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Emergence of Zebrafish as a Biomarker for Pesticide Poisoning in Forensic Toxicology Research

Saurabh Shukla, Reena C. Jhamtani and Rakhi Agarwal*

Laboratory of Analytical and Molecular Toxicology (Forensic Chemistry and Toxicology Laboratory), Institute of Forensic Science, Gujarat Forensic Sciences University, Sector 09, Gandhinagar – 382007, Gujarat, India; sau47bt@gmail.com, lamt.dct.gfsu@gmail.com , reenajhamtani@gmail.com

Abstract

Forensic toxicology is the branch of forensic science which utilizes toxicology, pharmacology and analytical chemistry to help in legal investigations of suicidal, homicidal and accidental poisoning cases. Accidental poisoning cases related to occupational exposure of pesticides are also very common. Zebrafish is a versatile model organism that has been used as a model organism in different field of science, has various practical advantages over other vertebrate models. Present review summarizes the effects of pesticides and the use of adult zebrafish as a model organism in forensic toxicology research to understand the symptoms/effects and mechanism of pesticide poisoning.

Keywords: Forensic Science, Forensic Toxicology, Oxidative Stress, Pesticide Poisoning, Zebrafish

1. Introduction

Forensic toxicology can be defined as the study of poisons i.e., how poisons encounter, how individuals respond and how to develop strategies for the clinical and regulatory management of toxic exposures. It involves the use of toxicological sciences, analytical chemistry and pharmacology to help in the legal investigations of fatal and survival poisoning cases. The primary aim of a forensic toxicologist is to provide outcome of poisoning investigations for legal procedures which involves the interpretation of findings. Poisons can be generally defined as biologically active substances causing toxic effects in living organism. Any biologically active molecule, capable of altering normal physiology within a living system is a poison. It causes bioaccumulation leading to quantity that is sufficient to produce toxic effects which ultimately leads to death. Pesticidal poisoning, is one of the oldest methods for committing suicide[1]. Residue of pesticides can be seen in almost all environmental components, fresh water sources and food samples. In addition to this, pesticides are the major cause of environmental pollution and there is a global concern over aquatic pollution because it shows harmful effects which are not only to aquatic animals but also affects human being[2, 3]. Pesticide can be defined as any substance or mixture of substances intended for preventing, destroying, or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals that causing harm during or interfering with the production, processing, storage, transport, or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs[4]. The field of forensic toxicology research also involves the assessment of mechanism of toxicity of pesticide on the target organ. Different pesticides have different mode of action and selected target organ thus it is necessary to understand the effects of pesticides on their target organ including whole body system.

2. Medicolegal Aspect of Pesticide Poisoning

Poisoning may be intentional viz. homicidal and suicidal or unintentional viz. accidental (survival or fatal) and occupational (survival with side effects).

*Author for correspondence

2.1 Suicidal Case

A 20 years male student was admitted in ICU with an episode of seizure and altered sensorium. Earlier he had no signs of illness and had travelled to Mumbai before four days. At the time of admission, he was restless with the pulse rate of 62/minute, blood pressure of 120/80 mmHg, with plenty of oral secretions. Different Neurological tests were carried out which shows Glasgow Coma Scale (GCS) of 6/15. Pupil's movements were accurate with Doll's eye movement absent. At the time of admission normal renal and liver function were observed with standard serum levels of sodium, potassium, calcium, and magnesium. Blood examination shows leukocytosis while chest X-ray showed acute respiratory distress. Computed tomography and magnetic resonance imaging scan of the brain were done and found to be normal. On second day he showed suffering with carbon dioxide retention and kept on ventilator for next 12 days. On sixth day he gave in written that he had injected metacid (methyl parathion) while travelling in a train with suicidal intention. Methyl parathion an organophosphate insecticide is known to be highly toxic to human[5].

2.2 Occupational Exposure

A 60 years old male farmer was admitted to hospital in feeble minded condition suffering from vomiting, diarrhoea and unable to stand properly. According to his family members, he was spraying the pesticides in fields and had difficulty in breathing. Later on developed nausea, vomiting, abdominal cramps and muscle twitching. There was no significant co-morbid medical illness and relatives also denied consumption of any drugs, poison or medications except for the use of pesticide fumigation. On physical examination, his vitals were normal with oxygen saturation of 60%. There was no pallor, cyanosis or injury marks. On neurological examination he was drowsy, having GCS of 8/15 with no focal neurological deficit. Chest X-ray was hazy but the ECG was normal[6].

The mentioned incidences are not limited, many pesticide poisoning cases could not be identified due to lack of diagnosis methods or awareness about the toxic symptoms develops by pesticides. Therefore, there is a need to understand the biochemical changes which occur due to acute pesticide poisoning so that diagnosis of pesticide exposure can be performed. Pesticide can cause multiple toxic manifestations in poisoning cases, thus present review provides baseline data for understanding the toxic manifestations due to commonly and widely used pesticides. Along with the banned pesticide aldrin and lindane, which is however still present in the environment.

Atrazine (ATZ) is one of the most commonly used herbicides in the world. Atrazine is odourless, white powder, less volatile and water soluble, some of the trade names of atrazine are aatrex, aatram, atratol and gesaprim[7]. Atrazine is widely used on corn, sorghum, sugarcane and pineapples to kill weeds; it can enter the air, while some may be washed away by rainfall and enter surrounding area like lake and stream. ATZ is one of the most commonly detected herbicide in rivers worldwide. It is resistant to degradation with half-life in surface water about >200 days; due to high persistency in nature it has been detected in high amount i.e. upto 22 mg L^{-1}. As per World Health Organization (WHO) guidelines, Acceptable Daily Intake (ADI) of ATZ is 0-0.02 mg/kg body weight (bw) and permissible limit in drinking water is 2 μg L^{-1}. It is rated as moderately toxic to aquatic animals[8]. Many studies suggested that atrazine act as an endocrine disruptor in animals and zebrafish[9]. Reports are available on atrazine poisoning, as seven family members were died and it was found that they have consumed cooked beans. Later on, atrazine was detected significantly in high amount in remaining food material, stomach content, blood and liver samples of the victims[10]. Atrazine is primarily excreted in urine in the form of dealkylated metabolites and mercapturic acid derivatives. Mercapturic acid derivatives are considered as useful biomarkers of atrazine exposure. The primary target organs of atrazine toxicity are the female reproductive system and liver. The reproductive effects in animals include transformed estrus cycle[11], serum and pituitary hormone levels[12], reduced fecundity, reduced ovarian and uterine weight[13]. The hepatic effects caused by atrazine exposure include increased serum lipids and liver enzyme[14], changes in liver weight, triglycerides and globulin levels[15]. All these effects may serves as a useful biomarker of atrazine exposure. Glyphosate is an herbicide, applied on the broadleaf plants and grasses. The trade names of glyphosate are Roundup, Rodeao, Pondmaster, etc. Glyphosate is a non-selective herbicide, it prevents the plants from making certain protein that are needed for plant growth. Berets et al. (2005) have observed increased Lipid Peroxidation (LPO) and antioxidant enzyme activity due to glyphosate in the maternal serum and liver of pregnant rats[16].

Dichlorvos (DIC) is an organophosphate and it was introduced in 1960s as a replacement of organochlorine pesticides. Dichlorvos is usually used as an agricultural insecticide on crops and stored products but is also used as and anti-helminthic (worming agent) for dogs, swine and horses, as a botacide agent that kills flies larvae. Dichlorvos is poisonous if swallowed, inhaled, or absorbed through the skin. The permissible limit of DIC in drinking water is 0.02 mg L^{-1}. ADI of DIC is 0-0.004 mg/kg bw. DIC has been listed by International Agency for Research on Cancer (IARC), WHO as Class, B2 "probably carcinogenic to humans"[17]. Dichlorvos is reported to be neurotoxic due to its irreversible inhibitory effects on acetylcholinesterase enzyme. This inhibition provokes an accumulation of acetylcholine in synapses with disruption of the nerve function that can end in the death of the organism[18]. In a rare case of accidental poisoning, a 25 day old infant diagnosed with the sign of pesticide poisoning who was further treated accordingly and recovered[19]. Dichlorvos at high doses causes symptoms of toxicity such as miosis, tremor, increased salvation, lacrimation, pulmonary secretions and perspiration. Dichlorvos can reduce the activity of two enzymes in the blood, serum cholinesterase and erythrocyte acetylcholinesterase. Serum cholinesterase appears to be more sensitive to inhibit by dichlorvos than erythrocyte acetylcholinesterase[20]. However, the activity of serum cholinesterase enzyme recovers rapidly than erythrocyte acetylcholinesterase because of the higher turnover rate of serum proteins as compared to erythrocyte[21]. The major metabolic products of dichlorvos are dimethyl phosphate and dichloroethanol. These compounds are rapidly excreted into the urine and eliminate from the body within 24-48 h[22]. Methyl parathion is an organophosphate class insecticides used to control biting or sucking insect pests of agricultural crops. These chemicals acts by interfering with the activities of cholinesterase, an enzyme that is essential for the proper working of the nervous systems in humans, animals and insects. Methyl parathion is highly toxic by inhalation and ingestion while moderately toxic by dermal adsorption. Because methyl parathion has a short half-life (1 hour on cotton) when applied to crops, the risk of exposure to agricultural workers is low. Factory workers who handle quantities of concentrated methyl parathion are at a higher risk. Exposure may occur during mixing, spraying or application of methyl parathion, during cleaning and repair of equipment or during early re-entry into fields. Celik and Suzek, (2008) have reported increased Malondialdehyde (MDA) levels and altered antioxidant enzymes activity (SOD, CAT, GST and GR) in all the examined tissues in rats with mild dose of methyl parathion[23].

Imidacloprid is a nicotinyl class insecticide introduced in the market in 1991 as a replacement of organophosphorus pesticide[24]. Imidacloprid is used to control aphids, plant hoppers, and whiteflies. It affects insect's nicotinic acetylcholine receptor and results in killing of insect[25]. Nicotinyl insecticides interact with nicotinic acetylcholine receptors (nAChR) at the central and peripheral nervous systems, resulting in excitation and paralysis followed by death. It simulates the activity of acetylcholine which results from the inhibition of acetylcholinesterase by organophosphorus compounds. Imidacloprid interacts with nAChR in a structure-activity relationship and shows a strong affinity to the insect receptor with a relatively low affinity to the vertebrate receptor, indicating a high selectivity towards insects as compared with the first compound in nicotine series. Research findings have raised concern over the use of imidacloprid, as it effects egg shell thinning, reduction in egg production and hatching time[26]. Kapoor et al.,[27] observed that a dose of 10 mg/kg/day for 90 days has not produced any major biochemical alterations on treated animals although changes. Imidacloprid is also known to affect levels of testosterone, sperm mortality and increased apoptosis of germ cell[28]. Imidacloprid is quickly absorbed by the oral route and rapidly distributed in nearly all organs. Imidacloprid metabolites were excreted primarily in the urine as glutathione, glycine conjugates of mercaptonicotinic acid and hippuric acid[29]. The liver is found to be the main target organ of imidacloprid exposure; the alterations include induction of hepatic microsomal enzymes activity. Imidacloprid toxicity leads to increased activity of cytochrome P-450 enzyme, with hepato-cellular hypertrophy, necrosis, swollen cell nuclei and increased liver weight[30]. Significant changes were observed in GPT, GOT, glucose and BUN levels due to imidacloprid induced toxicity[31]. Velisek, (2018) have observed suppressed SOD and GR activity in embryos and larvae of common carp, Cyprinus carpio due to thiacloprid exposure[32]. Chronic thiacloprid exposure affected ontogeny and growth rate in Cyprinus carpio. Yan et al., (2016) have observed elevated ROS, MDA levels, DNA damage and altered antioxidant enzyme activity (SOD, CAT and GST) due to thiamethoxam exposure in zebrafish[33].

Aldrin and dieldrin are synthetic organochlorine pesticide that acts as effective contact and stomach poisons for insects. Under most environmental conditions, aldrin is largely converted via biological and/or abiotic mechanisms to dieldrin, which is significantly more persistent[34]. Most environmental releases of aldrin and dieldrin are directly to soil. Because of low water solubility and tendency to bind strongly to soil, both compounds migrate downward very slowly through soil or into surface or ground water. Most surface water aldrin/dieldrin has been attributed to particulate surface run-off. Over time, it is possible that significant volatilization of aldrin/dieldrin might occur, with subsequent atmospheric photodegradation and/or rainfall "washout"[35]. Collectively, these characteristics will foster low levels of aldrin/dieldrin water contamination over comparatively extended periods of time. Dieldrin's extreme apolarity results in a high affinity for organic matter such as animal fats and plant waxes, which could lead to its bioaccumulation in the food chain. Due to hydrophobic nature, the highest concentrations of aldrin/dieldrin and their metabolites are typically found in the adipose tissues of both humans and other animals. Reported studies indicate that oral exposure to aldrin/dieldrin can induce a range of adverse systemic, neurological, reproductive/developmental, immunological, genotoxic, and tumorigenic effects in humans and/or animals. Aldrin is known to cause significant LPO and increases antioxidant enzyme activity in exposed zebrafish[36]. Lindane (08 mg/kg bw), an organochlorine class insecticide known as neurotoxin and interferes with normal functioning of GABA neurotransmitter. Radosavljevic et al., (2009) have observed elevated liver and plasma Thiobarbituric Acid Reactive Substances (TBARS) concentration in rats. In the same work they have reported elevated SOD and CAT activity[37].

Cypermethrin is a synthetic pyrethroid used as an insecticide in agriculture and in domestic purpose. It is known to cause oxidative stress in animals. Kanbur et al., (2008) have investigated the effects of cypermethrin, propetamphos and their mixtures on antioxidant enzyme activity in mice. They reported significant alterations in the MDA levels, SOD, CAT, and GPx activities of the exposed groups[38].

For assessing acute toxicity testing rodent, rabbit and other model animals have been widely used. However, toxicity testing in these animal model is costly, time consuming and cumbersome with introduction of so many new chemicals in the market. Therefore, new screening methods are needs to be employed to keep pace with the opening out of new chemicals. Zebrafish (*Danio rerio*), have emerged as a feasible vertebrate organism for the testing of chemicals. Zebrafish are small shoaling cyprinid fish native to the flood plains of the Indian subcontinent. The natural range of zebrafish is centered around the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh, and Nepal. It is a small, robust fish and large numbers can be kept easily in the laboratory, where it breeds all around year. Zebrafish genome-sequencing project have pointed out that at least 70% of human protein coding genes, including disease-associated genes, have an ortholog in fish[39]. Zebrafish, is one of the most important vertebrate model organism in toxicological research, genetics, developmental biology, neurophysiology and biomedicine[40-42]. Zebrafish also have several advantages for biomedical research studies as it offer relatively bulky amount of embryos suitable for performing genetics and cell biology experiments, and overall low maintenance cost. Its genome has been fully sequenced, and it will allow researchers to understand how human genes work and how genetic variants cause disease in ways that cannot be conducted in humans or other organisms. Some of the important advantages of zebrafish embryo toxicity testing include ease of maintenance, drug administration, short reproductive cycle, and embryo transparency that allow visual assessment of developing embryo. They also provide advantage over cell lines allowing metabolic and systemic interactions to imitate the processes in the human body. Hence, it can be used for diverse human disease like, cancer, cardiovascular diseases, neurodegenerative diseases, etc.

Some studies have demonstrated the reproducibility of rodent toxicity with that of zebrafish embryo toxicity testing. Woudenberg et al., (2013) have evaluated the effects of organotin compounds on morphology, histopathology and swimming activity on zebrafish embryos[43]. They observed concordance between the toxic effects observed in zebrafish embryo and rodent study. Similarly, De Jong et al., (2011) have assessed the developmental toxicity of six 1,2,4-triazole compounds in zebrafish embryotoxicity test, mouse embryonic stem cell test and rats postimplantation whole embryo culture[44]. Overall, the results of zebrafish embryotoxicity test gave excellent correlation with the relative in vivo developmental toxicities of tested compounds than other two methods. In another study the authors showed a correlation between the zebrafish

embryo LC_{50} and rodent LD_{50} of 60 compounds (Ali et al., 2011)[45]. Parng et al., (2002) have assessed the effects of 18 chemicals on zebrafish embryo and observed zebrafish bioassays are cheaper and faster than mouse assay[46]. They established that toxic response, teratogenic effects and LC_{50} in zebrafish are corresponding with the mice results. Extrapolation of zebrafish with human being and its application in different area are shown in Figure 1.

Pesticides are known to induce Reactive Oxygen Species (ROS) and cause oxidative stress leading to in-activation of antioxidant enzymes[47]. The oxidative stress is an inexorable component of aerobic life, an imbalance between the ROS production and the system to protect cells from ROS. Ascendancy of the ROS production results in defects that may cause cell or organism damages or death. This imbalance is referred as oxidative stress and it is a trigger factor for any diseased condition in body[48]. LPO is the comprehensively investigated process in oxidative stress induced by ROS. The most widely used assay for lipid peroxidation is MDA formation as a secondary product. Lipid peroxidation is the oxidative degradation of lipids, in these process free radicals steals electrons from the lipids membranes of the cell[49]. MDA generation due to pesticide exposure has been widely used biomarker. Lipid peroxidation has been widely accepted and reported phenomenon caused by pesticide exposure. A number of authors have reported lipid peroxidation caused by pesticide exposure using zebrafish as a biomarker[50-54]. Endogenous antioxidant defence system includes antioxidant enzymes which are capable of stabilizing, or deactivating free radicals before they attack cells thus, the activity of antioxidant enzymes

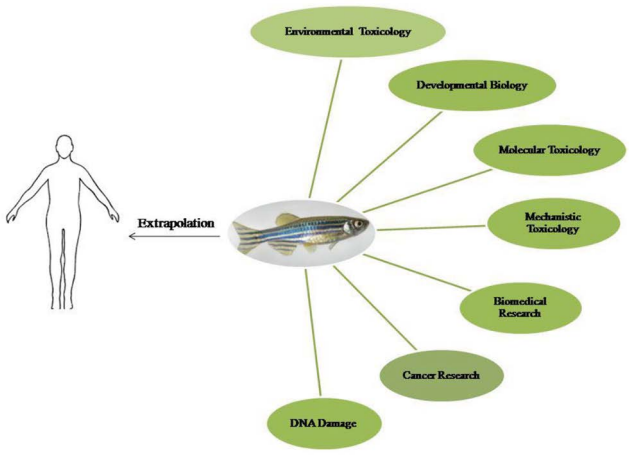


Figure 1. Pesticide induced oxidative stress and biomarkers for oxidative injury.

in fish have been extensively used as an early warning marker of fresh water pollution therefore served as biomarker for oxidative injury due to pesticide exposure. Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx) were found in different cells and these enzymes play important role in protecting the cells against the potentially toxic effects of environmental pollutants[48]. These enzymes served as biomarker to assess oxidative stress in tissue. Further, they protect the cells against reactive oxygen species by neutralizing free radicals[47]. The activity of AChE, SOD, CAT, GPx, levels of MDA and GSH are used for the assessment of oxidative damage by free radicals in experimental animals. Biomarkers are helpful in monitoring the status of various antioxidant defence mechanisms against oxidative stress[55]. There has been an increase in the application of molecular biomarkers of oxidative stress induced by various environmental xenobiotics. Endogenous and exogenous oxidative mechanism has made living cells with enzymatic and nonenzymatic antioxidant systems to regulate oxidative stress.

The activity of antioxidant enzymes suggest that the antioxidant status of the organisms and can also serve as biomarkers of oxidative stress. Cellular antioxidant defence system in living organism, when get exposed to environmental pollutants, are either depleted or elevated, but activity of antioxidant enzymes in living organisms may rise to restore the oxidative stress caused by xenobiotic[56]. A measurement normally detects an alteration (increase or decrease) of antioxidant enzyme activity under exposed conditions. Antioxidant defence system, (enzymetic and non enzymetic) which is generally ubiquitous in animal species and different tissue types, are found widely in aquatic organisms[49]. Antioxidant enzymes like SOD, CAT and GPx are the most important enzymes to maintain ROS balance as they scavenge the excess ROS and can be the first line of defence against ROS. SOD and CAT are the enzymes which convert superoxide (O^{2-}) into H_2O_2 and then into H_2O and O_2. Glutathione peroxidase is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. GPx protects the cell against xenobiotic at the expense of GSH. The main function of GPx is to reduce lipid hydroperoxides to their consequent alcohols and to reduce free hydrogen peroxide[55].

Zebrafish as a biomarker for pesticide induced toxicity has been widely used and well reported. Blahova et al.,

(2010) have reported the activity of antioxidative enzyme viz. SOD and GPx which was found to be elevated while the activity of CAT enzyme decreases due to atrazine exposure[50]. The work includes the evaluation of enzyme activity in whole body tissue of zebrafish exposed with atrazine for 28 days. In another work atrazine is found to damage DNA and effects antioxidant enzymes activity in zebrafish[51]. Sukritha and Usharani, (2013) observed an increase in the activity of SOD, CAT and GPx enzyme due to dichlorvos exposure[57]. In another study dichlorvos induced significant change in the activity of CAT enzyme in zebrafish[58]. The activity of CAT enzyme found to be elevated due to imidacloprid exposure[59]. The activity of SOD enzyme found to be depleted significantly, whereas CAT show enhanced activity due to aldrin exposure[53]. In our previous reported work, we observed significant alterations in the activity of antioxidant enzymes (biomarkers) due to pesticide exposure[47]. Acetylcholinestrase Enzyme (AChE) is also widely employed as a biomarker in pesticide induced toxicity especially organophosphate pesticides. It is a primary enzyme that catalyses the hydrolysis of acetylcholine into choline and acetic acid (Rodríguez-Fuentes G., 2015)[60]. Pesticide causes oxidative stress which in-turn alters AChE enzyme activity. DIC causes the irreversible inhibition of AChE in the central and peripheral nervous systems resulting in the accumulation of acetylcholine and activation of muscarinic and nicotinic receptors, which may even leads to death[23]. The activity of AChE enzyme has been widely utilized in assessing aquatic pollution. Effects of dichlorvos and other organophosphates pesticides on AChE activity have been widely studied. Rodríguez-Fuentes et al., 2015, reported decreased AChE activity in zebrafish embryos after 72 h of chlorpyrifos exposure[60]. Likewise, Assis et al., (2007) observed decrease in AChE activity in Tambaqui due dichlorvos exposure[61]. A part from antioxidant enzymes pesticide exposure is also known to cause mitochondrial dysfunction, initiation of apoptosis leading to cell death. Pesticide exposure also disrupt oxidative phosphorylation and influence the genetic polymorphisms in cell (PON1-155, 108), such as BCl2, Bax, Caspases-3 (Bloom et al., 2006; Chan et al., 2006; Kumar et al., 2015)[62-64].

Metallothioneins (MTs) are also widely regarded as a biomarker of aquatic pollution. It is low molecular weight proteins which are helpful in scavenging ROS[49]. MT is known to reduce the dangerous effects of free radicals by releasing zinc to the neuronal membrane. They contain zinc/cadmium ions, which helps in scavenging superoxide and hydroxyl produced by xanthine oxidase reaction. MTs as a biomarker have been extensively studied in heavy metal and pesticide toxicity[65-67]. Erdogan et al., (2011), Ceyhun et al., (2012) and Selcuk et al., (2018) have reported up-regulated MT-II mRNA expression due to deltamethrin, dichlorvos and imidacloprid exposure in rats, rainbow trout and common carp respectively[66,68,69]. MT as a biomarker for aldrin induced alterations was also assessed by Jhamtani et al., (2018) and reported significant alterations in the MT-II mRNA expression[36]. In our unpublished work, we have observed significant alterations due to atrazine, dichlorvos and imidacloprid in the MT-II mRNA expression in liver, kidney and brain of zebrafish.

3. Conclusion

Present review summarizes the potential role of zebrafish as a biomarker to evaluate pesticide induced poisoning incidences. Zebrafish have been used as model organism in different field of science such as environmental science, toxicology and human diseases. Pesticides are the major chemical agents, which pose health threat particularly to young people and depressed individuals. Pesticides have become widely used for committing suicide. Thus, there is a need to observe the biochemical alterations to evaluate the acute pesticide toxicity so that further treatment can be provided to manage pesticide poisoning incidence in occupational or environmental toxicity cases.

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5. Conflict of Interest

No conflict of interest.

6. References

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