolation in the chloride cells (broken arrow) under TEM (AL×7000). **2.9** Showing vacuolation (broken arrow) only under transmission electron microscopy (AF×2550).

fish compared to control fish is given in Table 1. Atrophy in secondary lamellae, hypertrophied gill epithelium and damage in chloride as well as pillar cells, detachment of chloride cells from gill epithelium and stunted growth of gill lamellae are the most common histological lesions observed under laboratory study (Figure 2.2). Contrary to laboratory findings, slight hypertrophy in gill epithelium and fusion in secondary gill lamellae are the only pathological lesions observed under field condition (Figure 2.3).

Under scanning electron microscopic study, gill epithelium showed fragmentation of microridges, swelling of microridges and loss of normal array of microridges (Figure 2.5), but under field condition hyper-secretion of

Table 1. Semi-quantitative assessment of frequency of pathological lesions in gill, liver and kidney of *H. fossilis* under laboratory and field conditions.

Pathological Lesion	Control	Laboratory condition	Field condition
Gill			
Histopathological			
Proliferated gill epithelium	–	++	+
Hypertrophy of the gill epithelium	–	+++	+
Hyperplasia of the gill epithelium	–	+++	+
Scanning electron microscopic			
Damage of microridge structures	–	+	–
Disappearance of normal array of microridges	–	+	–
Mucus secretion	–	++	–
Distortion of stratified epithelial cells	–	++	+
Swelling of stratified epithelial cells	–	+	+
Necrosis	–	++	+
Transmission electron microscopic			
Chloride cell damage	–	+++	–
Dilated mitochondria	–	+	++
Mitochondrial degeneration	–	+	–
Cytoplasmic vacuolation	–	+	–
Nuclear distortion	–	++	+
Liver			
Histopathological			
Disoriented hepatic cord	–	++	++
Hypertrophy of hepatocytes	+	+++	++
Degeneration of hepatocytes	–	++	+
Nuclear hypertrophy	+	+++	++
Cytoplasmic vacuolation	–	+++	++
Pyknotic nucleus	–	+	–
Detachment of hepatopancreatic acinar cells from hepatocytes	–	+++	++
Deformed hepatopancreas	–	++	–
Loss of zymogen granules	–	++	–
Transmission electron microscopic			
Cytoplasmic vacuolation	–	+++	+
Loss of rough endoplasmic reticulum	–	+	–
Loss of glycogen granules	–	++	+++
Dilated mitochondria	–	+	++
Kidney			
Histopathological			
Shrinkage of glomerulus	–	++	+
Lipid vacuoles in epithelial cells	–	++	+
Swelling in tubular epithelium	–	++	–
Hypertrophy in tubular epithelium	+	++	+
Fragmentation of glomerulus	–	+	+
Tubular degeneration	–	++	++
Loss of hematopoietic tissue	–	+	+
Transmission electron microscopic			
Vacuolation in epithelial cytoplasm	–	+++	+
Damage in proximal convoluted tubules	–	++	–
Dilated mitochondria	–	+	++

mucus, damage of microridges in few places were noticed after Almix exposure (Figure 2.6). Transmission electron microscopy analyses showed dilated mitochondria and endoplasmic reticulum, abnormal sized vacuolation in gill epithelium of *H. fossilis* under laboratory condition (Figure 2.8); however, in field condition gill epithelium showed almost normal appearance of pavement cells, chloride cells, mitochondria, apical pore except vacuolation in some places (Figure 2.9).

Liver

Semi-quantitative evaluation of frequency of pathological lesions in gill of laboratory and field condition fish compared to control fish is given in Table 1. The most expressive changes after Almix exposure in hepatocytes of the concerned fish species seen under light microscopy were distortion in hepatocytes with clumping of nuclei, hypertrophied and pyknotic nuclei, disarrangement of hepatic cords and cytoplasmic vacuolation under laboratory condition (Figure 3.2), while under field condition it showed only distended appearance of hepatocytes and short central vein and fat deposition in sinusoidal spaces in some places (Figure 3.3).

Ultrastructural alterations as viewed under TEM study showed severe degeneration in rough endoplasmic reticulum and mitochondria, vacuolation in cytoplasm and reduced amount of glycogen droplets in hepatocytes (Figure 3.5) under laboratory condition as compared to control (Figure 3.4), but in field condition, hepatocytes showed no significant changes in nucleus and rough endoplasmic reticulum but only dilated mitochondria in some places (Figure 3.6).

Kidney

Histologically, the kidney is made up of a large number of nephrons, each consisting of a renal corpuscle or the Malpighian body and renal tubules. Renal tubules consist of columnar epithelial cells and renal tubules which are spherical or oval in shape. Renal tubules are differentiated into proximal convoluted tubule (PCT), distal convoluted tubule (DCT) and collecting ducts (Figure 4.1).

Semi-quantitative evaluation of frequency of pathological lesions in gill of laboratory and field condition fish compared to control fish is given in Table 1. Nephropathic effects due to Almix toxicosis under laboratory condition included degenerative changes in PCT and DCT, distorted glomerulus in certain Bowman's capsules (Figure 4.2). However, under field condition no such significant alterations in PCT and DCT of *H. fossilis* were observed but aggregation and fatty deposition in hematopoietic tissues were prominent (Figure 4.3).

After 30 days of Almix exposure in laboratory condition, transmission electron microscopic study showed severe degenerative changes in mitochondria, deformity in nucleus, dilation, fragmentation and vesiculation in rough endoplasmic reticulum, severe vacuolation in cytoplasm and necrosis (Figure 4.5), while dilation in mitochondria, abundance of numerous mitochondria, lower amount of vacuolation were observed under field

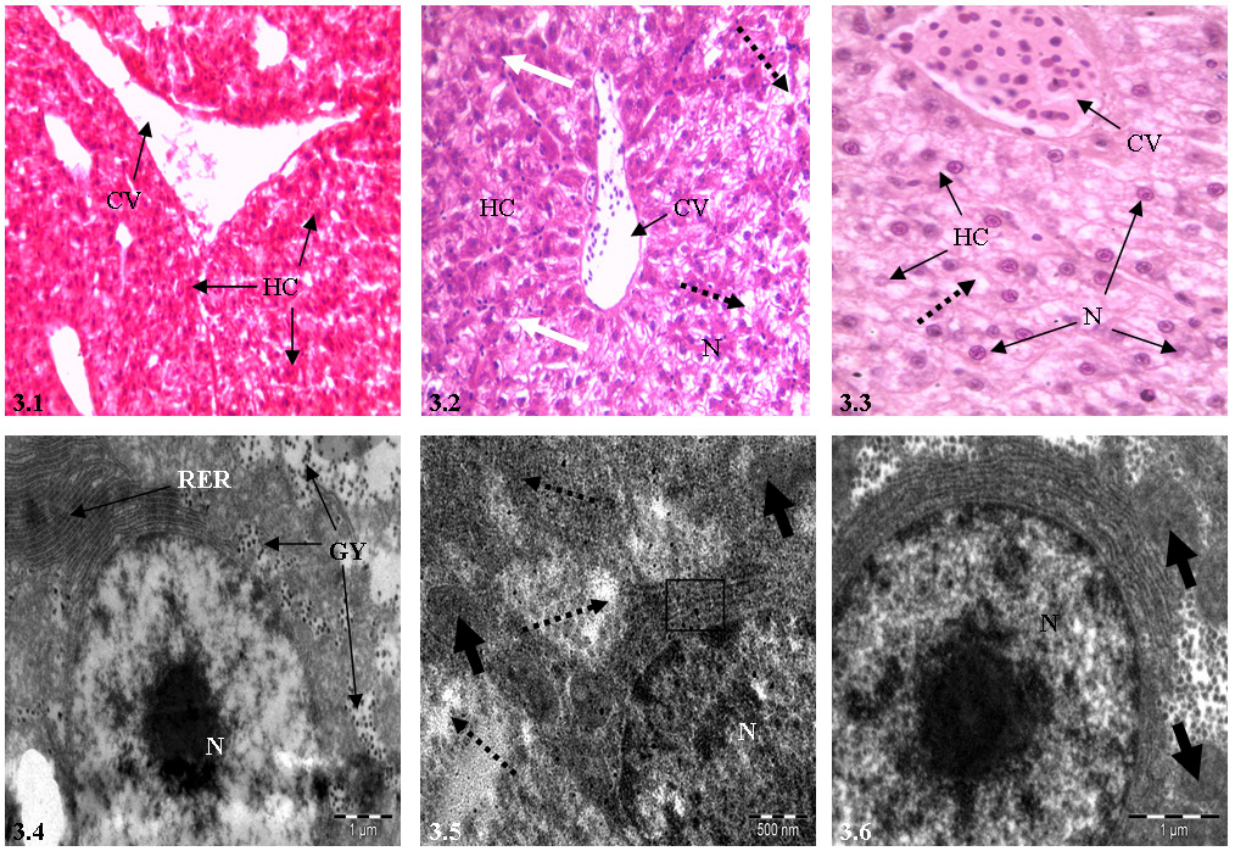

Figure 3. Histopathological photomicrographs of liver of *H. fossilis* under control condition (C), Almix treated laboratory condition (AL), Almix treated field condition (AF). **3.1** Showing normal appearance of hepatocytes (HC), compact arrangement around central vein (CV) with distinct nucleus (N) under light microscopy (C×400). **3.2** Showing hypertrophied and pyknotic nuclei (white arrow), vacuolation in hepatocytes (broken arrow) under light microscopy (AL×400). **3.3** Light microscopy showing vacuolation in HC (broken arrow) (AF×1000). **3.4** Normal appearance of hepatocytes with large number of mitochondria (M), rough endoplasmic reticulum (RER) and glycogen droplets (GY) under transmission electron microscopy (C×4000). **3.5** Hepatocytes with degenerated RER (square) and mitochondria (bold arrow) and vacuolation in cytoplasm (broken arrow) under transmission electron microscopy (AL×8000). **3.6** Under transmission electron microscopy hepatocytes showing almost normal nucleus (N) and vast amount of glycogen droplets (GY) with dilated mitochondria (bold arrow) (AF×6300).

study but damage was comparatively less compared with laboratory condition (Figure 4.6).

Discussion

The present study is a maiden attempt to report Almix toxicosis with regard to histological and ultrastructural observations in *H. fossilis*, although Senapati *et al.* (2012, 2013) reported histopathological alterations in stomach and intestine of *A. testudineus* exposed to Almix herbicide under laboratory condition.

Fish are considered a sentinel organism for ecotoxicological studies and play a significant role in evaluating the risk in aquatic ecosystem (Lakra & Nagpure, 2009). Simultaneously, cellular biomarkers including histological and ultrastructural study in tissues of pollutant-induced organism represent an intermediate level of biological organization between lower-level biochemical effects and higher-level population effects (Adams *et al.*, 2001). This will ultimately provide a better evaluation of the organism's health than a single biochemical response (Triebskorn *et al.*, 1997). They are now widely used as efficient biomarker of water quality, cellular state and mode of action of the xenobiotic contaminants under microscopic study as well as reflecting the overall health of the entire population in the ecosystem (Schwaiger *et al.*, 1997; Kammenga *et al.*, 2000).

The results of the present study showed that Almix intoxication caused serious pathological alterations in gill, liver and kidney of *H. fossilis* under laboratory study and less in field condition. Gills are considered the most vulnerable organ (Dutta *et al.*, 1996) because they are under direct contact with the surrounding contaminant medium and consequently are the first door of entrance for these contaminants (Machado & Fanta, 2003). Detailed description of each pathological lesion through light and electron microscopic observation in gills of *H. fossilis* helps to evaluate the degree of damage and potential consequences.

Hypertrophy and detachment of chloride cells from gill epithelium are the most profound alterations due

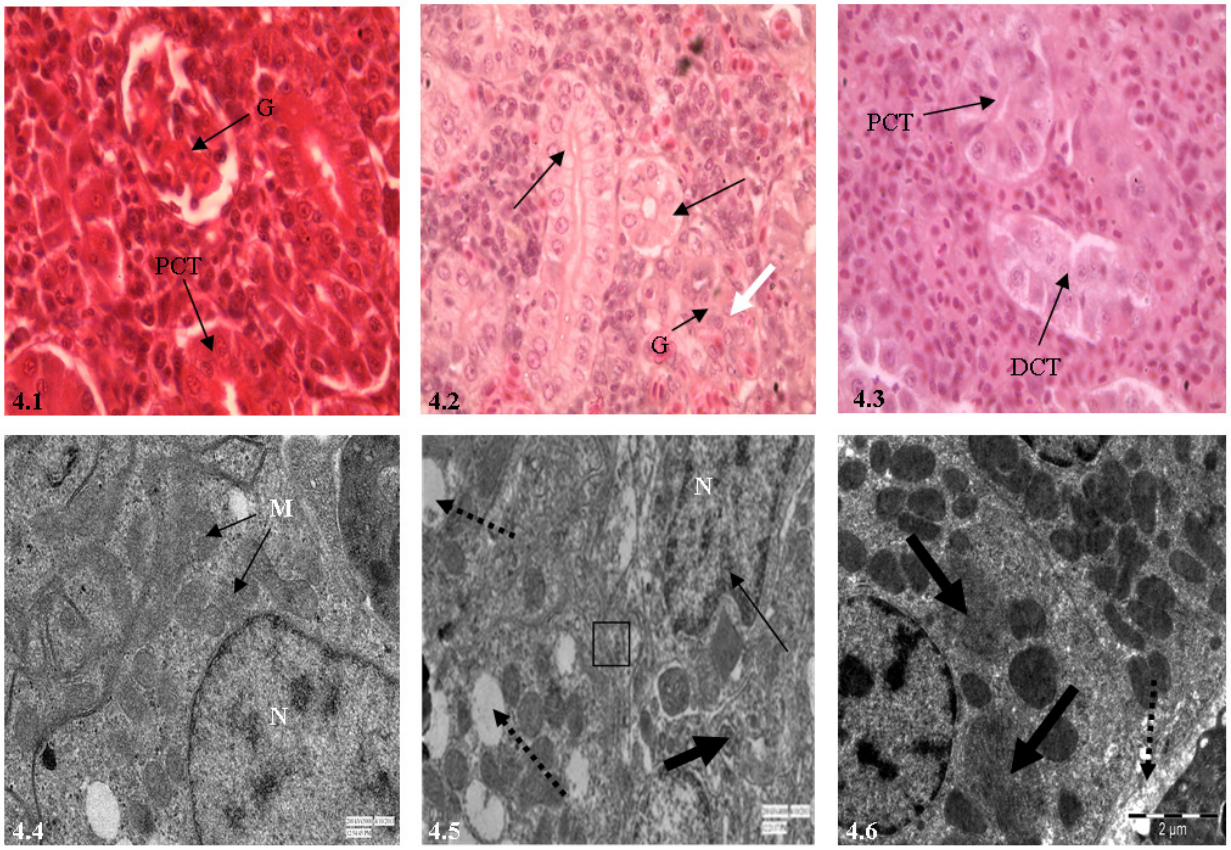

Figure 4. Histopathological photomicrographs of kidney of *H. fossilis* under control condition (C), Almix treated laboratory condition (AL), Almix treated field condition (AF). **4.1** Normal proximal convoluted tubule (PCT), distal convoluted tubule (DCT), Bowman's capsule and glomerulus (G) under light microscopy (C×1000). **4.2** Degeneration of PCT and DCT (arrow) and fragmentation of glomerulus (white arrow) under light microscopy (AL×1000). **4.3** Light microscopy showing normal structure of PCT and DCT (AF×1000). **4.4** Normal appearance of kidney with electron dense mitochondria (M), nucleus (N) and rough endoplasmic reticulum (RER) with abundant vesicular structures (V) under transmission electron microscopy (C×5000). **4.5** Degenerative mitochondria (bold arrow), deformed nucleus (arrow), dilated, fragmented and vesiculated of RER (square) and severe vacuolation (broken arrow) under transmission electron microscopy (AL×2550). **4.6** Showing dilated mitochondria (bold arrow) and lower amount of vacuolation (broken arrow) under transmission electron microscopy (AF×3200).

to Almix exposure. Similarly, Mallatt (1985) reported detachment of the gill epithelium as serious alteration. Fusion of secondary lamellae as observed in gills of *H. fossilis* indicated reduction of total respiratory area, which ultimately reduces oxygen uptake capacity (Karan *et al.,* 1998). Similar finding was also reported in *Lepomis macrochirus* after malathion exposure by Richmonds & Dutta (1989). Damage in chloride cells was also prominent under laboratory conditions. Similar results were also reported by van der Heuvel *et al.* (2000) along with loss of structural integrity of secondary lamellae and accumulation of blood cells. Stunted growth of gill lamellae is another most conspicuous change observed under the present study after Almix exposure. Scanning electron microscopic study showed fragmentation of microridges, swelling of microridges and loss of normal microridge array under laboratory condition. Light microscopic evidence as observed under the present study could be correlated with strands of secreted mucus cells over the gill surface through SEM study. Similar results of hyperplasia, loss of microridge and excess mucus secretion was also reported by Pfeiffe *et al.* (1997) in gill of juvenile goldfish, *Carassius auratus,* induced by 1-naphthyl-N-methylcarbamate (carbaryl). Excess mucus secretion as observed under field study indicated compensatory mechanism as well as defensive mechanism by the fish species against herbicidal exposure. In the present study, transmission electron micrograph showed double layered nucleus, dilated mitochondria and endoplasmic reticulum, and abnormal vacuolation in the chloride cells of gill epithelium after Almix exposure. Similar observation of chloride cell damage was also reported by Schwaiger *et al.* (2004) and indicated that these alterations might interfere with normal respiratory functions and general fish health status. Additionally, chloride cell damage might increase blood flow inside the lamellae, dilatation of marginal channel, and blood congestion or even aneurism (Rostey-Rodriguez *et al.,* 2002; Camargo & Martinez, 2007). Vacuolations in gill epithelium might impede gas exchange capacity as well as indication of swelling of mitochondria and rough endoplasmic reticulum (Ultsch *et al.,* 1980; Pawert *et al.,*

1998). Mitochondrial damage observed under the present study was also reported by Perry & Laurent (1989) and Goss *et al.* (1995) in their study after exposure to different contaminants. Although significant ultrastructural differences were observed in gill epithelium both under laboratory and field study, pathological responses were more pronounced in laboratory conditions than in field study. These alterations in gill morphology could lead to functional anomalies as well as interfere with the fundamental process such as maintenance of osmoregulation and antioxidant defence mechanism of gill epithelium (Pandey *et al.,* 2008).

Hypertrophied and pyknotic nuclei in hepatocytes of *H. fossilis* are the most pronounced lesions observed in the present study. Similar observations along with nuclear hypertrophy and cellular atrophy were reported in liver of *Cyprinus carpio* after chlorpyrifos exposure by Pal *et al.* (2012). Additionally, vacuolation in cytoplasm, infiltration of leukocytes and pyknotic nuclei were also reported by Jiraungkoorskul *et al.* (2002) in liver of *Oreochromis niloticus* after Roundup exposure. In the present study, vacuolization in hepatocytes indicated an imbalance between the rate of synthesis of substances in parenchymal cells and the rate of their release into systemic circulation. Additionally, enhanced glycolytic activity as compensatory response imposed by enhanced metabolic activity or reduction of carbohydrate absorption by intestinal part were reported (Hanke *et al.,* 1983; Gluth & Hanke, 1985; Braunbeck & Appelbaum, 1999). Disarrangement of hepatic cord is another most important hepatic lesion observed under the present study. Cytoplasmic vacuolation in hepatocytes observed under TEM study was also reported by Li *et al.* (2001). Damage in rough ER is the common response to herbicide exposure. Braunbeck & Völkl (1993) and Au *et al.* (1999) correlated damage in rough ER with higher biotransformation capacity of hepatocytes, while Ghadially (1988) demonstrated dilation of ER cisternae as enhanced storage of proteins due to reduced secretory activity. Similar findings were reported in rainbow trout after exposure to endosulfan and disulfoton (Arnold *et al.,* 1995), and in demersal fish following intraperitoneal injection of benzo(a)pyrene (Au *et al.,* 1999). Mitochondrial degeneration observed under the present study indicated impaired hepatocyte oxidative capability due to inhibition of respiratory chain enzymes function through oxidation of ATP molecule during phospholipid metabolism and fatty acid synthesis. Marked ultrastructural changes including swollen mitochondria have already been reported in liver of catfish exposed to methyl parathion by Tripathi & Shukla (1990). Reduced glycogen content is another most important cytological change associated with herbicide exposure. Cytopathological responses observed under the present investigation were more pronounced in laboratory condition compared with field study as fish are in natural condition and quickly adapt under herbicide-induced aquatic environment.

In kidney, light microscopic observation showed degenerative changes in PCT and DCT, and damage in glomerulus. The results of the present study were also in agreement with the findings of Fischer-Scherl *et al.* (1991) and Nesković *et al.* (1993). Jiraungkoorskul *et al.* (2002) in their study also reported damage in PCT, dilation of Bowman's capsule along with accumulation of hyaline droplets in epithelial cells of renal tubule of *Oreochromis niloticus* after Roundup exposure. Additionally, alterations observed under the present investigation could be correlated with disruption of several biochemical and physiological pathways including endocrine disruption (Mekkawy *et al.,* 2011; Sayed *et al.,* 2012). Degenerative changes in mitochondria and deformed nucleus observed under the present study indicated impaired metabolic activity, in particular enzyme activity. Cytoplasmic vacuolation observed under both conditions have also been reported in gold fish kidney after hexachlorobutadiene exposure by Reimschüssel *et al.* (1989). Similarly, Fischer-Scherl *et al.* (1991) reported degeneration and vacuolation in epithelial cells of kidney after lethal and sub-lethal atrazine exposure. Additionally, Fischer-Scherl *et al.* (1991) also reported dilation, fragmentation and vesiculation of RER in kidney of rainbow trout. Moreover, Bucher & Hofer (1993) reported accumulation of hyaline droplets in kidney. Abundance of large number of mitochondria, and lower amount of vacuolation observed under field condition indicated that fish are under stress. Additionally, protection against the stress-imposed conditions was observed; however, severity of damage is more pronounced under laboratory conditions than field study due to dilution capability of the natural environment.

Conclusion

In conclusion, cytopathological responses observed due to Almix intoxication indicated that laboratory study displayed higher impacts than did field study. Therefore, marked histological and ultrastructural alterations observed in gill, liver and kidney of *H. fossilis* could be considered as biomarkers of herbicidal toxicosis and might be helpful to characterize the health status of the entire aquatic ecosystem.

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ORIGINAL ARTICLE

Blood biomarkers of common toad *Rhinella arenarum* following chlorpyrifos dermal exposure

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ABSTRACT

Chlorpyrifos (CPF) is a broad spectrum pesticide commonly used for insect control, has great affinity for lipids and is thus a potential for bioaccumulation in aquatic organisms. The aim of this study was to evaluate the toxicity of CPF using the common toad *Rhinella arenarum* via dermal uptake in plastic bucket to simulate their natural exposition in ponds. *R. arenarum* toads were exposed individually to solutions containing a nominal concentration of a commercial formulation of CPF insecticide (5 and 10 mg/L). Different enzyme biomarkers (BChE: butyrylcholinesterase, CbE: carboxylesterase, and CAT: catalase) were measured in blood tissue after exposition. The capacity of pyridine-2-aldoxime methochloride (2-PAM) to reverse OP-inhibited plasma BChE and the ratio of heterophils and lymphocytes (H/L) as hematological indicators of stress were also determined. The normal values of plasma B-sterases (BChE and CbE) were highly inhibited (until ≈ 70%) in toads 48 h after exposure to CPF. The results indicate that 2-PAM produced BChE reactivation as well. The activity of CAT was also inducted for dermal exposure at more than double of that in the control toads (CPF; 5 mg/L). H/L ratios did not reveal a significantly increased stress. The study suggests that CPF via dermal uptake induced neurotoxicity and oxidative stress in the common toad *R. areanum*. Thus, some blood biomarkers employed in our study (i.e. BChE, CbE, 2-PAM, and CAT) might be used as predictors in health and ecological risk assessment of amphibian populations exposed to CPF.

KEY WORDS: *Rhinella arenarum*; blood; chlorpyrifos dermal exposure; B-esterases; catalase

Introduction

Amphibians may be particularly susceptible to anthropogenic chemicals for a multiplicity of reasons. Fundamentally, their complex life cycles (aquatic and terrestrial life stages) expose them to potential chemical contamination in both habitats (Brühl *et al.,* 2013; Lajmanovich *et al.,* 2015; Van Meter *et al.,* 2015). Indeed, terrestrial anurans breathe and absorb water mainly through their hyper-vascularized skin in the ventral pelvic region in adults, and this reason explains the susceptibility to pesticide uptake from contaminated sediments, water, and soil (Sparling *et al.,* 2001). Dermal exposure presents a potentially significant but insufficiently studied route for pesticide uptake in amphibians (Van Meter *et al.,* 2014).

Chlorpyrifos (CPF) is a broad-spectrum organophosphate pesticide widely used around the world in agriculture and domestic use against harmful insects (Lee *et al.,* 2004). Large-scale manufacture and handling of CPF have led to contamination of soil, air, surface and groundwater in many countries (Eaton *et al.,* 2008). Thus different concentrations of CPF have been detected in ground and surface waters worldwide (Turner, 2003). The major route of CPF to aquatic ecosystems is through rainfall runoff and air-drift (Xing *et al.,* 2012). Nowadays, large amounts of CPF are used in Argentine for the agriculture of crops, i.e. an estimate of 6.8 million kilograms per year based on import records, especially on soybean crops, fruits, grains and vegetables for local consumption (SENASA, 2011). Likewise, Marino and Ronco (2005) detected CPF residues in Argentine surface waters at concentrations ranging among 0.2 to 10.8 µg/L. However, in other regions the peak concentration (>10 mg/L) occurred in a drainage ditch; stream residues had declined to undetectable levels within 44 days (NRA 2000). In some cases, the CPF commercial formulations were found to be more toxic than

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the active ingredient, particularly to aquatic organisms (Ali *et al.,* 2009).

The integrated use of a measurable indicator such as cholinesterases (ChEs) may be necessary for biomonitoring programs to assess the risk of pesticide exposure in amphibians living in agroecosystems (Lajmanovich *et al.,* 2008; Mann *et al.,* 2009; Attademo *et al.,* 2011).The use of several biomarkers (i.e. pollutant-induced biological responses at sub-individual level measured by nonlethal methodologies) is one of the first ecotoxicity phases in the risk characterization of pollutants. Therefore, the measurement of blood butyrylcholinesterase (BChE) activity, such as carboxylesterase (CbE) (for neurotoxicity), catalase (CAT) (for oxidative stress) and the ratio of heterophils and lymphocytes (H/L) (for immune response to stress) are used as biomarkers to monitor CPF exposures in amphibian species (Attademo *et al.,* 2015; Lajmanovich el at. 2015). The case of monitoring anti-ChE pesticide exposure in the field using ChEs activity levels has some limitations. Detection of OP-exposed individuals is highly dependent on a well-represented reference group or an intense sampling effort to increase the likelihood of capturing exposed individuals (Rodríguez-Castellanos & Sánchez-Hernández, 2007). As Wilson & Ginsburg (1955) discovered, mono-pyridinium oximes were effective reactivators of OP-inhibited ChE. Several mono-pyridinium and bis-pyridinium oximes have been synthesized and tested (Jun *et al.,* 2008). Since then, the use of oximes such as pyridine-2-aldoxime methochloride (2-PAM) can partially solve the problem of identifying OP-exposed individuals in wild fauna (Sánchez-Hernández, 2007). One option to solve this problem was the use of oximes (Wilson & Ginsburg, 1955). Oximes such as pyridine-2-aldoxime methochloride (2-PAM) revert the OP-inhibited ChE (Jun *et al.,* 2008) and can partially solve the problem of identifying OP-exposed individuals in wild fauna (Sánchez-Hernández, 2007).

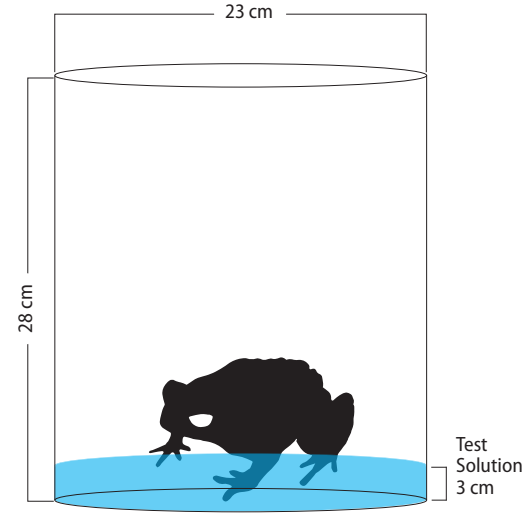

Figure 1. Design of the experimental semi-transparent plastic water bucket used for *Rhinella arenarum* toad dermal absorption.

Several studies have documented an apparent connection between the presence of CPF residues and reductions in amphibian populations, at both local (Fellers *et al.,* 2004) and landscape scales (Davidson *et al.,* 2001). Considering the world average application rate of CPF formulations and that the main part of ecotoxicological research for amphibians was done on aquatic life stages, it is important to emphasize the need of ecotoxicological risk assessment on adult terrestrial non-target amphibians. Following these assumptions and exposing *Rinella arenarum* toads to CPF formulation, we can state that the aims of this study were: (i) to determine the anticholinesterase responses (BChE and CbE activities as well as the ability of 2-PAM to reverse OP-inhibited plasma BChE), and (ii) to determine the oxidative stress response (CAT activities) and immune response (H/L ratio).

Materials and methods

Animals studied

Sixteen adult male of *R. arenarum* were collected by hand from temporary ponds (31°39'52.90"S - 60°42'50.20"W, South Park Lake, Santa Fe, Santa Fe province, Argentina). These sites had not been treated with chemical pesticides as determined by the laws to protect human and wildlife health. *R. arenarum* is used as a good model in ecotoxicology for several blood parameters (Cabagna *et al.,* 2005; Lajmanovich *et al.,* 2008). This toad is frequently found in forests, wetlands, agricultural land and urban territories (Peltzer *et al.,* 2006; Bionda *et al.,* 2015) and it has an extensive Neotropical distribution (IUCN, 2015). It feeds mainly on a variety of arthropods and thus it plays an important role as biological control, particularly in soybean crops (Attademo *et al.,* 2005).

After capture, the toads were quickly transported to the laboratory in darkened buckets containing water to minimize stress. Snout-vent length (SVL) (mm) and body weight (g) were recorded with digital calliper (precision 0.01 mm). The toads were acclimated for 24 h before experiment initiation (individually in semi-transparent plastic water buckets; size: Ø 23 cm × 28 cm) under laboratory conditions with a photoperiod 12-12 h (light 07:00–19:00 h), humidity (65±10%) and temperature 24±2 °C.

Experimental design

The commercial formulation of CPF (48 % active ingredient [a.i.], O,O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate, log K_{OW}=4.7), NUFARM®, Nufarm S.A. (Argentina), was used in this experimental study. The pesticide was tested in this form to mimic the way in which it is applied to cultivated fields and introduced into the environment (Sparling *et al.,* 2010). Likewise, Brühl *et al.* (2013) exposed terrestrial amphibians to pesticide formulations that contain additives.

The animals were randomly distributed into three experimental groups. A control group (CO, n=4) with 500 mL of dechlorinated tap water (DTW; pH 7.4±0.05;

conductivity 165±12.5 µmhos/cm; dissolved oxygen concentration 6.5±1.5 mg/L; and hardness 50.6 mg/L $CaCO_3$), and two groups treated with CPF pesticide formulations containing nominal concentration of 5 and 10 mg/L (n=6). Doses were chosen in ranges usually lacking overt toxicity, mimicking a scenario with no altering clinical signals that may erroneously lead to the assumption of absence of danger (Muller *et al.,* 2014; Lajmanovich *et al.,* 2015). Toads were randomly placed individually into a sterile bucket with 500 mL test solutions (DTW or pesticides) equivalent to 3 cm deep for 48 h, and under the same laboratory conditions as described above (Figure 1).

Blood samples (500 µl approximately) were collected by a minimal cardiac puncture using a heparinized syringe after 48 h of dermal exposure. Toads were maintained in the laboratory of Ecotoxicology in the Faculty of Biochemistry and Biological Sciences – National University of Litoral during a certain period of time for recovery and after a general revision of body condition, they were released to the same sites where they had been captured. To do this, we had the approval of the animal ethic committee of the same institution and followed the guidelines of ASIH *et al.,* (2004).

B-esterases

Plasma BChE activity was determined colorimetrically following the method proposed by Ellman *et al.* (1961). The reaction medium included 930 µl of 25 mMTris-HCl, 1 mM $CaCl_2$ (pH=7.6), 50 µl of 5,5′-dithiobis-2-nitrobenzoic acid (3×10^{-4} M, final concentration), 10 µl of butyrylthiocholine iodide (2×10^{-3} M, final concentration) and 10 µl of plasma. The optical density variation was recorded at 410 nm for 1 min at 25 °C using a Jenway 6405 UV-VIS spectrophotometer. Kinetic experiments were carried out in duplicate. Plasma BChE activity was expressed as µmol of substrate hydrolyzed $min^{-1}ml^{-1}$ of plasma using a molar extinction coefficient of $13.6\times10^{-3}\ M^{-1}cm^{-1}$. We did not determine plasma acetylcholinesterase (AChE) activity because BChE is the enzyme that primarily contributes to total plasma cholinesterase activity in many vertebrate species (Sánchez-Hernández & Moreno-Sánchez, 2002).

Plasma CbE activity was measured by the Gomori method (1953) as adapted by Bunyan *et al.,* (1968). The assay was carried out with 25 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$ (pH=7.6) and 10 µL of plasma at 25 °C. The reaction was initiated by adding 50 µL of *α*-naphthyl acetate (1.04 mg/mL in acetone – α-NA) as substrate, and stopped after 10 min by addition of 500 µL of 2.5% SDS and subsequently 500 µL of 0.1% Fast Red ITR in 2.5% Triton X-100 in water (freshly prepared). Samples were left in darkness for 30 min and the complex absorbance was read at 530 nm. Hydrolysis of α-NA was expressed as nmol of substrate hydrolyzed $min^{-1}ml^{-1}$ of plasma using a molar extinction coefficient of $33.225\times10^3\ M^{-1}cm^{-1}$.

Chemical reactivation of BChE

Two aliquots of each plasma sample were used for assaying reactivation of BChE activity in the presence of 2-PAM (dilution factor 1/5). An aliquot was spiked with 1.7×10^{-3} mol/L 2-PAM and the other was diluted with an equal volume of distilled H_2O (control). After incubation for 30 min at 25 °C, BChE activity of both aliquots was measured. The activity of BChE of control samples was compared to BChE activity of CPFs treatments. Inhibition of BChE activity by CPFs formulations was assumed when the increase of esterase activity was higher than 5% compared with the corresponding controls (Laguerre *et al.,* 2009).

CAT activity

Plasma CAT activity was measured using the method described by Aebi (1984), and was expressed as H_2O_2 µmol $min^{-1}mg^{-1}$ plasma using a molar extinction coefficient of H_2O_2 $40\times10^{-3}\ M^{-1}cm^{-1}$. The reaction medium was composed of 50 mM phosphate buffer (pH=7.2) and 30 mM H_2O_2, and the absorbance was read on the spectrophotometer at a wavelength of 240 nm at 25 °C (quartz cuvette).

Hematological indicators of stress

Two blood smears for each toad were prepared on clean slides, fixed, and stained by the May-Grunwald-Giemsa method (Dacie & Lewis 1984). To determine the counts of heterophil and lymphocyte, 1000 cells per film were examined by light microscopy. All blood counts, including granulocytes (heterophil, basophil and eosinophil) and non-granulocytes (lymphocyte and monocyte) were examined by the same analyst. The results are presented as the percentage of each cell occurring in each film. The heterophil/lymphocyte (H/L) ratio was examined as a response estimator of stress caused by the experimental assay (Davis *et al.,* 2008).

Data analyses

All biomarker data were expressed as mean ± SEM. The influence of pesticide treatments on each variable (B-esterases, CAT and H/L ratio) was analyzed with Kruskal-Wallis and Dunn's tests for post hoc comparisons (Lajmanovich *et al.,* 2013). The differences in the percentage increase of BChE activity after 2-PAM treatments among two CPF exposition doses were calculated using the Mann Whitney-U test for each data set. These statistical analyses were performed using the BioEstat software 5.0 (Ayres *et al.,* 2008). A value of $p<0.05$ was considered to be significant.

Results

Mean (±SEM) length and body mass of toads were 94.18±2.16 mm and 98.10±7.95 g, respectively. No signs of general behavioral disorders (hyperactivity, loss of coordination in hindlimbs and forelimbs, erratic swimming) were observed in toads exposed to CPF as a response to a possible severe acute neurotoxicity. The mean value of BChE activity in the non-pesticide exposure toads was 6.49±1.01 µmol/min/mL plasma at 48 h. BChE activity

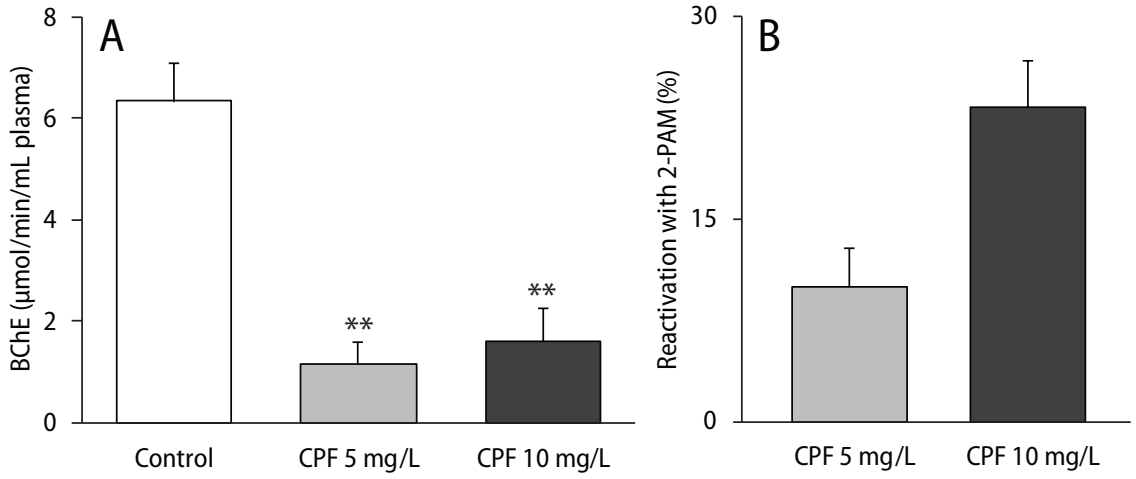

Figure 2. Effects of commercial pesticides exposure (48-h) on butyrylcholinesterase (BChE) activity in *Rhinella arenarum* toads; significantly different form control (** $p<0.01$; Kruskal-Wallis test followed Dunn's post-test) (**a**), and percentage of reactivations after addition of 2-PAM ($p>0.05$; Mann-Whitney test) (**b**). CO: control ($N = 4$), CPF: chlorpyrifos ($N = 6$). Data are expressed as mean ± SEM.

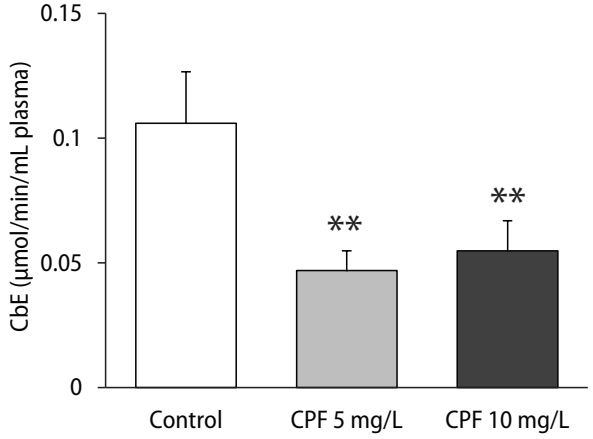

Figure 3. Effects of commercial pesticide exposure (48-h) on carboxylesterase (CbE) activity in *Rhinella arenarum* toads; significantly different from control (**$p<0.01$; Kruskal-Wallis test followed Dunn's post-test). CO: control (n=4), CPF: chlorpyrifos (n=6). Data are expressed as mean ± SEM.

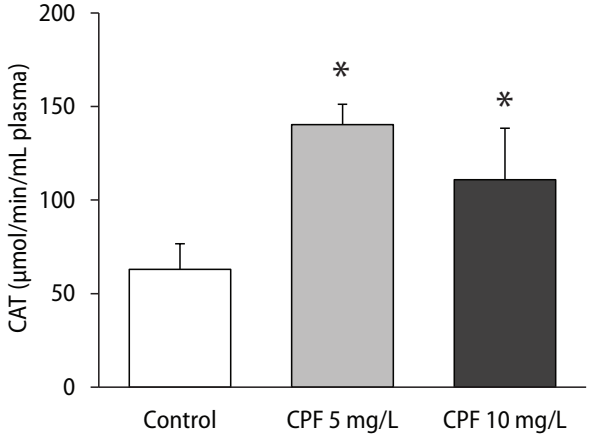

Figure 4. Effects of commercial pesticides exposure (48-h) on the catalase (CAT) activity in *Rhinella arenarum* toads; significantly different from control (*$p<0.05$; Kruskal-Wallis test fallowed Dunn's post-test). CO: control (n=4), CPF: chlorpyrifos (n=6). Data are expressed as mean ± SEM.

varied among groups exposed to CPF formulation (percentage of inhibition of 82.58% [5 mg/L] and 72.72% [10 mg/L]) with respect to the control group ($p<0.01$) (Figure 2a). Oxime-induced reactivation of plasma BChE activity was observed in all toads exposed to CPF formulations. The mean percentage increase of plasma BChE activity in samples containing 5 mg/L of CPF was 11.93% (±2.39), whereas for the samples with 10 mg/L of CPF it was 21.23% (±2.65). The differences between the two groups of samples were not significant (U=12; $p=0.39$) (Figure 2 b).

The CbE activity (mean ± SEM) in the control group was 0.1095±0.009 µmol/min/mL plasma at 48 h. CPF formulation inhibited CbE enzyme activity significantly ($p<0.01$) with respect to control in toads exposed at 5 mg/L (56.65%) and 10 mg/L (43.23%) (Figure 3).

The mean value of CAT activity in control toads was 60±8.10 µmol/min/mL plasma at 48 h. The induction of CAT enzymatic activity across dermal exposure at the two concentrations tested was significant ($p<0.05$) with respect to the control samples (Figure 4). Our results show that a mean H/L (48-h) ratio in control groups was 0.23±0.04. No differences were found in H/L ratio between toads exposed to both CPF formulations and the control group (KW=2.063; $p=0.35$).

Discussion

Greater than 70% of the worldwide amphibian species present decline (Hayes *et al.,* 2010). These vertebrates are important animal models in toxicology and they often

represent both aquatic and terrestrial forms within the life history of the same species (Helbing 2012). Certainly, frogs and toads have been considered as bio-indicators of aquatic and agricultural ecosystems (Peltzer *et al.,* 2006).

In the present study, BChE and CbE activity was significantly inhibited by dermal exposure to CPF formulations. Previous experiments showed similar results in these B-esterases (Lajmanovich *et al.,* 2015). In the same sense, it is well known that CPF suppresses the activity of ChEs in amphibian aquatic stage (e.g. Widder & Bidwell, 2006; Robles-Mendoza *et al.,* 2011; Liendro *et al.,* 2015). BChE and CbEs are important to reduce OP toxicity (Wheelock *et al.,* 2004; Laguerre *et al.,* 2009) and these isozymes may contribute to pesticide tolerance due to their capability of binding to OPs. Moreover, CbE was reported to comprise a group of isoenzymes that play a role in OP detoxification by acting as an alternative target protecting ChE from inhibition (Wheelock *et al.,* 2008). In the *R. arenarum* individuals used in this study, BChE and CbE may considerably diminish the effective concentration of the pesticide before they reach the blood-brain barrier in order to protect AChE from inhibition in the central nervous system (Walker, 1998).

Oxime-induced reactivation of plasma BChE activity was observed in all toads exposed to CPF formulations. Chemical reactivation of plasma phosphorylated ChEs activity has been used for complementary diagnosis of OP intoxication in birds (McInnes *et al.,* 1996; Parsons *et al.,* 2000; Iko *et al.,* 2003), reptiles (Sánchez-Hernández 2003; Sánchez-Hernández *et al.,* 2004), and amphibians (Attademo *et al.,* 2007; Lajmanovich *et al.,* 2008). In this respect, we performed the 2-PAM reactivation assay, which enabled us to identify organophosphates (OPs) as the agrochemicals responsible for plasma BChE depression in the exposed toads. Plasma BChE reactivation in the presence of 2-PAM appeared to be a more sensitive indicator of exposure to CPF than plasma BChE activity levels.

An antioxidant biomarker, i.e. CAT, was also included in this study, which would help evaluate the impact and risk of sub-lethal concentrations of CPF on *R. arenarum* toads. Indeed, exposure of freshwater gastropod to CPF for 48 h also resulted in a significant increase in CAT activity (Cacciatore *et al.,* 2015). *R. arenarum* larvae exposed to CPF for 96 h also showed changes in CAT activity (Liendro *et al.,* 2015). Oxidative stress may occur if the equilibrium between oxidants and antioxidants is interrupted either by the reduction of antioxidant defences or by the excessive increase of reactive oxygen species (ROS) (Valavanidis *et al.,* 2006). From earlier research it is understood that CPF produces oxidative stress resulting in the accumulation of lipid peroxidation products in different tissues (Verma *et al.,* 2003; 2007). Nevertheless, we cannot conclusively say that similar oxidative stress is caused also in toads. In this sense, four-week exposure of rats to CPF caused noticeable decrease in CAT activity in erythrocytes (Barski *et al.,* 2011).

On the other hand, there is evidence of changes in leukocyte profiles in amphibians under stress (Davis *et al.,* 2008). Severe stress may decrease heterophils (heteropenia) and increase lymphocytes in the periphery (lymphocytosis), resulting in a low H/L-ratio (Müller *et al.,* 2011). However, we did not find significant relations between leukocyte profiles and exposure to CPF. The lack of differences between toads exposed to CPF and control groups can be related to the fact that CPF in water degrades quickly in the first 24 hours (Wu *et al.,* 2003), or the time lag associated with the leukocyte response to stress may be the longest in ectothermic animals (Pough, 1980).

In conclusion, CPF formulations uptake in toad blood at 48 h produced BChE and CbE inhibition (BChE reactivated by oximes) and oxidative stress. Possibly the multiple effects observed were not restricted to CPF active ingredients and could be influenced by the commercial formulation adjuvants. The experiment proposed of *in vivo* dermal toxicity is a good method to characterize the risk exposures of native adult amphibians exposed to OP insecticides.

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ORIGINAL ARTICLE

Washing soda induced alteration of the differential cell count, nonself surface adhesion efficacy and nuclear morphology of the polyphenotypic cells of a freshwater sponge of India

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ABSTRACT

Washing soda has been identified as a precarious contaminant of the freshwater ponds and lakes, the natural habitat of *Eunapius carteri*. Treatment of sublethal concentrations of washing soda for 384 hours exhibited a significant decrease in the densities of blast like cells, small and large amoebocytes. The percentage occurrence of granular cells and archaeocytes yielded a marked increase against the experimental concentrations of washing soda. Washing soda mediated alterations in the differential cell densities of *E. carteri* indicative of a state of physiological stress and an undesirable shift in the cellular homeostasis of the organism distributed in polluted environment. Experimental exposure of washing soda yielded a significant increase in the cellular dimensions of large amoebocytes and archaeocytes. Prolonged treatment with washing soda presented a gross reduction in nonself surface adhesion efficacy of *E. carteri* cells. Experimental concentrations of washing soda resulted in a dose dependent increment in the frequencies of binucleation and micronucleation in the cells of *E. carteri*. The data were indicative of a high level of genotoxicity of washing soda in *E. carteri*. The present investigation provides an important information base in understanding the toxin induced chemical stress on the archaic immune defense of a primitive urmetazoa.

KEY WORDS: cellular adhesion; differential cell count, *Eunapius carteri*, genotoxicity, sodium carbonate

Introduction

Sponges, the members of the phylum Porifera, evolved before Cambrian and overcame the harsh and stressful conditions of the environment (Müller *et al.*, 2007). Freshwater sponges of the Indian subcontinent are a relatively little researched group of metazoans and demands a special scientific attention of toxicologists and environmentalists. The freshwater ecosystem of India is an abode of a wide range of aquatic bioresources, including members of freshwater sponge. *Eunapius carteri* (Porifera: Demospongiae: Spongillidae), an inhabitant of Indian freshwater ecosystem, is reported as an effective biomonitoring organism of aquatic pollution (Kakavipure and Yeragi, 2008) and an important component of the biota (Mukherjee *et al.*, 2015a). It is considered a neglected bioresource of Indian freshwater ecosystem which bears the potential to act as a source of bioactive and biomimetic molecules (Manconi *et al.*, 2013). Porous mode of structural organization and ability to generate microcurrent by the ciliary beating of the flagellated choanocytes permit sponges to filter a large volume of water that corresponds to several times their own body volume (Leys *et al.*, 2011). Nonselective filter feeding mode of adaptation enabled sponges to distribute food particles, minerals and gases and thus subjected to exposure of environmental contaminants of natural and anthropogenic origin and can accumulate toxins within their body.

The freshwater ecosystem of India has been facing a serious ecotoxicological risk of contamination by diverse groups of environmental compounds of anthropogenic origin including washing soda, chemically identified as

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anhydrous sodium carbonate (CAS registry number: 497-19-8) (Ray *et al.*, 2015; 2017). Washing soda, synonymous to 'soda ash', is a precipitating builder (Bajpai and Tyagi, 2007) and is a component of laundry detergent (Warne and Schifko, 1999) which is capable of increasing the alkalinity of water. Washing soda has been reported as a popular brand of cleaning agent used by the rural and semiurban human populations of India for the purposes of cleaning of apparels, washing of utensils and bathing of cattles (Mukherjee *et al.*, 2015b). Glass, paper, metal and mining industries have been identified as major sources of washing soda which contaminates the global environment (UNEP, 2003). Household cleaning products containing washing soda effluents are generally disposed through drain (HERA, 2005) and silently contaminate the freshwater ponds and lakes, the natural habitat of *E. carteri*. On the basis of buffering potential of the aquatic ecosystem, the acceptable concentration of sodium carbonate which can be discharged to the environment varies from 2 to 20 mg/L (HERA, 2005). Thus our experimental concentrations appear to be rational and environmentally realistic. The report of toxicity of washing soda in the aquatic invertebrates is scanty in current scientific literature (Mckee and Wolf, 1963; Warne and Schifko, 1999). However, washing soda has been identified as an 'immunotoxin' in *E. carteri* (Mukherjee *et al.*, 2015b; 2015c; 2016c). Authors reported washing soda mediated alteration in the phagocytic response, generation of cytotoxic molecules, activities of antioxidant enzymes, lysozyme activity and apoptotic response in the cells of *E. carteri*.

Poriferans mostly depend on the innate immune system (Wiens *et al.*, 2007) to combat the invasion of environmental pathogens, parasites and toxins. For their evolutionary primitiveness, sponges are often considered a model group to study the evolution of metazoan immunity (Wiens *et al.*, 2005). Unlike the vertebrate blood cells, identification, classification and morphofunctional characterization of sponge cells appeared to be non uniform and raised confusions and contradictions among different spongologists and evolutionary biologists. Differential cell density of aquatic invertebrates has been reported as an established immunotoxicological marker for the evaluation of health status of an organism distributed in contaminated environment (Oliver and Fisher, 1999; Chakraborty *et al.*, 2008). Mookerjee and Ganguly (1964) microscopically identified several morphological variants such as archaeocytes, amoebocytes, choanocytes, scleroblasts, pinacocytes and reproductive cells in the freshwater sponge *Ephydatia* sp. While examining the internal defense mechanism of the marine sponge *Terpios zeteki*, Cheng *et al.* (1968) classified the cell types as collencytes, archaeocytes, chromocytes, thesocytes and scleroblasts. Smith and Hildemann (1991) microscopically recorded pinacocytes, choanocytes, archaeocytes, spherulous cells, acid mucopolysaccharide-positive cells, acidophilic granulated cells, sclerocytes and germ cells as the major cell categories of Indo-Pacific marine sponge, *Callyspongia diffusa*. While studying the morphofunctional characterization of *E. carteri* cells, Mukherjee *et al.* (2015a) microscopically identified the archaeocytes as the predominant cell population followed by large amoebocytes and granular cells in the dissociated cell suspension. However, report of toxin mediated alteration in the differential density of sponge cells is almost absent in current scientific literature.

Cellular adhesion is considered a fundamental prerequisite for the establishment of tissue and organ architecture in metazoans. Shift in the adhesive property of the cells might lead to migration of cells from one place to another leading to metastasis (Zetter, 1990; Albelda *et al.*, 1990). Studies on the mechanism of cellular adhesion in sponge revealed that sponge cell adhesion is mediated by large, calcium dependent proteoglycan like molecules termed as "aggregation factors" (Varner, 1995). Ferandez-Busquets *et al.* (2002) demonstrated the role of cellular adhesion in the allogenic recognition process in a marine sponge, *Microciona prolifera*. Sponges have been considered 'biotools' to study cellular recognition process in biomedical research (Ferandez-Busquets *et al.*, 2002) and model organisms to study cellular adhesion response (Ferandez-Busquets and Burger, 1999). Moreover, the self-nonself recognition ability of sponges has been presented as a window to speculate the evolution of histocompatibility system in metazoans. However, a report of toxin mediated alteration of the nonself surface adhesion efficacy of sponge cells is absent in the existing scientific literature.

The micronuclei assay has been considered a biomarker of environmental genotoxicity in monitoring the health of aquatic organisms (Dailianis *et al.*, 2003; Chakraborty and Ray, 2009). Micronuclei are generated from chromosome fragments which may occur due to the problem in cytokinesis or damage in the centrometric region (Heddle *et al.*, 1991). According to Hsu (1982), toxic chemicals can exhibit genotoxic impacts on biological organisms by altering the structure of the DNA, thus resulting in irreversible damage to the structural integrity of chromosome. Correia *et al.* (2017) reported a significant increase in the frequency of micronuclei formation in the coelomocytes of the earthworm *Eisenia andrei* under experimental exposure of titanium silicon oxide nanomaterials, indicating its genotoxicity. Furthermore, report of toxin induced dose dependent augmentation of the frequency of micronucleation is in report in molluscs (Scarpato *et al.*, 1990; Wrisberg *et al.*, 1992), with no information available in sponge. In the present study, genotoxic effects of washing soda were assessed by enumerating the frequencies of binucleation and micronucleation in the cells of *E. carteri*.

The aim of the present investigation is to analyze and quantitate the magnitude of washing soda induced physiological stress in *E. carteri* at cellular and subcellular levels. The toxicity of washing soda in *E. carteri* was examined with reference to differential cell density, nonself surface recognition efficacy, micrometry of cells and genotoxicity. Furthermore, the current analyses would provide an important set of ecotoxicological information regarding the physiological stress of washing soda in *E. carteri* and evaluation of toxicity of washing soda in the freshwater ecosystem of West Bengal.

Materials and methods

Collection, transportation and laboratory maintenance of experimental sponge specimens

Live specimens of *E. carteri* were manually collected from the selected freshwater ponds (22° 86'N, 88° 40'E) of the state of West Bengal of India without a history of pisciculture, anthropogenic activities and toxin contamination. Pieces of freshwater sponge were surgically dissected from the submerged plant twigs by sterile scalpel and were immediately transported to the laboratory with ample volume of freshly collected pond water obtained from its natural habitat. The dissolved oxygen, pH and temperature of the pond water were routinely screened during the collection of sponge specimens and maintained accordingly during acclimation of sponge specimens in the controlled laboratory condition (Mukherjee *et al.*, 2015b). Sponges were acclimated in glass aquaria fitted with electrically operated aerator for 7 days and the water of the experimental glass aquaria was replenished routinely at every 24 hours with freshly collected pond water to supplement suspended food and for avoidance of toxicity due to accumulation of excretory products and metabolites (Mukherjee *et al.*, 2015c). Proper illumination and a uniform light rationing of 12:12 hours dark-light cycle were monitored throughout the experiment. The entire experiment on *E. carteri* was designed according to the guidelines and institutional norms of animal ethics and maintenance of the Department of Zoology of the University of Calcutta.

Experimental design and treatment methodology of *E. carteri* with washing soda

The body mass of experimental *E. carteri* was dissected into pieces each with an approximate dimension of 2 cm^3 containing at least one osculum (Hansen *et al.*, 1995). The dissected body masses of *E. carteri* were maintained in aerated glass aquaria in controlled laboratory conditions for 7 days to minimize the physiological stress and to reorganize their aquiferous system (Duckworth and Pomponi, 2005). Each experimental set consisted of 5 replicates of *E. carteri*, immersed in a volume of 20 l of pond water taken in different glass aquaria (Mukherjee *et al.*, 2016c). Specimens of *E. carteri* were treated with sublethal and environmentally realistic concentrations of 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours for toxicological analyses. Parallel control sets with similar replicates of healthy *E. carteri* were maintained in sodium carbonate free water. The highest experimental concentration of 16 mg/L of washing soda was less than one third of the median lethal concentration of the toxin determined in *E. carteri* for 384 hours of treatment. Hydrological parameters like pH, total alkalinity, carbonate ion, bicarbonate ion, calcium, magnesium, sodium and chloride ions of the experimental pond water with and without sodium carbonate were assessed following APHA (1998) (Mukherjee *et al.*, 2015c).

Mechanical dissociation of *E. carteri* cells and preparation of free cell suspension

Pieces of *E. carteri* with an approximate dimension of 0.5 cm^3 were surgically excised from the healthy specimens and subsequently rinsed with sterile phosphate buffered saline (PBS, pH 7.4) to remove clay, sand and other adhered particles prior to experimentation. Dissociated cell suspension of *E. carteri* was prepared by mechanical squeezing of the dissected body fragment through a meshed cloth (Ganguly, 1960) into sterile mineral medium (M–medium: 1 mM $CaCl_2$. H_2O, 0.5 mM $MgSO_4$. $7H_2O$, 0.5 mM $NaHCO_3$, 0.05 mM KCl, 0.25 mM Na_2SiO_3. $9H_2O$; pH 7.5) (Funayama *et al.*, 2005; Mukherjee *et al.*, 2015a) taken in a prechilled glass vial. The resultant cell suspension was passed through a 50 µm nylon mesh to eliminate spicules and other undissociated masses (Chernogor *et al.*, 2011). The filtrate with suspended cells, was centrifuged (Hermle Z323 K; Hermle Labortechnik, Wehingen, Germany) at 650×*g* for 10 min, pellets were resuspended in sterile M–medium and stored at 4 °C to minimize cellular aggregation. The viability of sponge cells was routinely examined by staining the cells with 0.4% trypan blue (HiMedia, India) employing the principle of vital dye exclusion (Mukherjee *et al.*, 2016a) and did not exhibit significant alteration.

Enumeration of differential cell density

An aliquot of cell suspension (both control and washing soda treated *E. carteri*) was smeared on separate glass slides using micropipette and incubated for 1 hour. Postincubated cells were air dried for 10 min, fixed in methanol and stained with Giemsa. Differential cell count of washing soda treated *E. carteri* along with the control was carried out microscopically following the field count method and was expressed as relative percentages of the various cell types (Mookerjee & Ganguly, 1964; Mukherjee *et al.*, 2015a). At least 200 sponge cells per glass slide were examined under bright field optics for determination of percentage of different cell types.

Micrometric analyses of *E. carteri* cells

The micrometric analyses of the different types of cells (*E. carteri* exposed to 8 and 16 mg/L of washing soda for 384 hours along with the control) and their nuclei were determined by estimating the longest axis using an ocular micrometer (Erma, Tokyo, Japan) attached with the microscope (BH-2; Olympus, Tokyo, Japan) (Mukherjee *et al.*, 2015a).

Estimation of nonself surface adhesion efficacy of cells

The nonself surface adhesion efficacy of *E. carteri* cells was assessed after the protocol of Chen and Bayne (1995) with necessary modifications. Freshly prepared sponge cell suspension with a density of 10^6 cells/ml from control and washing soda treated *E. carteri* was placed on clean and sterilized glass slides and incubated in a humid chamber at 25 °C for 180 minutes for adequate adhesion. Supernatants of postincubated cell suspensions were carefully removed by a micropipette and the number of

non-adherent cells was enumerated using a Neubauer hemocytometer attached to the microscope (Axiostar Plus; Zeiss Microscopy, Jena, Germany).

For analysis of the morphology of adherent sponge cells, the *E. carteri* cells were examined under a scanning electron microscope (Zeiss EVO 18 special edition, Germany). The sponge cells were fixed in 3% glutaraldehyde (Sigma, USA) dissolved in 0.1 M sodium cacodylate buffer containing 12% glucose (pH 7.8) for 2 h at 4 °C (Mukherjee *et al.*, 2015b). This was followed by postfixation in 1% osmium tetroxide (Sigma, USA) for 1 h. Fixed cells were dehydrated with graded ethanol, air dried, sputter coated with gold in an ionic coater for scanning electron microscopy.

Detection of nuclear anomalies in cells of *E. carteri*

Relative abundance of micronucleated and binucleated cells of *E. carteri* was estimated after the modified protocol of Fenech *et al.* (2003) and Bolognesi and Fenech (2012). The Giemsa stained cells were examined under light microscope (Axiostar Plus; Zeiss Microscopy, Jena, Germany) for different kinds of nuclear aberrations. Digital photodocumentation and analyses of different nuclear anomalies of *E. carteri* cells was carried out using a CCD camera (ProgRes C5; Jenoptik, Jena, Germany) attached to the light microscope (BH-2; Olympus, Tokyo, Japan). Round or ovoid shaped non-refractory particles with the color and structure identical to chromatin with diameter equaling 1/3–1/20 of the main nucleus and its clear detachment from macronucleus were interpreted as 'micronuclei' (Chakraborty & Ray, 2009). On the other hand, binucleated cells consisted of two nuclei with intact nuclear membrane and identical staining intensity and were found within the same cytoplasmic boundaries. At least 450 cells were examined per slide for the enumeration of micronucleated and binucleated cells in *E. carteri* treated with washing soda.

Statistical analyses

The experimental data were initially checked for normality and homogeneity using Bartlett's test (Mukherjee *et al.*, 2016c). Since all data were normal, parametric statistics were applied, following one-way analysis of variance (ANOVA). The statistical data analysis was carried out using one way ANOVA followed by Dunnett's post hoc test (GraphPad Prism version 5 for windows, GraphPad Software, La Jolla California, USA) among the samples of each experimental hour to determine the level of significance between control and treatment sets (Gopalakrishnan *et al.*, 2009; Mukherjee *et al.*, 2015c). Each experiment was repeated 5 times. Data were presented as the mean ± standard deviation (S.D). Differences were considered significant at $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Results

Washing soda mediated shift in the differential cell count of E. carteri

Light microscopical analyses of dissociated *E. carteri* cells revealed the existence of eight distinct cellular variants, *i.e.* blast like cells, choanocytes, small amoebocytes, granular cells, pinacocytes, large amoebocytes,

Table 1. Micrometric estimations of the cell types of *E. carteri* exposed to 8 and 16 mg L^{-1} of washing soda for 384 hours.

Cell types	Dimensions	Control for 384 h	Exposure to washing soda for 384 h	
			8 mg/L	16 mg/L
Blast like cells	Cell (C) diameter; µm Nuclear (N) diameter; µm N/C ratio	5.85±0.36 3.91±0.22 0.66±0.05	6.11±0.32 4.01±0.59 0.65±0.02	6.09±0.51 3.67±1.14 0.58±0.08
Choanocytes	Cell (C) diameter; µm Nuclear (N) diameter; µm N/C ratio	5.52±1.16 1.65±1.05 0.28±0.10	5.66±0.73 1.29±0.11 0.23±0.06	5.74±0.45 1.78±0.33 0.31±0.03
Small amoebocytes	Cell (C) diameter; µm Nuclear (N) diameter; µm N/C ratio	7.67±2.98 2.93±1.50 0.38±0.01	8.97±2.00 3.93±0.48 0.43±0.01	9.45±2.27 3.91±0.53 0.39±0.06
Granular cells	Cell (C) diameter; µm Nuclear (N) diameter; µm N/C ratio	18.79±3.15 5.68±0.89 0.30±0.01	19.11±0.68 5.90±1.86 0.31±0.04	21.46±1.71 5.92±1.61 0.29±0.08
Pinacocytes	Cell (C) diameter; µm Nuclear (N) diameter; µm N/C ratio	16.44±5.57 6.49±0.56 0.43±0.11	15.14±2.72 6.78±0.66 0.44±0.15	16.98±1.31 7.13±0.95 0.42±0.12
Large amoebocytes	Cell (C) diameter; µm Nuclear (N) diameter; µm N/C ratio	20.81±0.92 6.16±1.15 0.30±0.02	22.49±0.80 7.26±1.29 0.32±0.03	23.45±0.50* 6.91±1.32 0.28±0.04
Archaeocytes	Cell (C) diameter; µm Nuclear (N) diameter; µm N/C ratio	22.35±0.96 6.75±0.84 0.31±0.05	22.32±0.88 6.53±1.11 0.29±0.01	24.9±0.30* 7.10±0.95 0.28±0.03
Sclerocytes	Cell (C) diameter; µm Nuclear (N) diameter; µm N/C ratio	11.02±1.81 3.12±0.43 0.28±0.07	10.48±1.48 2.86±0.47 0.25±0.11	11.21±1.28 3.10±0.44 0.24±0.09

Data were presented as mean ± S.D. (n=5). The asterisks indicate the values that were significantly different from the control ($^{*}p<0.05$).

archaeocytes and sclerocytes (Mukherjee *et al.*, 2015a). The authors microscopically identified and characterized the sponge cells on the basis of their shapes, dimensions, cytoplasmic inclusions and nuclear-cytoplasmic ratios. Differential count of cellular subpopulations in *E. carteri* exposed to sublethal concentrations of washing soda along with the control was enumerated microscopically employing bright field optics. Upon treatment with 2, 4, 8 and 16 mg/L of washing soda for multiple spans of time, *i.e.* 24, 48, 96, 192 and 384 hours, the cellular subpopulations exhibited differential changes in density distribution (Figures 1–3). The relative percentage of blast like cells of control *E. carteri* ranged from 10.28±0.73 to 10.21±1.10 over a time span of 24 to 384 hours respectively (Figure 1A). The density of blast like cells was significantly increased against all the experimental concentrations of washing soda for 48 hours and 16 mg/L of toxin for 24 and 96 hours. The highest density of blast like cells was

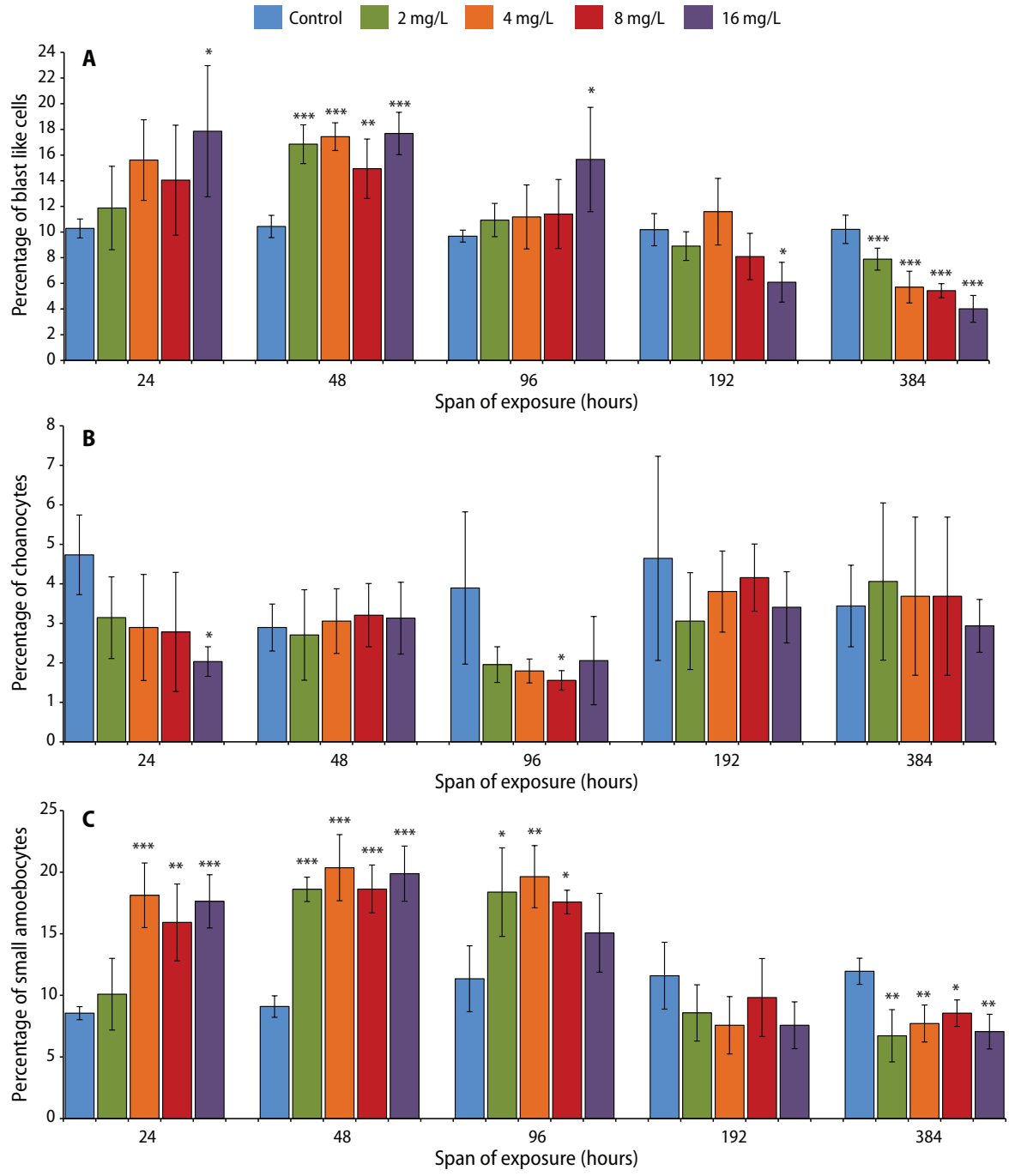

Figure 1. Dynamics of densities of blast like cells (A), choanocytes (B) and small amoebocytes (C) of *E. carteri* under the exposure of 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours. Data were presented as mean ± S.D. (n=5). The asterisks indicate the values that were significantly different from the control (*p<0.05, **p<0.01, ***p<0.001).

recorded as 17.85±5.11% under 16 mg/L of washing soda treatment for 24 hours. However, prolonged exposure of washing soda for 384 hours exhibited a dose dependent decrease in the density of blast like cells. The maximum inhibition in the relative percentage of blast like cells was recorded to be 4.01±1.04 under 16 mg/L of washing soda for 384 hours.

The relative density of choanocyte subpopulation of control *E. carteri* ranged from 4.73±1.01% to 3.44±1.03% over a time span of 24 to 384 hours (Figure 1B). A nonlinear dose independent fluctuation in the density of choanocytes was recorded under all experimental concentrations of washing soda for different time spans. Experimental exposure of 16 mg/L of washing soda for 24 hours and 8 mg/L for 96 hours exhibited a significant decrease in the relative percentage of choanocyte subpopulation. In control *E. carteri*, the percent density of small amoebocyte subpopulation varied from 8.56±0.52 to 11.95±1.06

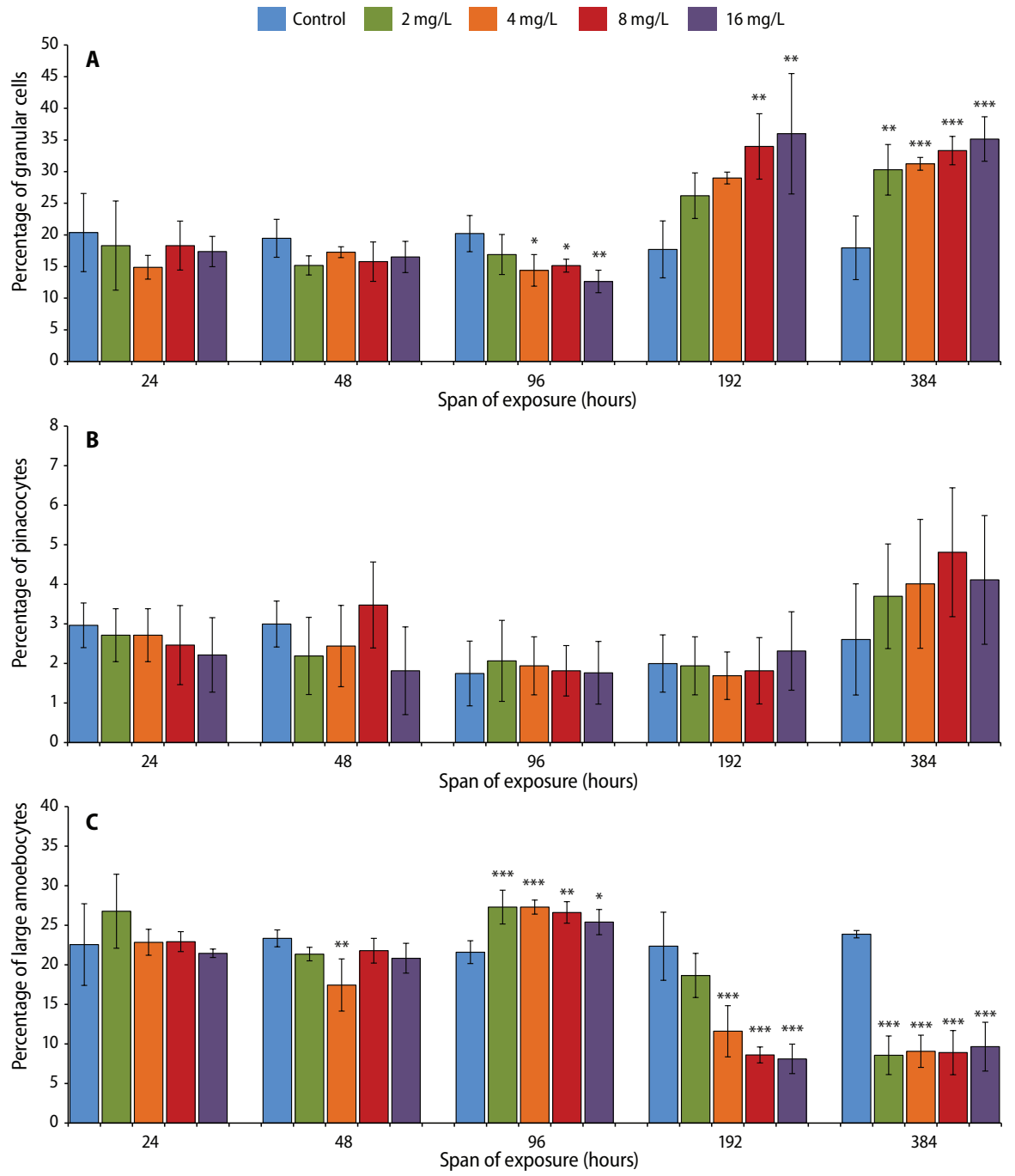

Figure 2. Dynamics of densities of granular cells (A), pinacocytes (B) and large amoebocytes (C) of *E. carteri* under exposure to 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours. Data were presented as mean ± S.D. (n=5). The asterisks indicate the values that were significantly different from the control (*p<0.05, **p<0.01, ***p<0.001).

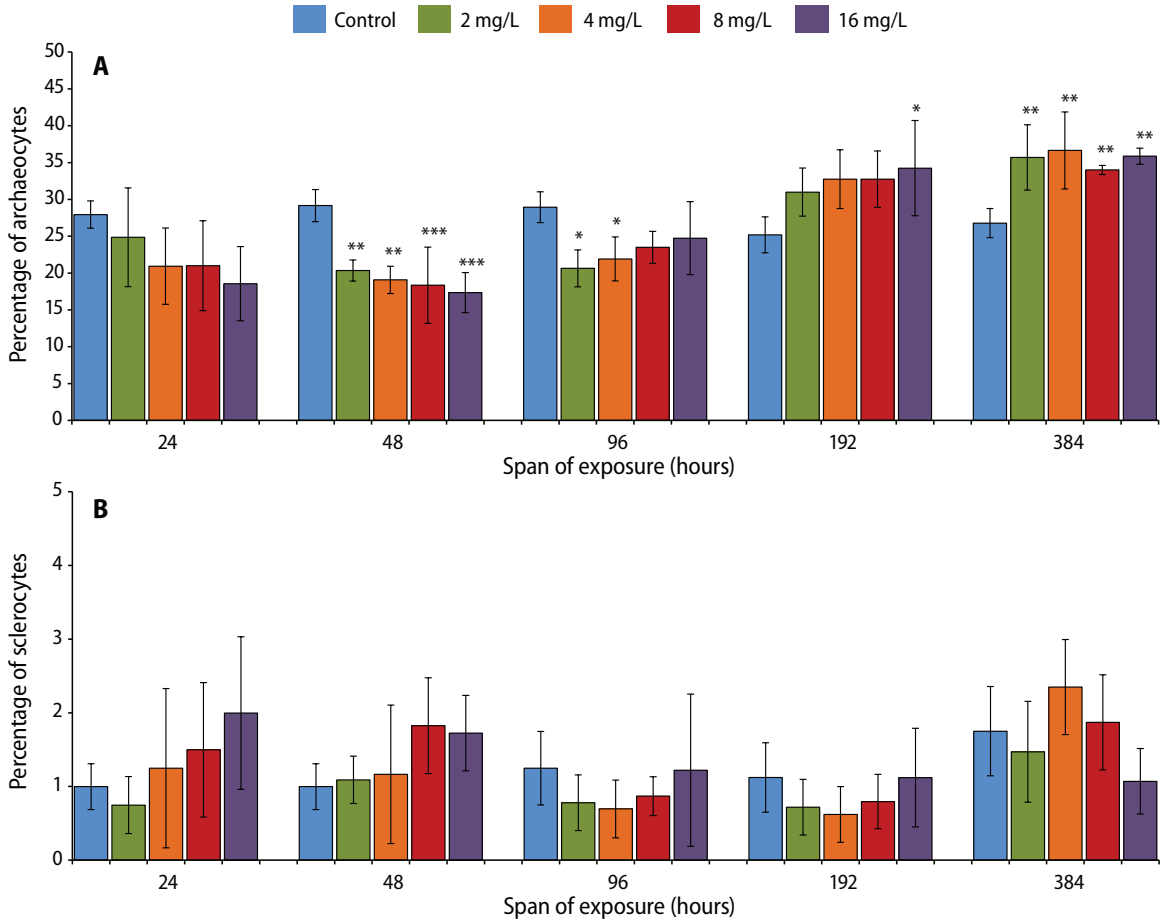

Figure 3. Dynamics of densities of archaeocytes (A) and sclerocytes (B) of *E. carteri* under the exposure of 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours. Data were presented as mean ± S.D. (n=5). The asterisks indicate the values that were significantly different from the control (*p<0.05, **p<0.01, ***p<0.001).

for different time spans (Figure 1C). A trend of increase in the percentage occurrence of small amoebocytes was recorded against the experimental concentrations of washing soda up to 96 hours. The highest density of small amoebocyte subpopulation was recorded as 20.36±2.68% in *E. carteri* treated with of 4 mg/L of washing soda for 48 hours. However, long-term exposure of washing soda for 384 hours presented an overall decrease in the relative density of small amoebocyte subpopulation against all experimental concentrations. The highest inhibition in the percentage of small amoebocytes was recorded as 6.71±2.11 against 2 mg/L of washing soda for 384 hours of exposure. The percentage of granular cells ranged from 20.36±6.17 to 17.96±5.02 in untreated *E. carteri* over a time span of 24 to 384 hours respectively (Figure 2A). A trend of decrease in the density of granular cells was recorded under 4, 8 and 16 mg/L of washing soda treatment for 96 hours. The lowest density of granular cells was recorded as 12.64±1.78% in *E. carteri* exposed to 16 mg/L of washing soda for 96 hours. However, treatment of sublethal concentrations of washing soda for 192 and 384 hours exhibited a trend of increase in the density of granular cells. The maximum density of granular cells was recorded as 35.98±9.52% under 16 mg/L of washing soda exposure for 192 hours. The percent density of pinacocyte subpopulation of untreated *E. carteri* ranged from 2.96±0.56 to 2.60±1.40 for multiple time spans (Figure 2B). Experimental exposure of washing soda did not exhibit any significant alteration in the percentage occurrence of pinacocytes.

The relative density of large amoebocytes in the control *E. carteri* ranged from 22.56±5.16 % to 23.87±0.45% over a time span of 24 to 384 hours (Figure 2C). Experimental exposure of washing soda up to 48 hours did not exhibit any significant alteration in the percentage occurrence of large amoebocytes except in *E. carteri* treated with 4 mg/L of washing soda for 48 hours which exhibited a significant decrease in comparison to that of the control. A significant increase in the percentage of large amoebocytes was recorded under 2, 4, 8 and 16 mg/L of washing soda for 96 hours. The maximum density of large amoebocytes was recorded as 27.31±2.13% under the experimental exposure of 2 mg/L of washing soda for 96 hours. A dose dependent decrease in the relative percentage of large

amoebocytes was estimated in *E. carteri* for 192 hours of washing soda exposure. However, prolonged treatment of sodium carbonate for 384 hours presented an overall depletion in the percentage of large amoebocytes in all experimental concentrations. The maximum inhibition in the relative percentage of large amoebocytes was recorded as 8.11±1.86 against 16 mg/L of washing soda for 192 hours of exposure. The percent density of archaeocytes of untreated *E. carteri* varied from 27.94±1.85 to 26.77±1.97 for different time spans (Figure 3A). An inhibition in the percentage of archaeocyte subpopulation was recorded under the exposure of all experimental concentrations of washing soda for 48 hours and 2, 4 mg/L of toxin for 96 hours. The maximum inhibition in the percentage occurrence of archaeocytes was recorded as 17.34±2.72 under the treatment of 16 mg/L of washing soda for 48 hours.

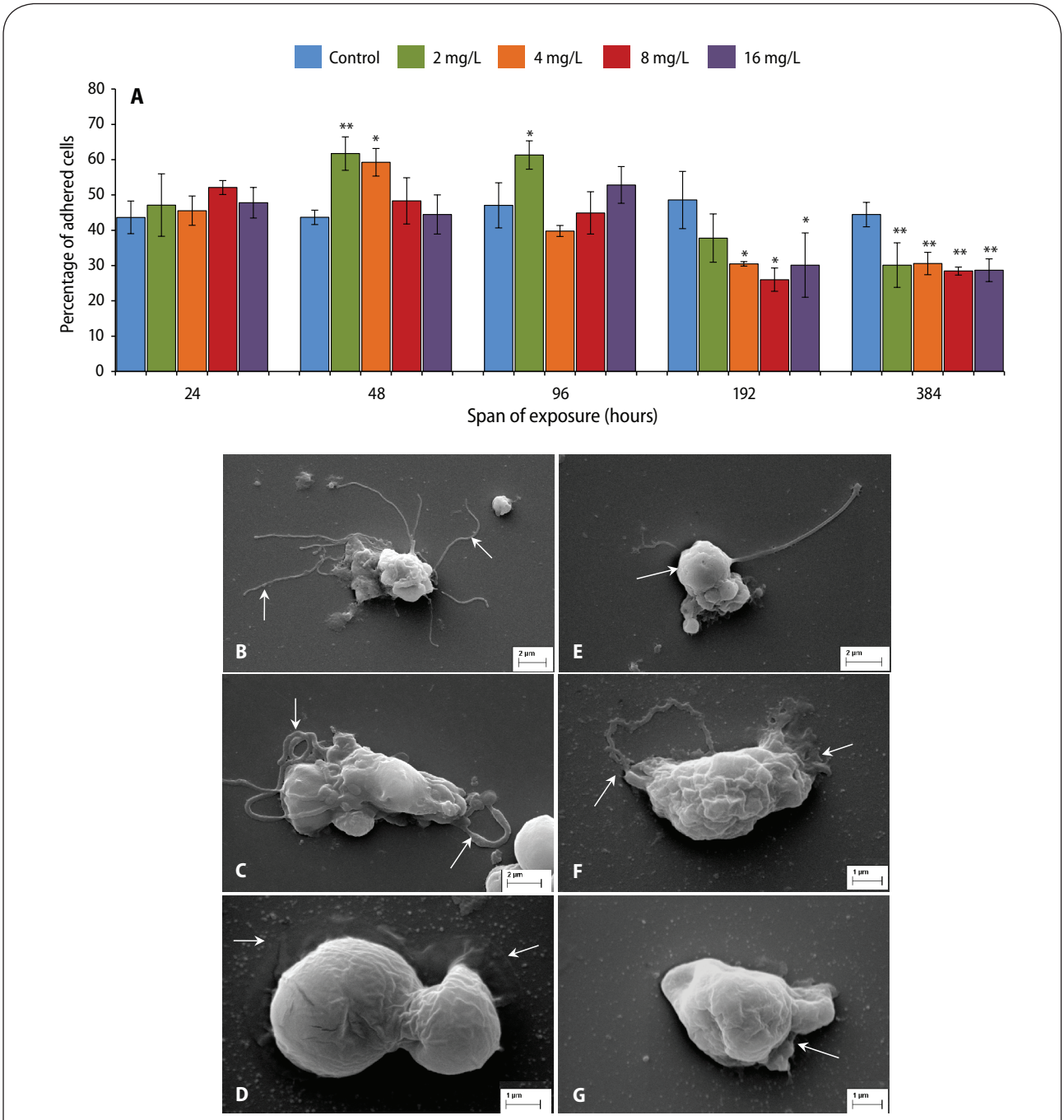

Figure 4. Percentage of adherent cells of *E. carteri* exposed to 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours (A). Data were presented as mean ± S.D. (n=5). The asterisks indicate the values that were significantly different from the control (*p<0.05, **p<0.01, ***p<0.001). Scanning electron micrographs of adherent cells of *E. carteri* on the glass surface (B–G). B, C, D represent the cells of the control *E. carteri* and E, F, G exhibit the sponge cells treated with 16 mg/L of washing soda for 384 hours. The cells of the control *E. carteri* exhibited extensive membrane involutions (B) as well as longer cytoplasmic projections (B, C, D). Exposure of washing soda yielded membrane smoothening (E) and relative decrease of pseudopodial projections (E, F, G) in the cells of *E. carteri* as evident from ultrastructural analyses.

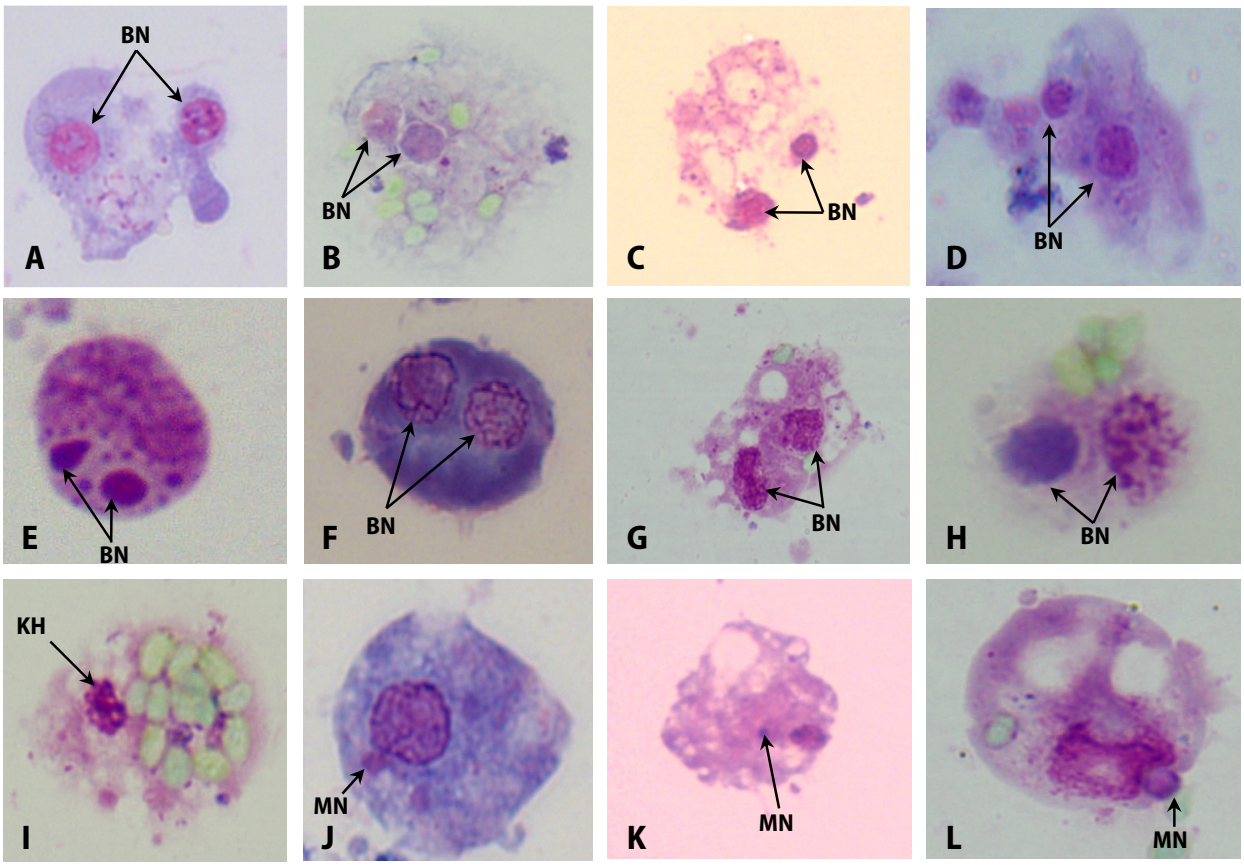

Figure 5. Light microscopical identification of nuclear anomalies in the cells of *E. carteri* exposed to 16 mg/L of washing soda for 384 hours. Washing soda yielded binucleation (BN) (A–H), karyorrhexis (KH) or nuclear fragmentation (I) and micronucleation (MN) (J–L) in the cells of *E. carteri*. Scale bar: 10 µm.

However, a significant increase in the relative percentage of archaeocytes was recorded under 16 mg/L of washing soda for 192 hours and all experimental concentrations of sodium carbonate for 384 hours. The maximum elevation in the density of archaeocyte subpopulation was recorded as 36.64±5.22% against 4 mg/L of washing soda for 384 hours of exposure. The relative density of sclerocyte subpopulation was recorded to be the least among other subpopulations of cells. Sclerocytes were rarely encountered in the dissociated cell suspension of *E. carteri*. The percent density of sclerocytes of control *E. carteri* ranged from 0.99±0.31 to 1.75±0.60 over a time span of 24 to 384 hours respectively (Figure 3B). Treatment of washing soda did not present any significant alteration in the relative density of sclerocyte subpopulation.

Micrometric alteration of *E. carteri* cells under the exposure of washing soda

Micrometric analyses of different types of cells were estimated in *E. carteri* treated with 8 and 16 mg/L of washing soda for 384 hours along with controls (Table 1). Cell and nuclear diameters and nuclear-cytoplasmic (N/C) ratios were microscopically estimated in blast like cells, choanocytes, small amoebocytes, granular cells, pinacocytes, large amoebocytes, archaeocytes and sclerocytes of control and washing soda exposed specimens employing bright field optics. Micrometric analyses of sponge cells revealed significant swelling in the cellular dimensions of large amoebocytes and archaeocytes of *E. carteri* exposed to 16 mg/L of washing soda for 384 hours in comparison to the respective controls.

Washing soda mediated shift in the nonself surface adhesion efficacy of *E. carteri* cells

When the cells of *E. carteri* were allowed to settle over the glass surface, cells got adhered, flattened and exhibited pseudopodial extensions as evident from their scanning electron microscopic images (Figure 4B–G). Morphologically, the adherent cells of control *E. carteri* were characterized by their pseudopodial projections over the glass surface (Figure 4B–D). Cells exposed to sublethal concentrations of washing soda exhibited distinctive morphological alterations and relative reduction in the cytoplasmic projections, membrane involutions and natural spreading behavior (Figure 4E–G).

Percentage occurrence of adherent cells of *E. carteri* was microscopically enumerated under the experimental exposure of 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours of time span (Figure 4A). The percentage of adhered cells of control *E. carteri* ranged

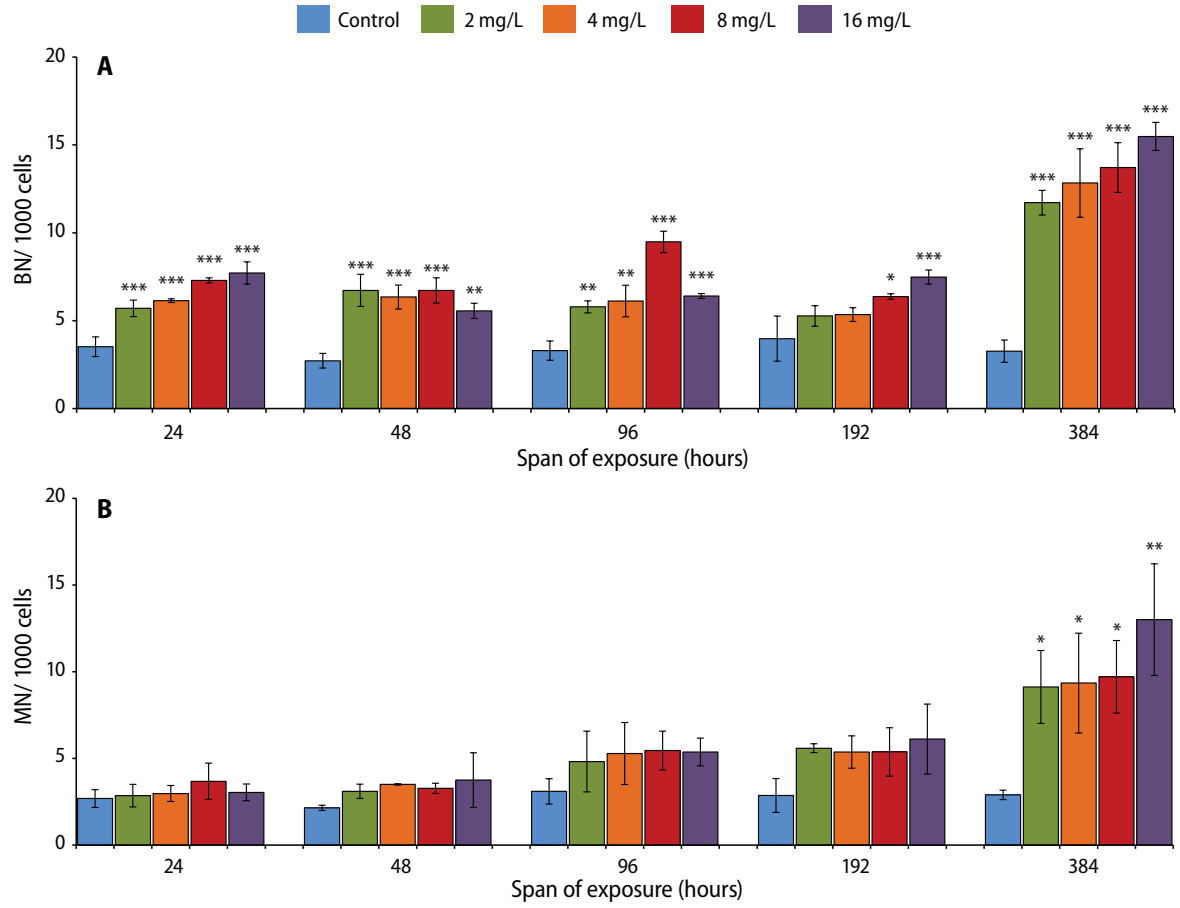

Figure 6. Frequencies of binucleation (A) and micronucleation (B) in the cells of *E. carteri* treated with 2, 4, 8 and 16 mg/L of washing soda for 24, 48, 96, 192 and 384 hours. Data were presented as mean ± S.D. (n=5). The asterisks indicate the values that were significantly different from the control (*p<0.05, **p<0.01, ***p<0.001).

from 43.63±4.62 to 44.64±3.46 over a time span of 24 to 384 hours respectively. A significant increase in the percentage of adherent cells was recorded in *E. carteri* treated with 2 mg/L of washing soda for 48 and 96 hours and 4 mg/L of toxin for 48 hours. The highest percentage of adherent cells was recorded to be 61.71±4.72 in *E. carteri* exposed to 2 mg/L of washing soda for 48 hours. A significant decrease in the percentage of adherent cells was recorded in *E. carteri* treated with 4, 8 and 16 mg/L of washing soda for 192 hours. Long-term exposure of washing soda for 384 hours yielded an overall depletion in the cellular adhesion response of sponge cells under all experimental concentrations. The maximum inhibition in the percentage of adherent cells was recorded as 26±3.28 against 8 mg/L of washing soda for 192 hours of exposure.

Washing soda induced nuclear anomalies in the cells of *E. carteri*

Light microscopical observation revealed that experimental exposure of washing soda appears to be genotoxic in *E. carteri* as evident from the appearance of nuclear anomalies, *i.e.* binucleation (Figure 5A–H), micronucleation (Figure 5J–L) and karyorrhexis or nuclear fragmentation (Figure 5I) in the sponge cells. The frequency of binucleation (Figure 6A) and micronucleation (Figure 6B) in the cells of *E. carteri* were estimated microscopically under 2, 4, 8 and 16 mg/L of washing soda for multiple spans of exposure along with the respective controls. Normal physiological levels of binuclei frequencies ranged from 3.51±0.55 to 3.26±0.63 per 1000 cells during 24 to 384 hours of observations made in the control *E. carteri*. A marked increase in the frequency of binuclei was recorded against the exposure of all experimental concentrations of washing soda for 24, 48 and 96 hours in comparison to the controls. Washing soda exposure of 8 and 16 mg/L for 192 hours exhibited a significant increase in the frequency of binucleation in the cells of *E. carteri*. However, all the experimental concentrations of washing soda for 384 hours of treatment presented a dose dependent increase in the frequency of binucleation in the cells of *E. carteri*. The highest frequency of binuclei was recorded as 15.48±0.79 per 1000 cells in *E. carteri* treated with 16 mg/L of washing soda for 384 hours. The micronuclei frequencies in the cells of control *E. carteri* ranged from 2.68±0.51 to 2.89±0.27 per 1000 cells over a time span of 24 to 384 hours. Experimental exposures of washing soda up to a

span of 192 hours did not yield any significant alteration in the frequencies of micronuclei. However, long term exposure of washing soda for 384 hours exhibited a significant increase in the frequency of micronucleation under all experimental concentrations. The highest frequency of micronuclei was recorded as 13.01±3.21 per 1000 cells in *E. carteri* treated with 16 mg/L of washing soda for 384 hours.

Discussion

For their developmental characteristics, sponges are often considered candidate species in evaluating the toxicity of aquatic ecosystem (Goh, 2008). From a scientific point of view, sponges are important because of their unusual cellular organization, their ability to regenerate the damaged parts and their biochemical uniqueness. Wagner *et al.* (1998) proposed sponges as a model organism to evaluate the toxicity of environmental compounds of anthropogenic origin. *E. carteri* is considered an "ecological hotspot" (Mukherjee *et al.*, 2016b) and is able to establish a wide array of functional relationships with other aquatic organisms, including small crabs, shrimps, insect larvae, water mites, nematodes, oligochaetes, polychaetes, ciliates, molluscs (Soota, 1991) in their shared natural habitat. Many of these organisms utilize the colony of *E. carteri* as suitable sites for reproduction, parental care and shelter.

The impact of detergents and their allied compounds on the aquatic ecosystem and human health have been an issue of concern for many decades due to their widespread production and use (Pedrazzani *et al.*, 2012). Warne and Schifko (1999) revealed the toxicity of sodium carbonate in the freshwater cladocera, *Ceriodaphnia dube.* Azizullah *et al.* (2011) reported the toxicity of laundry detergent in the freshwater flagellate *Euglena gracilis*. Sublethal concentrations of pesticides, rogar and endosulfan were reported to increase the protein content of the freshwater sponge *Spongilla lacustris* (Ingle *et al.*, 2003). According to Cebrian *et al.* (2003), Mediterranean sponge *Crambe crambe* has been reported to accumulate moderate concentrations of lead, copper and vanadium within its body. Contamination of natural habitat under the face of metal pollution might lead to an alteration in the physiological growth, fecundity and survival of the experimental species.

Due to apparent nonexistence of well constructed tissues and organs, sponges present morphofunctional diversities of heterogenous populations of cells to perform diverse physiological functions including nonself surface adhesion, cell-cell aggregation, phagocytosis and generation of cytotoxic molecules, etc. (Smith & Hildemann, 1991; Varner, 1995; Philip, 1997; Peskin *et al.*, 1998; Leys & Eerkes-Medrano 2006; Mukherjee *et al.*, 2015a). Russo and Lagadic (2000) reported hexachlorobenzene and atrazine mediated induction in the hyalinocyte population of mollusc, *Lymnaea palustris.* Chakraborty *et al.* (2008) reported inorganic arsenite induced modulation in the relative densities of hemocyte subpopulations of freshwater bivalve *Lamellidens marginalis,* which shares the same habitat with *E. carteri*. In the present investigation, treatment of 2, 4, 8 and 16 mg/L of washing soda for 48 hours yielded a significant increase in the percentage occurrence of blast like cells (Figure 1A). However, persistent exposure of washing soda for 384 hours yielded a dose dependent decrease in the relative percentage of blast like cells. Treatment of sublethal concentrations of washing soda up to a span of 96 hours resulted in an overall increase in the relative density of small amoebocytes of *E. carteri* followed by a decrease at 384 hours (Figure 1C). Washing soda exposure for 192 and 384 hours resulted in a dose dependent induction in the percentage occurrence of granular cells in *E. carteri* (Figure 2A). Treatment of all experimental concentrations of washing soda for 96 hours exhibited a significant increase in the density of large amoebocyte subpopulation (Figure 2C). However, washing soda exposure for 192 and 384 hours resulted in a gradual depletion in the percentage occurrence of large amoebocytes in comparison to that of the controls. Washing soda treatment of 2, 4, 8 and 16 mg/L for 384 hours yielded a significant increase in the relative percentage of archaeocytes in *E. carteri* (Figure 3A).

The blast like cells are relatively smaller in size, with large central nuclei which resemble mammalian lymphocytes. Funayama *et al.* (2010) proposed the blast like cells as immature choanocytes or choanoblasts and demonstrated their involvement in the development and differentiation processes in the freshwater sponge *Ephydatia fluviatilis.* The archaeocytes, on the other hand, played a pivotal role in the reconstitution and reorganization of the dissociated cells to form an adult functional sponge and bore the potential of stemness (Funayama, 2010). The granular cells are a special type of sponge cells with intense cytoplasmic granulation and play a pivotal role in glycogen synthesis. Sclerocytes are responsible for the formation of siliceous sponge spicules by accumulating and depositing silicate in an organized fashion. Archaeocytes, large amoebocytes and granular cells were identified as chief phagocytes of *E. carteri* which were capable of engulfing foreign particles (Mukherjee *et al.*, 2015a). Archaeocytes and large amoebocytes were recorded as major generators of superoxide anion and nitric oxide in *E. carteri*. Washing soda mediated undesirable shift in the differential cell density of *E. carteri* might adversely affect the cell mediated innate immunological status and physiological homeostasis of the organism distributed in a polluted environment. Moreover, the present data are indicative of possible interference of multiple functional attributes like phagocytosis, nonself recognition efficacy and generation of cytotoxic molecules in different cell types of *E. carteri* distributed in washing soda contaminated habitat.

Sublethal and environmentally realistic concentrations of washing soda yielded substantial morphological damage in the heterogenous cell populations of *E. carteri* (Mukherjee *et al.*, 2015c). The authors identified cytoplasmic disintegration, nuclear dissolution, cell surface smoothening, hypervacuolation and membrane blebbing as principal morphological damage of *E. carteri* cells

under washing soda exposure. According to them, washing soda induced morphological damage of *E. carteri* cells was indicative to impairment of overall morpho-functional status of the organism inhabiting detergent contaminated habitat. Mukherjee *et al.* (2015a) reported the micrometric data of different cell types of control *E. carteri*. However, report of toxin induced micrometric alteration of the cell types of sponge is absent in current scientific literature. Chakraborty *et al.* (2013) reported micrometric alteration and significant swelling in the hemocytes of freshwater bivalve, *L. marginalis* under sublethal concentrations of sodium arsenite, a geogenic toxin. Calisi *et al.* (2008) recorded cadmium chloride induced morphological alterations in the granulocytes of mollusc, *Mytilus galloprovincialis*. The authors reported enlargement of granulocyte morphology and rounding up of cells due to loss of pseudopodial projections in *M. galloprovincialis* under the experimental exposure of cadmium. According to them, pollutant induced morphometric alterations in the granulocytes of mollusc might function as a biomarker for monitoring and assessment of the degree of environmental contamination. In a separate study, Calisi *et al.* (2009) demonstrated the enlargement of granulocytes of the earthworm, *Eisenia fetida*, under exposure to copper sulfate and methiocarb. The authors claimed the studied parameter as a sensitive biomarker for monitoring the health of earthworm distributed in the contaminated habitat. In this present investigation, washing soda exposure of 16 mg/L for 384 hours resulted in a significant increase in the cellular dimensions of the large amoebocytes and archaeocytes (Table 1). The washing soda induced micrometric alterations of the cells were suggestive of an impairment of the structural integrity and physiological activity of cells of *E. carteri* distributed in polluted environment.

Throughout the course of evolution, cell-cell and cell-substratum adhesion play a significant role for the purposes of different physiological functioning including food uptake, epithelium formation and self-nonself recognition, development as well as maintenance of body plan in metazoans (Varner, 1995). The degree of cell spreading and nonself surface adhesion efficacy of molluscan hemocytes had been modulated by environmental chemicals (Chen & Bayne, 1995). Canesi *et al.* (2001) demonstrated the surface interaction between the bacteria *Escherichia coli* and the hemocytes of the Mediterranean mussel, *M. galloprovincialis*. Anderson *et al.* (2011) reported modulation in hemocyte number and cellular adhesion in β-1, 3 glucan treated mollusc, *Crassostrea virginica*. Experimental exposure of cholera toxin modulated the hemocyte adhesion efficacy of wax moth, *Galleria mellanella* (Lapointe *et al.*, 2012). According to Wootton and Pipe (2003), phagocytosis is principally dependent on the membrane properties of hemocytes and alteration in the adhesive property of hemocytes may lead to a decrease in phagocytic activity. In the present investigation, sublethal concentrations of washing soda for 384 hours resulted in a significant inhibition in the nonself surface adhesion efficacy of cells of *E. carteri* (Figure 4A). The result was indicative to an undesirable shift in cellular integrity, morphogenesis and maintenance of body plan in *E. carteri* distributed in washing soda contaminated habitat.

The micronuclei assay is considered an established method to study the genotoxicity and mutagenicity of a chemical compound by estimating the structural and numerical aberrations of chromosomes (Bolognesi and Cirillo, 2014). An increase in the frequency of micronucleation was recorded in the hemocytes of moth, *Galleria mellonella* exposed to deltamethrin, a pyrethroid insecticide used in agricultural fields (Kurt and Kayiş, 2015). Experimental treatments of organotin compounds, arsenic, polyaromatic hydrocarbons and polychlorinated biphenyl exhibited a significant alteration in the micronuclei frequency of clam, *Mya arenaria* (Debenest *et al.*, 2013). According to them, augmentation in micronuclei frequency was physiologically associated with oxidative stress, which in turn affected the phagocytic potential of hemocytes of clams. Several xenobiotics can cause oxidative stress in living organisms through the generation of reactive oxygen species (ROS) and alter the antioxidant buffering potential of the cells in invertebrates (Livingstone, 1993). ROS can react with biological macromolecules to cause lipid peroxidation, DNA damage and protein oxidation (Shi *et al.*, 2005). Thus, it can be inferred that oxidative DNA damage under the exposure of environmental pollutants can be considered to be the most putative mechanism of genotoxicity (Hu *et al.*, 2010).

While studying the toxicity of sodium arsenite in freshwater bivalve *Lamellidens marginalis*, Chakraborty and Ray (2009) identified micronucleation, binucleation, pyknosis and nuclear disintegration as principal nuclear aberrations of hemocytes. The authors proposed the nuclear anomalies of molluscan hemocytes as an effective biomarker of environmental genotoxicity and an "early warning tool" to assess the health status of freshwater ecosystem. Stefanoni and Abessa (2011) reported genotoxicity of anionic surfactant, linear alkylbenzene sulphonate in brown mussel *Perna perna* by determining the degree of micronucleation. In the present study, treatment of washing soda resulted in an overall increase in the frequency of binucleation in *E. carteri* cells (Figure 6A). On the other hand, all experimental concentrations of washing soda for 384 hours yielded a significant increase in the frequency of micronucleation in the cells of *E. carteri* (Figure 6B). The frequencies of micronucleation and binucleation were recorded to be the highest in the cells of *E. carteri* treated with 16 mg/L of washing soda for 384 hours. Washing soda induced induction in the frequencies of both binuclei and micronuclei formation were suggestive to a high degree of genotoxicity of this aquatic contaminant in *E. carteri*. The result was indicative of a substantial damage in the genetic makeup of *E. carteri* distributed in washing soda contaminated waterbodies.

Unrestricted contamination of the natural aquifers by washing soda is thus apprehended to cause physiological and immunological stresses and adversities in diverse groups of aquatic invertebrates, including the sponge. Such a situation might lead to a shrinkage in the

population density and depletion in reproductive efficacy and survival fitness of *E. carteri* inhabiting the polluted environment. A special "sponge watch programme" (Hansen *et al.*, 1995) was launched in USA to monitor the health of aquatic ecosystem. Similarly, initiation of *E. carteri* as a biomonitoring organism might be an effective biological measure for evaluating the toxicity of environmental xenobiotics in the freshwater ecosystem of India. Furthermore, the cellular parameters like differential count, nonself surface adhesion and nuclear aberrations appeared to bear the potential to qualify as biomarkers of washing soda toxicity. The present investigation might thus help spongologists, environmental managers and aquaculturists to conserve this important bioresource for its rational utilization in a chemically safe environment.

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ORIGINAL ARTICLE

Prophylactic efficacy of some chemoprotectants against abrin induced lethality

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ABSTRACT

Abrin is a highly toxic protein produced by *Abrus precatorius*. Exposure to abrin, either through accident or by act of terrorism, poses a significant risk to human health and safety. Abrin functions as a ribosome-inactivating protein by depurinating the 28S rRNA and inhibits protein synthesis. It is a potent toxin warfare agent. There are no antidotes available for abrin intoxication. Supportive care is the only option for treatment of abrin exposure. It is becoming increasingly important to develop countermeasures for abrin by developing pre- and post-exposure therapy. The aim of this study is to screen certain pharmaceutical compounds for their chemoprotective properties against abrin toxicity *in vivo* in BALB/c male mice. Twenty-one compounds having either antioxidant, anti-inflammatory and cyto-protective properties or combination of them, were screened and administered as 1h pre-treatment followed by exposure of lethal dose ($2\times LD_{50}$, intraperitoneally) of abrin. To assess the protective efficacy of the compounds, survival and body weight was monitored. Fifteen compounds extended the survival time of animals significantly, as compared to abrin. The following five of these compounds, namely: Epicatechin-3-gallate, Gallic Acid, Lipoic Acid, GSH and Indomethacin extended the life time ranging from 6 to 9 days. These compounds also attenuated the abrin induced inflammation and enzymes associated with liver function, but none of them could prevent abrin induced lethality. The compounds offering extension of life could be useful to provide a time-window for other supportive treatment and could also be used as combinatorial therapy with other medical countermeasures against abrin induced lethality.

KEY WORDS: Abrin; antidote screening; medical counter-measures; ribosome inactivating protein; ricin

Introduction

Abrin and ricin are potent phytotoxins belonging to the family of ribosome inactivating proteins (RIPs) that inhibit protein synthesis either directly by inactivating the ribsome or indirectly by modifying factors involved in translation of protein synthesis (Olsnes & Pihl, 1973).

Abrin shows significant similarities to ricin at the sequence and structure level, but abrin is several times more potent than ricin (Stirpe *et al.,* 1992). Abrin, like ricin, is currently considered a threat to public safety because of potential application in biological warfare or terrorist attacks (Olsnes *et al.,* 1978).

Both toxins are polypeptide toxins comprised of two dissimilar polypeptide chains, A chain and B chain held together by disulfide bond. The B chain is a galactose specific lectin and hence it binds to cell surface glycosylated receptors, which allows toxin entry, while the A chain having RNA N-glycosidase activity that irreversibly inactivates the 28S rRNA of the mammalian 60s ribosomal unit and arrests host cell protein synthesis (Endo *et al.,* 1987). In addition to its ability to inhibit protein synthesis, abrin is believed to adopt alternative mechanisms to trigger apoptosis. Inactivation of antioxidant proteins resulting in increased production of reactive oxygen species are also proposed to cause toxicity by abrin (Shih *et al.,* 2001). Abrin causes apoptosis in caspase dependent manner along with loss of mitochondrial membrane potential (Bora *et al.,* 2010). Ricin has been shown to induce lipid peroxidation, glutathione depletion and DNA damage in mice (Muldoon *et al.,* 1992). Abrin and ricin are also shown to induce localized and systemic inflammation (Dickers *et al.,* 2003; Griffiths, 2011). Currently, there is no FDA-approved therapeutics available for ricin and abrin exposure. Treatment is purely supportive and

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symptomatic. Thus the development of abrin countermeasures is urgent and important.

Since abrin has been shown to induce oxidative stress, inflammation, and cytotoxicity, we investigated the efficacy of a number of compounds with properties of inhibiting oxidative stress, inflammation or cytotoxicity. These included Celastrol, Sulforaphane, Galangin, Pinocembrin, Gossypin, N-acetyl Cysteine (NAC), Epicatechin-3-gallate (EGCG), Gallic Acid, Lipoic Acid, Ebselen, Naringin, Bay 11-7085, Amifostine, DRDE-07, Caffeic Acid, Melatonin, GSH, Quercetin, Prednisolon, Minocycline hydrochloride and Indomethacin (Saxena *et al*., 2014).

We administered a lethal dose of abrin which causes consistent lethality in mice. Using this condition with death as an end point, twenty one compounds or known drugs were screened against abrin toxicity. Fifteen compounds exhibited the ability to extend the survival time, of them five compounds extended the survival time up to or beyond 6 days. Though none of them prevented abrin induced death but at least these compounds were able to provide extension of life span up to a certain extent allowing to use other medical countermeasures.

Materials and methods

Chemicals

All kits for biochemical assessment were obtained from Erba Mannheim. Cytokine levels were estimated by using ELISA kit from R & D Systems. All other chemicals were obtained from Sigma Chemicals Co (St Luis, Missouri, USA), unless otherwise mentioned.

The following drugs were used for their potential as an antidote:

Celastrol, Sulforaphane, Galangin, Pinocembrin, Gossypin, NAC, EGCG, Gallic Acid, Lipoic Acid, Ebselen, Naringin, Bay 11-7085, Amifostine, DRDE-07, Caffeic Acid, Melatonin, GSH, Quercetin, Prednisolone, Minocycline hydochloride, Indomathacin. All compounds were obtained from Sigma-Chemical Co (St Luis, Missouri, USA) except DRDE-07. DRDE-07 is an amifostine analogue and synthesized in the Synthetic Chemistry Division of the Establishment.

Isolation of Abrin

Abrin was isolated from seeds of the white variety of *Abrus precatorius* using sepharose 6B affinity column chromatography and purified as described in a previous study (Kumar *et al.,* 2008). The purity and molecular weight of abrin protein was confirmed by coomassie blue staining and MALDI-TOF (data not shown). The stock protein solution was diluted with phosphate buffered saline (PBS, pH 7.4) to a concentration of 2 mg/ml.

Animals

Balb/c male mice randomly bred in the Institute's animal facility, weighing between 22–25 g were used in this study. The animals were housed in standard conditions of temperature and humidity. The animals were fed standard pellet diet (Ashirwad Brand, Chandigarh, India). Food and water were given *ad libitum*. The animals were handled according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC) approved the experiment with approval number Tox-57/55/NS.

Treatment regimen

Each treatment group consisted of six animals. The median lethal concentration (LD_{50}) of abrin with 95 percent confidence limits for intraperitoneal (ip) route was determined by the Gad and Weil method (Gad & Weil, 1989). For each dose (log dose) six mice were used and three to four doses were administered. After administration of abrin, the animals were observed for toxicity related symptoms and mortality till the 14th day post exposure. The LD_{50} of abrin with 95 percent confidence limit was calculated from table values and was found to be 1 µg/kg with confidence limit of 0.7–1.5 µg/kg. Only the abrin treated group was administered a single dose of 2×LD_{50} of abrin (2 µg/kg body weight respectively) by ip route. The compounds were tested as 1h pre-treatment followed by abrin exposure. The doses of the compounds and route of administration were chosen based on previous published literature and at least 3 doses were used to observe their efficacy. In cases where no previous published doses were available the dose was established based on preliminary study conducted at our lab. Those compounds which offered some protection were further repeated to confirm their protective efficacy at the minimum dose offering maximum protection. All compounds were administered either ip or intragastric (ig) (Table 1). Control animals received the same volume of vehicle control as the experimental group.

Assessment of efficacy of compounds

Screening of compounds was based on mean survival time. Change in body weight was also observed till the animal survived. All mice administered abrin 2×LD_{50} dose died within 2 days. Those antidotes which extended the life time beyond 5 days were further evaluated for other parameters. For further estimation of parameters, another set of groups was formed. In one group mice were treated with abrin 2×LD_{50}. Other groups were 1h pre-treated with compounds followed by abrin 2×LD_{50} exposure. Here we used the minimum dose of compounds offering maximum protection on the basis of survival time. All animals were anesthetized and euthenized on the 2nd, 4th and 6th day of treatment.

Determination of liver body weight index (LBI)

After sacrifice on day 2, 4 and 6 liver samples were quickly removed and washed to make free for adhering material, blotted and weighed to determine liver body weight index (LBI=liver weight ×100/body weight). The dissected liver was immediately frozen in liquid nitrogen and stored at –80 °C for further studies. In abrin only treatment groups all animals were sacrificed on day 2.

Table 1. Details of the compounds evaluated for protective efficacy against abrin toxicity.

S No	Compound	Doses used (mg/kg)	Solubility	Route of administration	Category	Property
1	Celastrol	2.5, 5, 10	Ethanol	ip	Triterpenoid	Antioxidant, anti-inflammatory
2	Sulforaphane	0.05, 0.5, 1, 10	DMSO	ip	Organosulfur compound	Antioxidant, anti-inflammatory
3	Galangin	1, 10, 20	DMSO	ig	Flavonoid	Antioxidant, anti-inflammatory
4	Pinocembrin	5, 20, 40	Ethanol	ig	Dihydroxyflavanone	Antioxidant, anti-inflammatory
5	Gossypin	10, 20, 30	Ethanol	ig	Pentahydroxyflavone glucoside	Antioxidant, anti-inflammatory
6	NAC	200, 400, 800 250, 500, 1000	Water	ig ip	Acetylated variant of L-cysteine	Antioxidant , free radical scavenger
7	EGCG	0.5, 2, 10 0.5, 2, 10	Water	ig ip	Bioflavonoids,	Antioxidant , free radical scavenger
8	Gallic Acid	50, 100, 150	Water	ig	Phenolic acid	Antioxidant ,free radical scavenger
9	Lipoic Acid	50, 100, 150	Ethanol	ig	Cyclic disulfide	Antioxidant , free radical scavenger
10	Ebselen	10, 50, 100	CHCl3	ip	Organo-selenium	Antioxidants , free radical scavenger , cytoprotectants
11	Naringin	1, 2	Ethanol	ip	Flavonoid	Anti-oxidant, anti-inflammatory ,anti-apoptotic
12	Bay 11-7085	1, 2.5, 5, 10	Ethanol	ip	Nitrite containing sulfonyl group	Anti-inflammatory, anti- apoptotic,
13	Amifostine	50, 100, 200	Water	ip	Organic thiophosphate prodrug	Antioxidants , Cytoprotectants,
14	DRDE-07	100, 200, 250	Water	ip	Amifostine analogue	Antioxidants ,anti-inflammatory Cytoprotectants,
15	Caffeic Acid	5, 10, 200 5, 10, 200	Ethanol	ip ig	Phenolic compound	Antioxidant, cytoprotectant
16	Melatonin	10, 20, 50	Ethanol	ip	Alkaloid	Antioxidant protects lipids, proteins, and DNA against oxidative damage.
17	GSH	50, 100, 200	Water	ig	γ-glutamylcysteinylglycine	Antioxidant, detoxification of xenobiotics
18	Quercetin	25, 50, 75	Ethanol	ig	Flavonoid	Antioxidant
19	Prednisolone	10, 15, 20, 25	Methanol	ip	Glucocorticoid corticosterone	Anti-inflammatory
20	Minocycline hydro-chloride	5, 25, 50	Water	ip	Tetracycline derivative	Anti-inflammatory
21	Indo-methacin	1, 5, 10	Ethanol	ip	Nonsteroidal anti-inflammatory drugs	Cyclooxygenase (COX) inhibitor

Liver lipid peroxidation assay

Measurement of malondialdehyde (MDA) was used as an index for lipid peroxidation in liver. It was carried out according to a previously described method of (Ohkawa *et al.,* 1979). The colorimetric reaction between MDA and TBARS was assayed (pH 2–3, 90 °C) for 15 min. The maximum absorption was recorded at 532 nm. The level of MDA was normalized with the total protein content.

Assessment of biochemical parameters

After the 2nd, 4th and 6th day of treatment blood was collected from retro-orbital plexus of mice before sacrifice. Serum harvested from each mouse at specified time points was used to determine serum activity of lactate dehydrogenase (LDH), alanine amino transferase (ALT) and aspartate aminotransferase (AST) and total bilirubin level by commercial diagnostic kits. In abrin only treatment groups all animals were sacrificed on day 2 and serum was stored.

Measurement of serum cytokines

Levels of inflammatory cytokines TNF-α, IFN-γ and IL-6 in serum samples were measured on day 2, 4 and 6 in compound treated group followed by abrin exposure, while on day 2 in abrin exposed group, using a standard sandwich ELISA according to the manufacturer's instructions.

Statistical analysis

Results are presented as mean±SEM. Values between control, toxin alone group and the antidote treated groups were compared using Student's t-test, with $p<0.05$ as the measure for significant differences.

Results

In the present study a number of compounds having antioxidant, anti-inflammatory, anti-apoptotic and cytoprotective properties or combinations of them were included. The details regarding solubility, dose administered and route of administration are given in Table 1. For each compound at least 3 doses were used. The LD_{50} of abrin in this study was calculated 1 µg/kg through ip route. Abrin at 2 µg/kg ($2\times LD_{50}$) consistently produced

Table 2. Protective efficacy of compounds against lethal dose of abrin in mice. Mice were treated with varying doses of compounds for 1h prior to abrin (2×LD_{50}) exposure.

S No	Compound	Dose and route of administration at which maximum protection offered (mg/kg)	Time to death (Days)
1	Control	NA	NA
2	Abrin (2LD_{50})	NA	1.9±0.50
3	Celastrol	All doses	2.2±0.62
4	Sulforaphane	0.5	4.0±0*
5	Galangin	10	2.6±0.50
6	Pinocembrin	All doses	2.0±0.50
7	Gossypin	20	4.1±0.80*
8	NAC	ig –all doses ip-250	2±0.7 3.6±0.25*
9	EGCG	ig - all doses - ip-2	2±0 7.5±1.5*
10	Gallic Acid	100	5.8±1.1*
11	Lipoic Acid	100	5.8±1.4*
12	Ebselen	All doses	2.4±0.5
13	Naringin	1	4.6±0.5*
14	Bay 11-7085	2.5	4.8±0.4*
15	Amifostine	50	3.3±0.5
16	DRDE-07	100	4.8±0.8*
17	Caffeic Acid	ig -all doses ip-all doses	2.60±.59 2.1±0.74
18	Melatonin	10	3±0.6*
19	GSH	50	6±1.1*
20	Quercetin	50	3.25±1.1
21	Prednisolone	20	3±0*
22	Minocycline hydrochloride	25	4±0.7*
23	Indomethacin	5	6.5±1.5*

Values are mean±SEM of six animals. *Significantly different from abrin group at $p<0.05$ by student's t test. The survival of mice was recorded daily and reported in days.

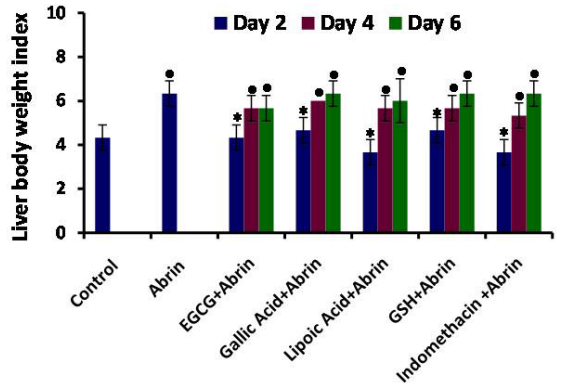

Figure 1. Protective potential of EGCG (2 mg/kg), Gallic Acid (100 mg/kg), Lipoic Acid (100 mg/kg), GSH (50 mg/kg) and Indomethacin (5 mg/kg) pre-treatment on liver body weight index after challenge with lethal dose (2×LD_{50}) of abrin on day 2, 4 and 6. Values are mean ± SEM of six animals in each group. * Significantly different from control for the same day and *significantly different from abrin at $p<0.05$ by student's t test.

100% lethality and the mean time to death varied from 1–2 days. Ip administration of abrin causes weight loss, drowsiness and diarrhea in mice. As mice were expected to die in about 1–2 days after abrin treatment, any significant delay in death by administration of this compound would be a protective effect. Table 2 summarizes the results on protective efficacy of the compounds. For each compound minimum dose offering maximum protection was calculated. A substantial number of compounds increased survival time but none of them protected from abrin induced lethality. Sulforaphane, Gossypin, NAC, EGCG, Gallic Acid, Lipoic Acid extended the survival time, but offered no protection from abrin induced lethality. Similarly Naringin, Bay 11-7085, Amifostine, DRDE-07, Melatonin, GSH, Predniosolone, Minocycline hydrochloride, Indomethacin extended the mouse survival but could not prevent lethality. Compounds having antioxidant, anti-inflammatory, antiapoptotic properties admistered alone or combinations of them provided extension of survival time suggesting no specific properties of compound responsible for protection. Among all the compounds which offered partial protection, five compounds EGCG, Gallic Acid, Lipoic Acid, GSH, and Indomethacin extended the survival time beyond 5 days. EGCG (2 mg/kg) by ip route was found to be the most potent antidote increasing the life time up to 7 days. Interestingly, EGCG did not provide any protection by ig route of administration. No toxicity was observed with any of the solvents or compounds when administered alone (data not shown). In the abrin treated group there was a drastic decrease in body weight on the second day, as compared to the initial weight, and all animals died on the 2nd day. On day 2 EGCG, Gallic Acid, Lipoic Acid, Naringin, Bay 11-7085, DRDE-07, Malatonin, GSH, Quercetin, Prednisolone, Minocycline, Indomethacin significantly protected against the body weight loss as compared to the decrease in weight in the abrin treated group. On day 4 and 6 there was further decrease in body weight in EGCG, Gallic Acid, Lipoic Acid, GSH, Indomethacin treated mice as compared to day 0, leading eventually to death (Table 3).

Effect of the compounds on LBI

Figure 1 shows the effect of pharmaceutical compounds on LBI. On day 2 there was significant increase in LBI in abrin treated animals as compared to control mice, but LBI in the groups treated with compounds were comparable to control. On day 4 and 6, LBI was found to be significantly increased in the group treated with compounds followed by abrin exposure as compared to control mice but still comparable to the abrin exposed group.

Effect of abrin and pre-treatment of compounds on liver lipid peroxidation

The deleterious effect of reactive oxygen species is measured by the amount of lipid peroxidation. MDA is commonly measured as a lipid peroxidation marker. There was more than a 3-fold increase in MDA formation in the abrin treated group compared to control.

Table 3. Effect of abrin and pre-treatment of compound against abrin toxicity on mice body weight.

Group	Day 0 Body weight(g)	Day 2 Body weight(g)	Day 4 Body weight(g)	Day 6 Body weight(g)
Control	23±0.5	24±0.8	26±0.8•	27±0.5•
Abrin(2×LD_{50})	24±0.5	19.6±0.3•	–	–
Sulforaphane(0.5 mg/kg)+Abrin(2×LD_{50})	23±0.9	21±0.7•	20±0.7•	–
Gossypin(20 mg/kg)+Abrin(2×LD_{50})	25±0.4	21±0.2•	18±0.5•	–
NAC(250 mg/kg)+Abrin(2×LD_{50})	23±1.0	18±0.4•	–	–
EGCG(2 mg/kg)+Abrin(2×LD_{50})	23±0.5	24±0.8*	23±0.64*	22±1.1
Gallic Acid (100mg/kg)+Abrin(2×LD_{50})	25±0.5	24±1.2*	23.5±0.3•*	21±0.6•
Lipoic Acid(100mg/kg)+Abrin(2×LD_{50})	25±0.2	25±1.2*	22±0.5*	20±0.3•
Naringin(1mg/kg)+Abrin(2×LD_{50})	24±1.5	23±1*	22±0.3•*	–
Bay 11-7085(2.5mg/kg)+Abrin(2×LD_{50})	25±0.4	23±0.8*	21±0.8•	–
Amifostine(50mg/kg)+Abrin(2×LD_{50})	24±0.7	19.7±1.5•	16±0.9•	–
DRDE-07(100mg/kg)+Abrin(2×LD_{50})	23±0.8	24±0.1*	23±0.35*	–
Melatonin(10mg/kg)+Abrin(2×LD_{50})	24±0.6	24.5±0.2*	20±1.2•	–
GSH(50mg/kg)+Abrin(2×LD_{50})	24±0.9	25±0.9*	23±0.5*	22±0.05•*
Quercetin(50mg/kg)+Abrin(2×LD_{50})	25±0.2	24±0.8*	22±0.1•*	–
Prednisolone(20mg/kg)+Abrin(2×LD_{50})	24±0.7	21±0.8*	–	–
Indomethacin (5mg/kg)+Abrin(2×LD_{50})	25±0.2	25±0.7*	23±0.9•*	20±1•

Significance $p<0.05$; *Abrin group (day 2) *vs.* treatment group (day 2, 4 and 6); • within same group day 0 *vs.* day 2, 4 and 6. The body weight of mice was recorded daily till the animal survived.

Pre-treatment of EGCG, Gallic Acid, Lipoic Acid, GSH caused significant reduction in lipid peroxidation as compared to the abrin treated group, but still their level was significantly higher than untreated mice at all three time points, suggesting partial protection offered by these compounds. Indomethacin pre-treatment was not able to suppress abrin induced MDA level at any time point studied (Figure 2).

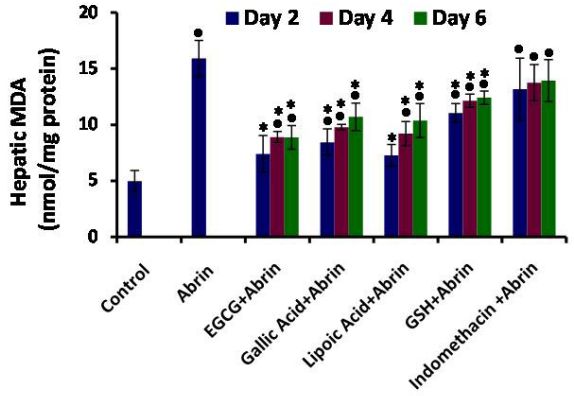

Figure 2. Protective effect of EGCG (2 mg/kg), Gallic Acid (100 mg/kg), Lipoic Acid (100 mg/kg), GSH (50 mg/kg) and Indomethacin (5 mg/kg) pre-treatment on hepatic MDA level after exposure to lethal dose (2×LD_{50}) of abrin on day 2, 4 and 6. Values are mean ± SEM of six animals each group. • Significantly different from control and *significantly different from abrin at $p<0.05$ by Student's t-test.

Effect of compounds on biochemical parameters

Serum enzymes AST, ALT, total bilirubin, LDH are the enzymes commonly used for liver cell integrity. Abrin exhibited toxicity as indicated by the significant increase in the level of these enzymes as compared to control. Pre-treatment of EGCG significantly brought down the level of serum AST, ALT, total bilirubin and LDH augmented by abrin but could not suppress them to the level comparable to control. Similar observation was found with gallic acid pre-treatment for serum AST, ALT and total bilirubin levels, except serum LDH. Serum LDH was found be the same as in abrin treated mice in Gallic Acid treated group on day 4 and day 6. Lipoic Acid pre-treatment also significantly attenuated serum AST, serum ALT, total bilirubin and serum LDH level as compared to levels in abrin treated mice. GSH pre-treatment also decreased the level of serum AST, ALT, total bilirubin and serum LDH activity increased by abrin. GSH and indomethacin pre-treatment could not inhibit serum AST activity on day 4 and 6, as compared to abrin. Indomethcin significantly decreased the total bilirubin level increased by abrin on all three days of the study. At the initial time point serum LDH and ALT were decreased by indomethacin pre-treatment but at a later time point there was further increase in the level, reaching the level of abrin treated mice (Figure 3).

Effect of compounds on pro-inflammatory cytokines

Serum IFN-γ, IL-6 and TNF-α are the cytokines associated with inflammation. We also examined the effect of pretreatment with these compounds on inflammatory cytokines. Abrin elevated the serum IFN-γ (339±46 pg/ml; 4 fold), IL-6 (364±60 pg/ml; 4 fold) and

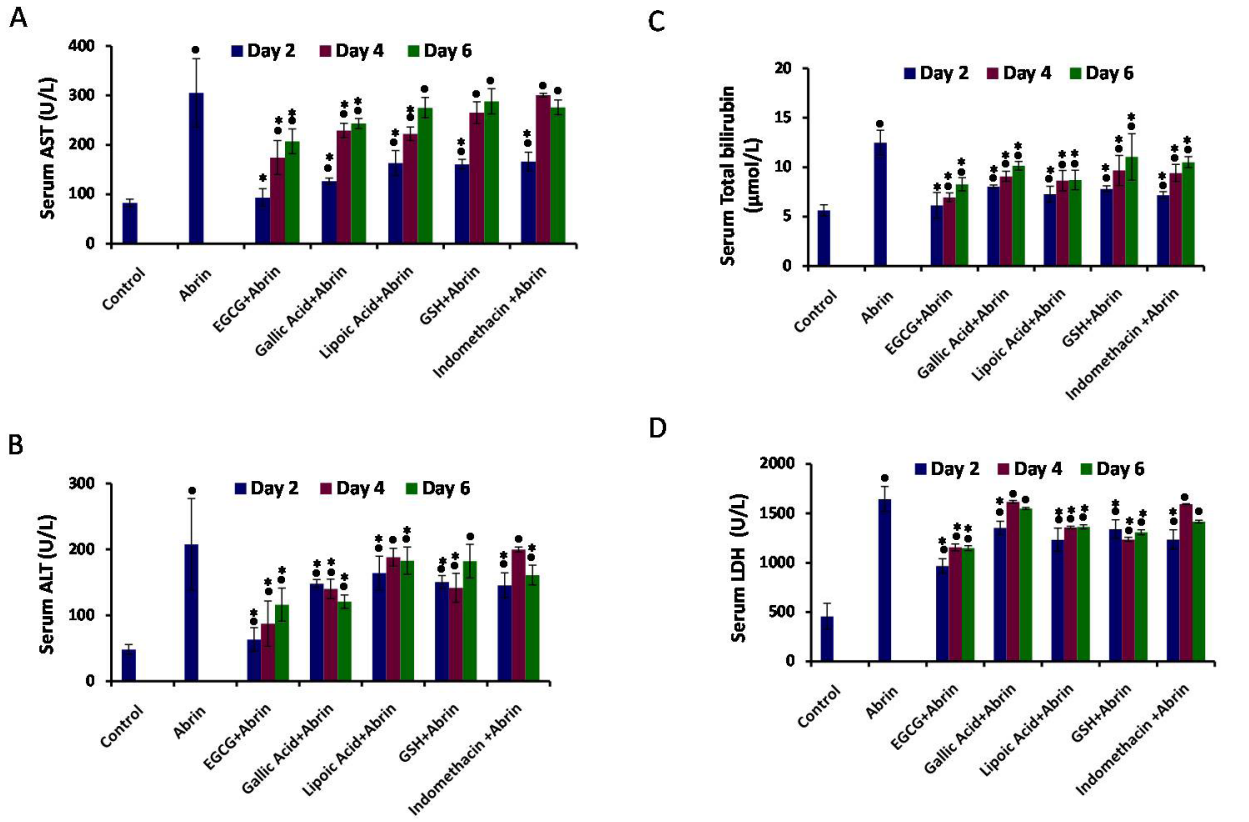

Figure 3. Protective efficacy of EGCG (2 mg/kg), Gallic Acid (100 mg/kg), Lipoic Acid(100 mg/kg), GSH (50 mg/kg) and Indomethacin (5 mg/kg) pre-treatment on (A) serum AST; (B) serum ALT; (C) serum total bilirubin and (D) serum LDH after challenge with lethal dose ($2\times LD_{50}$) of abrin on day 2, 4 and 6. Values are mean ± SEM of six animals each group. * Significantly different from control and *significantly different from abrin at $p<0.05$ by Student's t-test.

TNF-α (446±60 pg/ml; 5 fold) level as compared to control. All the compounds significantly decreased the level of all three cytokines as compared to abrin. Although gallic acid and lipoic acid pre-treatment on day 4 and 6 could not suppress TNF-α levels increased by abrin exposure.

Discussion

Abrin and ricin are most dangerous plant toxins and were classified as potential agents for biological warfare and bioterrorism by the Biological and Toxin Weapon Convention (BTWC, 2001). Though the mechanisms of toxicity of RIPs at cellular and molecular levels have been delineated previously, the development of antidote has proven elusive (Miller *et al.,* 2002). As one of the potential bioweapons, development of abrin countermeasure has received considerable attention. The immediate consequence of abrin poisoning is oxidative stress, inflammation, cytotoxicity. The mode of action at cellular level is the specific inhibition of protein synthesis. Studies aimed at finding an antidote for ricin have shown significant extension of survival time in mice (Muldoon & Stohs, 1994). Earlier studies also indicate a possible role for free radical scavenger in antagonizing abrin induced toxicity (Saxena *et al.,* 2014).

Few antioxidants, anti-inflammatory and cyto-protective compounds have been shown to counteract the oxidative damage and inflammation produced by toxicants (Muldoon & Stohs, 1994; Sorrenti *et al.,* 2013). The compounds of these categories were therfore screened for their ability to inhibit abrin induced toxicity by reducing oxidative stress and inflammation. Of the compounds tested, a few provided significant extension of survival time, while the other compounds had no effect at the doses used in the study.

Compared to abrin treatment where all animals were died within 2 days, 15 compounds extended the survival time ranging from 3 days to 7 days but their body weight decreased drastically. EGCG, Gallic acid, Lipoic Acid, GSH and Indomethacin increased protection beyond 5 days and maximally up to 7 days. All these 5 compounds suppressed the oxidative stress, inflammation and liver function associated parameters elevated by abrin exposure, but none of them could induce decrease up to the level of control animals. Among all the compounds studied, maximum life time extension was provided by EGCG. EGCG is the major catechin found in green tea and functions as a powerful antioxidant, preventing

oxidative damage in healthy cells. In the present report EGCG effectively decreased the abrin induced level of MDA, inflammatory cytokines, oxidative stress, serum AST, ALT, total bilirubin and serum LDH as compared to gallic acid, lipoic acid, GSH and indomethacin pretreatment. In our previous study, abrin has been shown to induce Fas pathway of apoptosis, It could be possible that EGCG suppresses abrin toxicity by inhibiting expression of the ligand of death receptor (Fas L), as shown in cisplatin induced nephrotoxicity. EGCG increases the activity of phase II detoxifying enzymes in mouse liver and blocks a wide array of signal transduction pathways, which may be another reason of highest protection achieved by EGCG. Protection by EGCG by ip route not by ig route suggested that the route of administration greatly matters and affects bioavailability. In concordance with previous reports it is suggested that ig administration of EGCG is not effective due to inefficient absorption or metabolism in the digestive tract (Yuan *et al.,* 2012). A similar observation was found with NAC where *ig* administration was not effective, while *ip* administration marginally increased the survival time (Saxena *et al.,* 2013; Zou *et al.,* 2014; Singh *et al.,* 2011). Via ig and oral route of administration, NAC undergoes deacetylation and produces cysteine, a precursor of glutathione. We thought it appropriate to present glutathione (Shalansky *et al.,* 2005) as this route may be beneficial to suppress abrin induced oxidative stress. But unfortunately, no protection was observed using the ig route of administration. Since abrin has also been shown to induce hepatotoxicity (Niyogi, 1977), we hypothesize that NAC via ip route may reach the liver and neutralizes the toxicity. Although the ip route of NAC has offered protection by extending the life span of mice against abrin toxicity but it was less significant as compared to EGCG, Gallic acid, Lipoic acid, GSH and Indomethacin. In the cisplatin induced nephrotoxicity model of the rat, NAC was tested by ip, oral, intravenous (iv) and intra-artrial (ia) route. There was no protection with oral and ip route but the iv and ia route of administration showed significant protection, suggesting that the route of administration can have a profound effect on the efficacy of chemoprotectants and an elaborated study is warranted with using the iv and ia route of administration against abrin toxicity (Dickey DT *et al.,* 2008).

In the present report, abrin is shown to deplete glutathione level and increase lipid peroxidation, similarly to previous studies where ricin treatment was shown to elevate lipid peroxidation (MDA), while GSH was decreased in both liver and kidney (Kumar *et al.,* 2003, Muldoon *et al.,* 1992). Keeping in mind the ability of GSH to replenish glutathione level and attenuate lipid per oxidation, it was tested for its ability to reduce abrin toxicity. GSH increased the life span up to 6 days. Surprisingly, NAC and amifostine, possessing a similar property of thiol modulation, were not able to protect the mice up to the same extent. DRDE-07, which is an amifostine analogue, significantly extended the survival time and partially better than amifostine, which may be due to the presence of an aryl group in DRDE-07 which increases its lipophilicity and thus its bioavailability (Kerksick & Willoughby, 2005; Vijayaraghavan *et al.*, 2001). Gallic acid and lipoic acid, well known antioxidants and free radical scavengers, extended the survival time up to 6 days, while Galangin, Pinocembrin, Ebselen Caffeic Acid, which are also having antioxidant property, did not offer any protection. Flavonoids are another group of cytoprotectans which donate the hydroxyl group to the free radicals, sparing GSH to interact with other free radicals. Naringin, belonging to the group of flavonoids occurring naturally in citrus fruit, extended the survival time up to 4 days, while quercetin, which is also a flavonoid, did not offer the same protection. Similarly to Naringin, Gossypin is

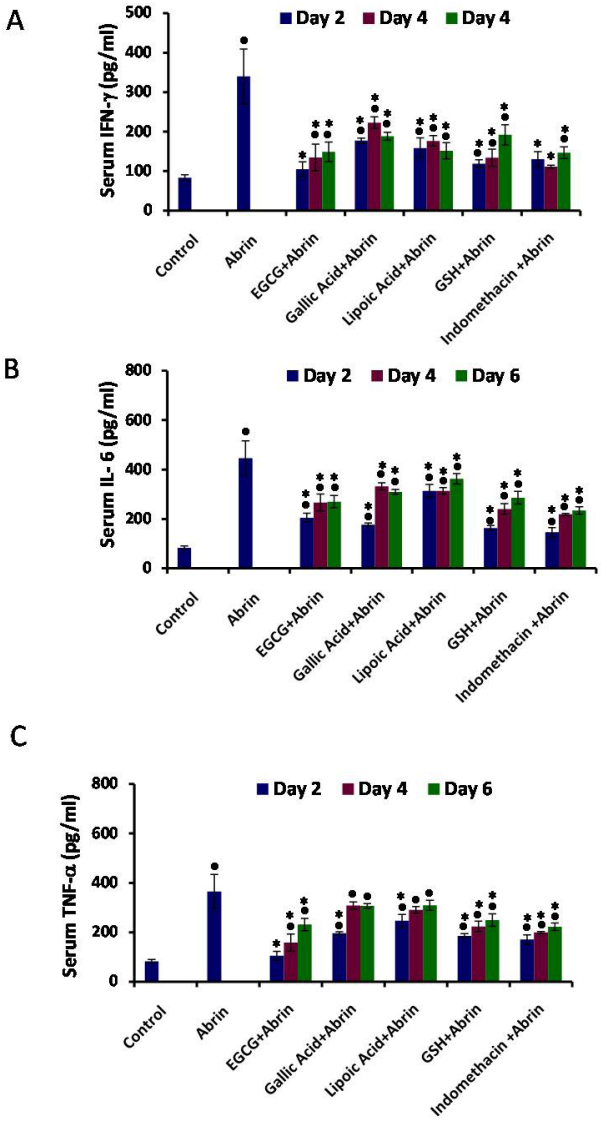

Figure 4. Protective potential of EGCG (2 mg/kg), Gallic Acid (100 mg/kg), Lipoic Acid (100 mg/kg), GSH (50 mg/kg) and Indomethacin (5 mg/kg) pre-treatment on inflammatory cytokine levels after exposure to lethal dose (2×LD_{50}) of abrin on day 2, 4 and 6. (A) serum IFN-γ level; (B) serum IL-6 level; (C) serum TNF-α level. Values are mean ± SEM of six animals each group. * Significantly different from control and *significantly different from abrin at $p<0.05$ by Student's t-test.

another flavonoid. It exhibited anti-inflammatory action and increased the life span up to 4 days. Indomethacin is a known non-steroidal anti-inflammatory drug which could extend the life span significantly, possibly by suppressing abrin induced inflammation. Bay11-7085 and prednisolone are further anti-inflammatory compounds which offered significant increase in life span but less than did indomathacin (Strickson *et al.,* 2013; Garg *et al.,* 1994).

Suforaphane, Melatonin and Minocycline also provide significant extension of survival time owing to antioxidant and anti-inflammatory activity, while celastrol did not provide any protection in spite of having similar properties.

A few compounds of antioxidant and anti-inflammatory activity are offering protection, while other compounds having the same property are not. This is suggesting that differences in chemistry between these compounds may be responsible for different efficacy. Another reason could be that the structural difference in the compounds leads to different mechanisms of action for protection or difference in bioavailability at the site of action. Further modulation of abrin toxicity may also depend upon pharmacokinetics, bioavailability and doses of compounds. Those compounds offering protection were also tried for repeated treatment but could not provide any additional significant protection (data not shown). In the present study, one of the limitations is lack of data of compound efficacy after abrin treatment. But no beneficial effects of repeated treatment of few compounds suggest these compounds may be not beneficial for therapeutic treatment. Since these compounds are offering extension of life, combination of these treatments among themselves as well with other treatment modalities may be useful to inhibit abrin toxicity.

Only life time extension for a few days and then death suggests at a later course of action of abrin toxicity some other pathways to be dominating, which are responsible for the death of mice. Abrin toxicity is shown to associate with multiple modes of cell death, as inhibition of protein synthesis leads to activation of MAPK pathway and caspase 3 activation. Abrin is also shown to exert cytotoxicity via other pathways like receptor mediated extrinsic pathway as a secondary consequence of toxicity. Further cross talk between these pathways may aggravate the toxicity. It can be concluded that our compounds are only neutralizing the consequences of toxin up to some extent but increase in all parameters associated with stress at later time points and death after some time suggest that direct neutralization of toxin alone or combination with these compounds may be ideal approach and good therapeutic option.

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SHORT COMMUNICATION

Multiple giant cell formation – A consequence of type II pyrethroid intoxication

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ABSTRACT

Pesticides are the main remedy for pest eradication, but their use has been found to be harmful also to various non-target organisms. In this study, giant cell formation was observed in hepatocytes of experimental albino rats following two type II pyrethroid pesticdes, Cypermethrin and Beta-cyfluthrin. Histopathological examination was done for this purpose and the results revealed the formation of giant cells and polyploidy condition following intoxication of these experimental compounds with Beta-cyfluthrin, with an edge over, and Cypermethrin, probably due to structural differences.

KEY WORDS: pyrethroids; toxicity; liver

Introduction

The use of pesticides and their designing has increased with great pace pertaining to increased global food demands as well as genetical modifications leading to resistance development in various pest species. Pyrethroid pesticides, covering of a large part of the world insecticide market, are an integral part of eradication programs and find their use both in domestic and large scale field purposes (Bian *et al.,* 2004; Bhalli *et al.,* 2006; Bhushan *et al.,* 2010; 2013, Saxena & Gaur, 2016). Pyrethroids are considered comparatively safe pesticides and therefore many new synthetic analogues have been added to the market. Contrary to their broad spectrum of use due to their low photostability and non-target toxicity, they can disturb various ecological balances existing on this planet (Rana *et al.,* 2008; Bhushan *et al.,* 2013; Pande *et al.,* 2014, Saxena & Gaur, 2016; Saxena & Bhushan, 2017). We have therefore evaluated and compared hepatocytic toxicity of two broadly used types pyrethroid pesticides, *i.e.* cypermethrin and beta-cyfluthrin, focusing on giant cell formation if any, through histological analysis. Beta-cyfluthrin was found to be designed from cypermethrin by introduction of a fluorine atom at position 4 of the phenyl ring in its structure (Figures 1 and 2).

The present study was conducted on 75 female albino rats, *Rattus norvegicus,* selected from an inbred colony. The animals were about two weeks in age and 100±20 g in weight. They were kept under appropriate temperature and light conditions, provided with standard rat pellet feed and water *ad libitum.* After one week of acclimatization to laboratory conditions, the rats were divided into three sets of equal number, *i.e.* 25 rats each. The experimental compounds cypermethrin and beta-cyfluthrin (technical grades of approximately 95% purity) were orally administered to the rats in acute ($1/10^{th}$ of LD_{50} for 1 d) and sub-acute($1/10^{th}$ of LD_{50} for 7, 14, 21 and 28 ds) doses. All these three sets, one corresponding to control and the other two, *i.e.* cypermethrin and beta-cyfluthrin, were subdivided into five subsets each comprising five rats. LD_{50} for cypermethrin and beta-cyfluthrin (Finney, 1971) were 416.98 and 354.8 mg/kg b.wt., respectively (Bhushan *et al.,* 2010; 2013; Bhushan & Saxena, 2017). The rats were then sacrificed at predetermined time intervals. Liver tissue was excised, washed in physiological saline, cut into small pieces, fixed in carnoys fixative (Gatenby & Beams, 1950) for 4 hours, dehydrated, embedded, sectioned (5 µ sections) and finally stained with hematoxylin and eosin (Humason, 1979). These sections were then observed at 400× and 1 000× and appropriate locations were photographed. Maximum incidence of hypertrophy and giant cell formation was found in albino rat liver intoxicated with sub-acute dose for 14 days. The beta-cyfluthrin intoxication was more expressive than that of cypermethrin (Figures 3–5).

The liver size of an organism is tightly regulated, yet stress conditions can increase its size markedly. The consequences of increasing liver size are primarily increase in cell division (hyperplasia) or increase in cell volume (hypertrophy). Giant cell formation as observed in the

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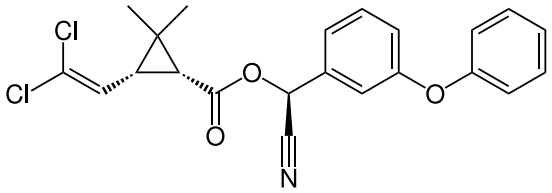

Figure 1. Structure of cypermethrin.

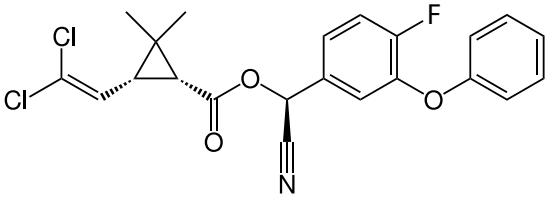

Figure 2. Structure of beta-cyfluthrin.

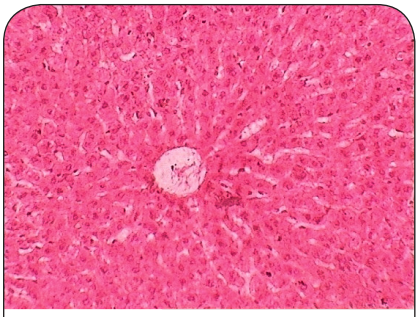

Figure 3. Histo-architecture of control albino rat liver (400×).

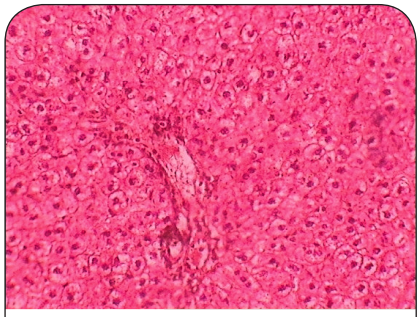

Figure 4. Histo-architecture of cypermethrin intoxicated albino rat liver (400×).

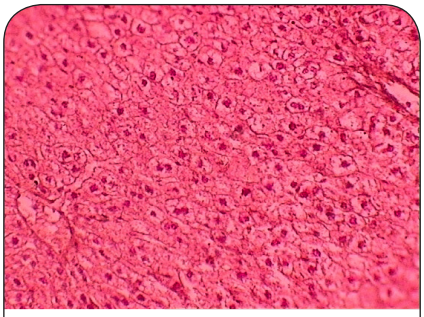

Figure 5. Histo-architecure of beta-cyfluthrin intoxicated albino rat liver (400×).

present study may be a consequence of hypertrophy, which may be a consequence of polyploidy condition. Polyploidy in turn is associated with altered DNA function. However, it is now broadly accepted that type II pyrethroids, including both our experimental compounds, are well capable of causing chromosomal as well as DNA damage and altering cell cycles, which may lead to consequences of tumor promotion (Singh & Saxena, 2002; Bhushan *et al.,* 2010; Madkour 2012). It is though noteworthy to mention cancer promotion by these experimental compounds. The previous results of excess DNA synthesis and now the presence of giant cell formation in subpopulations of hepatic lobule point towards the same aspects. Tumor promotion, is a very complex process having various mysterious and multistep pathways. But initially carcinogenesis induces nuclear enlargement which is generally associated with increase in DNA content. Increase in DNA content and altered cell cycle can also be justified by the fact in our study that we got hepatic ALP values lower in treated rats than in controls (Bhushan *et al.,* 2013). This fact concerns also altered DNA synthesis as ALP is an important enzyme catalyzing cleavage of phosphate groups, an integral part of DNA. This suggest formation of ploidy conditions in these hepatocytes, which further can get transformed into malignant.

Further, giant cell formation is more pronounced in case of beta-cyfluthrin than cypermethrin, probably due to structural differences in their chemistry. Beta-cyflthrin is a more recent pyrethroid product than cypermthrin and has been designed by modifying basic cypermethrin structure at the level of addition of fluorine group to position 4 of phenyl ring in beta-cyfluthrin, which seems to be responsible for its enhanced toxicity as C-F bond is one of the strongest bond and difficult to break (Bhushan *et al.,* 2013; Bhushan & Saxena, 2017).

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