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CARBENDAZIM INDUCED OXIDATIVE STRESS AND REDUCED BRAIN ACETYLCHOLINESTERASE ACTIVITY IN ZEBRAFISH

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ABSTRACT

Carbendazim (CBZ) is a widely used broad spectrum fungicide applied during the pre- and post- harvest times of food crops. It is mainly applied to control the *Ascomycetes*, *Fungi imperfecti* and *Basidiomycetes* fungal diseases. WHO categorized CBZ under hazardous chemicals and classified as a human carcinogen. It exhibits fungicidal activity by binding to the spindle microtubules causing the nuclear division blockade. As it is considered as an effective fungicide it has been widely applied and humans are mostly exposed to it. Besides its antifungal activity CBZ has toxic effects on humans who use it. In the current experimental study prolonged exposure (42 days) effects of CBZ on liver, gill and brain tissues of zebrafish has been observed. The toxic effect of CBZ on first line defense mechanisms like antioxidant enzymes i.e., catalase (CAT), Superoxide Dismutase (SOD), Glutathione Reductase (GR), Glutathione Peroxidase (GPx), Lipid Peroxidation (LPO) and brain acetylcholinesterase (AChE) has been studied at intervals of 1, 21 and 42 days. The results show that the activity of antioxidant enzymes was increasing with the exposure time but it was always less than the control groups. Lipid peroxidation has showing a significant increase and AChE was decreased throughout the exposure time in the treated groups.

KEYWORDS: Carbendazim, fungicide, zebrafish, Oxidative, Acetylcholinesteras.

INTRODUCTION

Carbendazim (CBZ) ($C_9H_9N_3O_2$; methyl 1-H-benzimidazol-2-yl carbamate) is a broad spectrum fungicide widely used to control the pre- and post-harvest infections caused by fungal species such as *Ascomycetes*, *Fungi imperfecti*, *Basidiomycetes* and many other fungal species infecting fruits and vegetables.[1] Carbendazim interferes the fungal activity by inhibiting the microtubule polymerization causing disruption in their assembly leading to impaired segregation of chromosomes during cell division.[2,3,4,5,6] This results in cell cycle arrest and an induction of apoptosis.[7]

Persistance of carbendazim in the environment is very high with a half-life of about 3 days to 12 months.[8,9,10] which is due to the formation of a potent and a highly toxic component, 2-amino-benzimidazole.[4] The wide usage and stable persistence of carbendazim results in the constant exposure of the human resulting in unknowing intoxications. A similar inhibitory effect of carbendazim was also observed in mammalian cells[1] with a transient dysfunction of the neuromuscular junction.[11] World Health Organization classified carbendazim under hazardous category of chemicals and considered as human carcinogen in combination with carbomyl.[12]

The extensive and repeated use of carbendazim placed it as major pollutant detectable in food, soil and water. Besides humans carbendazim induces toxic effects in non-target organisms like invertebrates, aquatic life forms and soil microorganisms. Previous studies of carbendazim toxicity showed significant effects on reproductive system, cause germ cell apoptosis, embryotoxicity, or teratogenesis in hamsters, mice and rats.[13] Carbendazim was found to be extremely toxic to fish inducing disruptive developmental changes in embryos of *Xenopus laevis*, Prussian carp and *Daneo rario*.[14,15,16,13]

Zebrafish is a well-documented, attractive model animal for toxicological studies for its advantageous features like small size, ease of culture, short life cycle, high reproductive capacity and transparent eggs.[17] Earlier studies of carbendazim exposure on zebrafish larvae showed induction of oxidative stress, immunotoxicity and endocrine disruption.[18] carbendazim induced the up regulation of genes regarding antioxidant enzymes, apoptosis and immune response. The present study is to observe the effect of carbendazim on adult zebrafish antioxidant enzymes which include catalase (CAT), Superoxide Dismutase (SOD), Glutathione Reductase (GR), Glutathione Peroxidase (GPx) and Lipid Peroxidation (LPO) and brain acetylcholinesterase (AChE) activity in a long term exposure, which can be

considered as a reference material for toxic effects of carbendazim on prolonged contact.

MATERIALS AND METHODS

Experimental Design

Mature healthy and adult male and female zebrafish in equal ratio (n = 8) were taken from our aquarium stock and kept in 20 L glass aquaria with a stocking density of one fish per 2 liters of water. Fishes were divided into 2 groups where Group 1 acted as control group (pesticide water), and fish in Group 2 were added with toxicant CBZ, 400 µg/L. All the groups were maintained in triplicates. The toxicant exposure in the present study was conducted for a period of 42 days. The antioxidant enzyme and brain AChE analysis was performed at a time interval of 1, 21 and 42 days. At the stipulated period both control and experimental fishes were dissected to collect the liver, gills and brain for experimental analysis.

Preparation of tissues for Antioxdant assays

Tissue sampling was done on 1, 21 and 42 days. On each sampling day, two fish from each replicate were randomly sampled (liver and gills) and pooled for analyses. Sample preparation for antioxidant enzyme assays was done as described by Reshma and Philip.[19] Catalase activity was assayed according to the method of Sinha,[20] SOD activity was assayed as described by Misra and Fridovich,[21] GPx activity was assayed by the Rotruck et al. method,[22] GR activity was estimated by the method of David and Richard[23] and LPO levels were studied by the method of Ohkaawa et al.[24]

Acetylcholinesterase (AChE) assay

The excised brain tissues on the corresponding analysis day were collected and placed in ice (4°C). Tissue preparation for AChE assay was done as described by Ezeoyili et al., [25] and the assay was performed according to the method of Ellman et al.[26]

Statistical analysis

Results obtained were reported as standard error mean (±SEM) of triplicate measurements using SPSS (version 17).[27]

RESULTS

The activity of antioxidant enzymes Catalase, Super oxide dismutase, Glutathione Reductase and Glutathione Peroxidase was found to be higher in both liver and gill tissues of control than treated groups throughout the experimental period, day 1, 21 and 42. The activity is increasing as the time of exposure progresses but it was lower than the control groups. The lipid peroxidation was elevated in both liver and gill tissues with concomitant decrease in the antioxidant enzymes by CBZ exposure. CBZ inhibited brain acetyl cholinesterase activity in treated groups in all 1, 21 and 42 days. The results of all the antioxidant enzymes, lipid peroxidation and AChE were shown in the Table 1. From the results it was clear that the activity of antioxidant enzymes was increasing with the exposure time but it was always less than the control groups. Lipid peroxidation was showing a significant increase and AChE was decreased (Table 2) throughout the exposure time in the treated groups.

Table I: Antioxidant enzyme activity in liver and gill tissues of zebrafish exposed to CBZ at 400 µg/L at different time interval.

Antioxidant Enzyme	Zebrafish tissues sampled after exposure of CBZ at 400µg/L	Duration in Days			
			1	21	42
Catalase	Liver	Control	38.71±0.22	39.72±0.14	54.88±0.38
		Treated	21.84±0.08	23.60±0.11	38.6±0.13
	Gills	Control	24.72±0.16	25.59±0.14	40.55±0.10
		Treated	15.49±0.08	16.77±0.46	29.83±0.10
SOD	Liver	Control	35.87±0.04	37.26±0.06	74.34±0.10
		Treated	16.74±0.15	17.72±0.11	52.7±0.02
	Gills	Control	15.99±0.01	16.77±0.04	35.64±0.13
		Treated	7.65±0.11	9.10±0.06	28.67±0.12
GPx	Liver	Control	8.98±0.03	9.69±0.03	20.59±0.15
		Treated	6.73±0.05	6.68±0.02	14.47±0.07
	Gills	Control	5.03±0.05	5.37±0.27	15.37±0.27
		Treated	4.88±0.02	4.79±0.04	10.1±0.08
GR	Liver	Control	9.81±0.06	10.81±0.02	13.8±0.03

		Treated	6.89±0.03	7.40±0.08	10.35±0.02
	Gills	Control	5.99±0.01	6.69±0.01	10.89±0.02
		Treated	4.64±0.07	5.47±0.03	9.89±0.02
LPO	Liver	Control	0.49±0.01	0.54±	0.97±0.02
		Treated	0.99±0.01	1.09±0.10	1.09±0.01
	Gills	Control	0.24±0.01	0.20±	0.46±0.02
		Treated	0.46±0.01	0.47±	0.99±0.01

Values are the mean with triplicates and expressed as standard error mean (SEM)

Table II: Brain AChE activity in of zebrafish exposed to CBZ at 400 µg/L at different time interval.

Brain AChE activity	**Duration in Days**		
	1	**21**	**42**
Control	0.99±0.01	1.59±0.03	1.66±0.02
Treated	0.80±0.01	0.86±0.02	0.83±0.02

Values are the mean with triplicates and expressed as standard error mean (SEM)

DISCUSSION

Carbendazim, for its widespread use in controlling fungal infections in different economically important sectors has importance in studying its toxic effects on living forms and humans as well. As zebrafish is a well-known research model for toxicological studies, experimental studies of carbendazim exposure on zebrafish have gained significance. Carbendazim induced changes in the antioxidant parameters and brain AChE activity.

Zebrafish possess a sophisticated antioxidant system, complete with oxygen- and electrophile-sensing signaling pathways and the enzymes that detoxify free radicals and replenish small molecule antioxidants which responds promptly on exposure to pesticides and insecticides.[28] Catalase, Super oxide dismutase, Glutathione Peroxidase act as the first line defense of the antioxidant enzymes converts superoxide radicals into hydrogen peroxide and then into water and molecular oxygen[29, 30, 31] SOD plays a key role in removal of superoxide radicals by converting them into H_2O_2, O_2 and Catalase enzyme degrades the H_2O_2 to water and oxygen.[32] CAT and SOD activity in the present experiment was showing a decreased activity in the CBZ treated liver and gill tissues of zebrafish. CAT and SOD were higher in treated liver tissue than gills, however both the enzymes in both the tissues were lower than control fishes. As the concentration of CBZ is higher i.e., 400µg/L it would generate a high and continuous flow of free radicals throughout the treated period. CAT and SOD could not balance the excessive generation of these free radicals and as a result the treated liver and gill tissues were showing a decreased enzyme activity than the control. Similar reduction in CAT, SOD activities were observed in CBZ exposed African giant rat,[33] *Clarias gariepinus.*[25]

Besides catalase GPx also reduces the H_2O_2.[34] Failure of GPx results in the oxidative stress and increased cytotoxicity.[35] In the present experiment decreased GPx activity is found in the CBZ treated zebrafish liver and gills but it was found to be higher in liver than gills. Significant decrease in GPx was found in the brain of *Clarias gariepinus.*[25] Decrease in GPx activity was found in the treated liver and gill tissues over control in Chlorpyrifos treated zebrafish.[36]

Glutathione reductase (GR) plays a significant role in the cellular metabolic pathways and antioxidant protection mechanisms.[37] Decreased GR activity was observed in the present study in both liver and gill tissue of CBZ exposed zebrafish but the activity was significantly reduced in liver than gill tissue. GR works together with GPx to fight against the ROS generated due to exposure of Xenobiotics.[38] Decreased GPx activity supports its combined action with GR to manage the oxidative stress. Significant decline in GR and GPx were observed in *Clarias gariepinus.*[25]

LPO is considered as biomarker for ROS production.[39] Zebrafish relatively have high level of unsaturated fatty acids to maintain the membrane fluidity and makes them more susceptible to oxidation.[28] Fishes use lipids as an energy source,[40] the excessive use of lipids results in the elevated lipid oxidation. In the present experiment elevated LPO was observed in the CBZ treated liver and gills. Zebrafish when exposed to CBZ due to overwhelmed production of ROS inorder to defense the oxidative stress rapidly uses the easily available lipids resulting in the increased lipid peroxidation.

Acetylcholinesterase is the first neurotransmitter discovered participates in transmission of nerve signals in the nervous system.[41] Significant decline in the AChE was observed in CBZ treated zebrafish. Inhibition of brain AChE indicates the damage in the enzyme

structure caused by CBZ. It inhibits AChE by forming less stable AChE-carbamate complex, from which the carbamyl moiety can be easily removed.[41] Inhibition of AChE by carbamates is considered as reversible because the enzyme activity was found to be recovered after removal of the toxicant.[25]

CONCLUSION

Significant decrease in the antioxidant enzymes and AChE activity indicate the toxic effect of CBZ on adult zebrafish for a prolonged exposure. The overwhelmed production of ROS lead to the failure of the antioxidant defense system and also disrupted the AChE structure. As zebrafish is considered as the best studied research model in toxicology CBZ exposure studies gain importance to know its toxic effects in mammals as well as aquatic animals.

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