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CARBENDAZIM INDUCED MORPHOLOGICAL ABNORMALITIES IN DEVELOPING ZEBRAFISH EMBROYS

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ARTICLE INFO	ABSTRACT
	Carbendazim is an extensively used fungicide for protection of crops, fruits and vegetables against fungal diseases, and also for protection of harvested products during their storage and transportation. Carbendazim inhibits the synthesis of nucleic acids and cell division in fungi. The present study is an attempt to examine the effect of non-lethal concentration of Carbendazim on potential developmental stages of Zebrafish (Danio rerio). Fertilized eggs of the same developmental stage, 4 hours post fertilization were exposed to three different concentrations of Carbendazim namely 600μg/L, 800 μg/L and 1000 μg/L. Standard growth parameters like hatching rate, mortality and morphological deformities were observed and recorded at regular intervals of 24 hrs upto 96 hpf. Delay in hatching was noticed at higher concentrations of Carbendazim and abnormalities like edema, heart rate, difference in yolk sac size and decrease in body pigmentation were observed in embryos before hatching, where as in larvae edema, shrinking of yolk sac and dorsal curvature of the body was identified. From the studies carried out Carbendazim was shown to delay hatching and also caused different morphological abnormalities. These two could be due to slower utilization of yolk.

INTRODUÇÃO

Carbendazim (methyl-1-H-benzimidazol-2-yl-carbamate) is one of the most widely used benzimidazole fungicides. It is highly toxic to target organisms, inhibiting the development of a wide variety of fungi even at low doses. It is used in agriculture, horticulture, forest and home gardening and as a preservative in paint, papermaking, textile, leather industry and fruits (Selmanoglu *et al*., 2001). Carbendazim is recognized as a major pollutant detectable in food, soil, water and also known to induce acute and delayed toxic effects on humans, invertebrates, aquatic life forms and soil microorganisms (Simranjeet *et al*., 2016). Recent toxicological studies of carbendazim exposure to *Caenorhabditis elegans* showed harmful effect on the locomotive behavior, development and growth, reproduction, lifespan, and antioxidant system of *C. elegans* (Jie *et al*., 2020). The higher concentrations of carbendazim are reported to show significant detrimental effects on the developing embryos of Prussian carp (Agnieszka *et al*., 2013). Zebrafish (*Danio rerio*) has emerged as a successful scientific platform for studies of toxicology and metabolic diseases (Schlegel A, and Gut P 2015). This animal model has great advantages like small size, easy maintenance, short breeding cycle, high fecundity, translucent embryos, and cheap cultivation. Studies of carbendazim on developing embryos of zebrafish aids to know the abnormal effects of the toxicant during development. The embryonic developmental stages of Zebrafish have been characterized by

Kimmel *et al*], while Parichy *et al*. described the normal table of post-embryonic Zebrafish development. These two excellent studies make it possible to assign individual Zebrafish to particular developmental stages, and describe the developmental changes in a variety of anatomical traits and how these traits vary over the Zebrafish life cycle. The advantages of Zebrafish studies are well established and include transparency of the fertilized embryos, rapid external development, tractable genetics, generation of embryos in large numbers and the availability of a large plethora of established scientific approaches. In addition, much of Zebrafish physiology and anatomy is homologous to mammalian physiology and anatomy (Eimon PM, and Rubinstein AL 2009). The genetic similarities with humans are another advantage of the Zebrafish as an animal model (Lieschke GJ, and Currie PD 2007). All these make zebrafish as the best choice of animal model for toxicology, metabolomics and developmental abnormality studies.

METHODS AND MATERIALS

Maintenance of Parental Fish: Wild type adult Zebrafish (*Danio rerio*) used in this study was bred in our aquarium facility for two generations. These were kept in aquaria filled with filtered tap water with the oxygen saturation of more than 80% and pH at 7.0 ± 0.3. The water temperature was maintained at 26 ± 1°C at a 14h-10h light/dark cycle. Fish were fed with freshly hatched live brine shrimp

(*Artemia nauplii*) once a day, supplemented with vitamin rich dried flake food once a day. The aquarium water was aerated continuously with stone diffusers connected to mechanical air compressor. Renewal of water was done daily by siphoning out 80% of water and refilling with fresh water and the aquaria screens were also cleaned daily. Eggs were collected from breeding stock of healthy, unexposed mature male and female Zebrafish which were above the age of six months. The spawning glass trays covered with a fine nylon net with an appropriate mesh size for eggs to fall through were placed in the aquaria on the evening before the collection of eggs was required. Plant imitations made of plastic serving as spawning substrate were fastened to the nylon mesh. The fish were left undisturbed over night. Eggs were spawned synchronously at dawn of the next morning. After the lights were turned on the next morning embryos were generated by natural mating and then collected within 30 minutes after spawning. Fertilized eggs were collected from the spawning trays and they were rinsed several times with filtered tap water and their quality was checked under the microscope to select the healthy fertilized eggs for the experiment. Unfertilized eggs were identified by their milky white color and discarded. The dead appear white because of the coagulation and precipitation of proteins. Technical grade Carbendazim (Methyl 1H benzimidazol-2-ylcarbamate) was obtained from S.L.Scientific chemicals.

Carbendazim: Procurement and Preparation of Stock: Stock solution of Carbendazim was prepared by dissolving 10mg of Carbendazim in 1ml of distilled water and stored at 4°C in darkness. Daily requirement was taken from this.

Experimental Design: Fertilized eggs at the same developmental stage 4 hpf were collected and exposure experiments were carried out by placing 100 eggs in 500 ml of filtered tap water in glass chambers. 600 µg/L, 800µg/L and 1000µg/L concentration of Carbendazim was added and stirred for uniform distribution of the toxicant. Controls were maintained only with tap water. All exposure experiments were carried out in triplicate. The toxicant was added everyday to maintain exact concentration. Embryos and larvae were observed after 24 hpf, 48 hpf, 72 hpf and 96 hpf of exposure under a stereomicroscope (Magnus MLX) for mortality, hatching and morphological/ developmental abnormalities. The magnification used for observation was 10X and 4X for eggs and larvae respectively. Thirty eggs from the above experiment were collected after 24 hpf , 48hpf, 72hpf and 96hpf to study the Vitellogenin cleavage pattern during development. Mortality/Survival was observed after exposure to carbendazim.

RESULTS

Mortality/Survival was observed after exposing the fertilized eggs to different concentrations of Carbendazim upto 96hpf. This was done by counting live/dead eggs/larvae in each of the exposure chambers and cumulative mortality was calculated. Carbendazim caused a dose related increase in mortality with significant death of embryos at a chemical threshold of 800 µg/L and 1000 µg/L and time threshold of 24 hpf and 48 hpf. In control group no mortality was observed up to 72 hpf stage, where as in 96 hpf 7% mortality was observed. In the treated groups mortality rate in 24 hpf were 5% in 600 µg/L , 9% in 800 µg/L and 17% in 1000µg/L, in 48 hpf exposed group 7% in 600 µg/L , 7% in 800 µg/L and 13% in 1000µg/L, in 72 hpf in exposed group 3% in 600 µg/L , 6% in 800 µg/L and 20% in 1000µg/L and in 96 hpf exposed group 3% in 600 µg/L , 5% in 800 µg/L and 16% in 1000µg/L respectively. The cumulative percent of Mortality in both Control and treated groups were represented in Fig. I. Zebrafish embryos hatched sporadically between 48 and 72hpf in control as well as in exposure groups with a difference in percentage of hatching. In control groups, hatching at 48h were 85%. The percentage of hatching observed at 600µg/L, 800µg/L and 1000µg/L of Carbendazim was 56%, 55% and 45% respectively. At 72hpf, the percentage of hatching rate was 100% in control group, and in Carbendazim treated group it was 97% in 600µg/L, 95% in 800µg/L and 91% in 1000µg/L respectively.
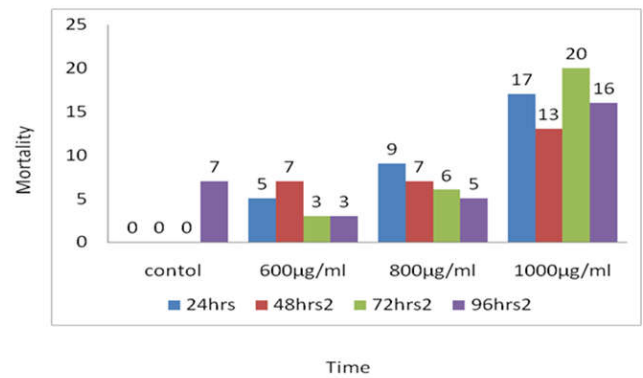

Fig. 1. Percent mortality observed at different time periods after exposing fertilized eggs to different concentrations of Carbendazim

The cumulative percent of hatching in both Control and treated groups were represented in Fig. II.
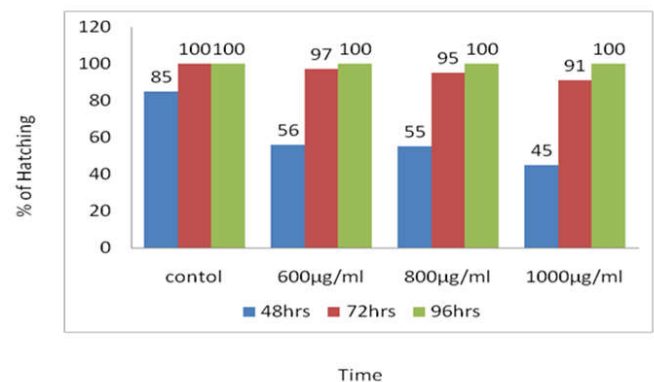

Fig. 2. Percent hatching observed at different time periods after exposing fertilized eggs to different concentrations of Carbendazim

In Zebrafish embryos heart beat was first noticed in 24 hpf. Embryos/larvae were monitored for heart rate daily in both control and Carbendazim treated groups (Table 1).

Table 1. The rate of heartbeat at different developmental stages exposed to Carbendazim The mean of eight individual observations is shown with SD. The experiment was performed in triplicate, and values mentioned are mean (n=6) SD. Decrement was statistically significant (P<0.05) over control

Heart rate (Beats/20seconds)				
		Carbendazim		
Observation made at	Control	600µg/L	800µg/L	1000µg/L
24hpf	52±2	47±2	48±1	49±2
48hpf	54±2.1	50±1	51±1	49±1
72hpf	59±1	52±1	48±1	44±1
96hpf	61±1	54±1	52±2	50±1

Heartbeat became more prominent and decreased as development progressed in experimental groups compared with control groups. In all the developmental stages, the rate of heart beat was significantly decreased at three concentrations of Carbendazim compared to control (Fig. 3). Zebrafish embryos developed normally, and no abnormal embryos were observed in control/solvent groups during the experimental period. Prominent morphological abnormalities noticed in the Carbendazim treatment groups are listed in Table. 2, which shows a clear concentration-response relationship. Edema (pericardial and yolk sac), tail deformity (detachment and undeveloped) and axial malformations (bending of notochord and dorsal curvature) were observed at different concentrations of Carbedazim tested, i.e. 600µg/L 800µg/L and 1000µg/L groups.
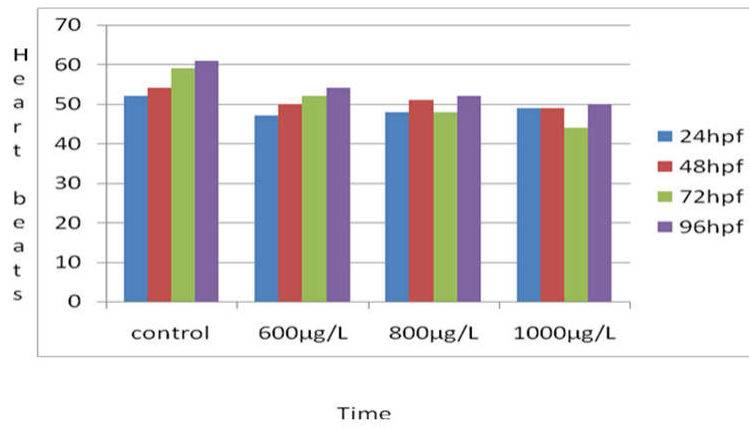
Fig. 3. The rate of heartbeat at different developmental stages exposed to different concentrations of Carbendazim

Table 2. Prominent morphological abnormalities in embryonic development parameters of Carbendazim exposed early Zebrafish embryos/larvae

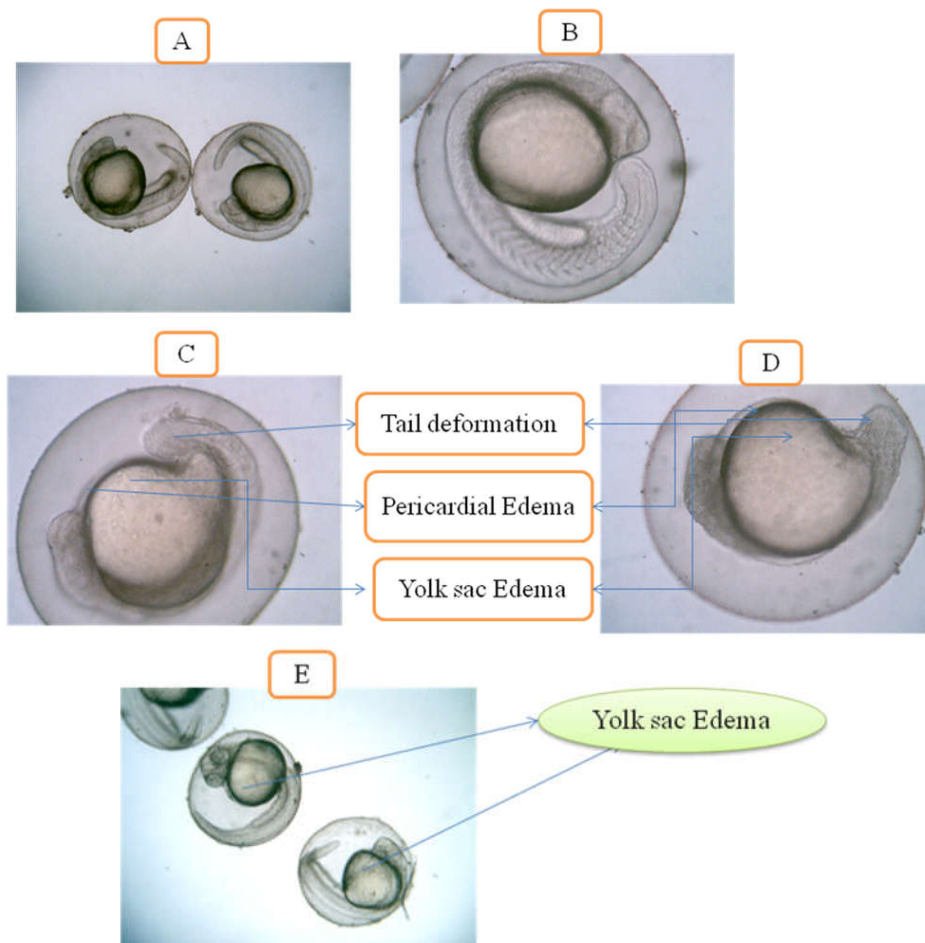| Morphological , behavioral and physiological abnormalities	Development stage (hpf)	Control	600µg/L	800µg/L	1000µg/L
Head Deformation	72	-	-	-	+
Pericardial edema	24	-	+	+	+
Yolk sac edema	24	-	+	+	+
Body and eye pigmentation	48	-	+	-	+
Growth retardation	48	-	+	+	+
Tail deformation	24	-	+	+	+
Touch response	48	-	+	+	+
Notochord bending	72	-	+	+	+
Tail curvature	48	-	+	+	+
Slow blood flowing	48	-	+	+	+

Fig. 4. Photo micrographs of embryos/larvae. 24hpf.. Control embryos. A & B. Experimental Embryos C (600 µg/L), D (800µg/L), E (1000µg/L).,

These deformities were noticed in all surviving larvae at three concentrations. At these concentrations, there was no response to touch and at the same time, blood flow was slow. Defects of swim bladder accompanied by abnormal swimming were also seen in these treated groups. Significant decrease in body and eye pigmentation compared to the control was evident only in larvae of 48hpf stage exposed to 800µg/L. Carbendazim treatment (600ug/L, 800ug/L and 1000ug/L) caused increase in yolk sac size and embryos/larvae length of all 100 treated embryo/larvae. Along with these, abnormalities like edema were also noticed. Yolk sac edema was noticed in more number of larvae than pericardial edema at 24hpf stage. At 48hpf stage only pericardial edema was observed. Decreased eye and body pigmentation was also observed in most embryos of 72hpf and 96hpf stages (90% and 98% respectively) at three concentrations (Fig. 6 & 7).
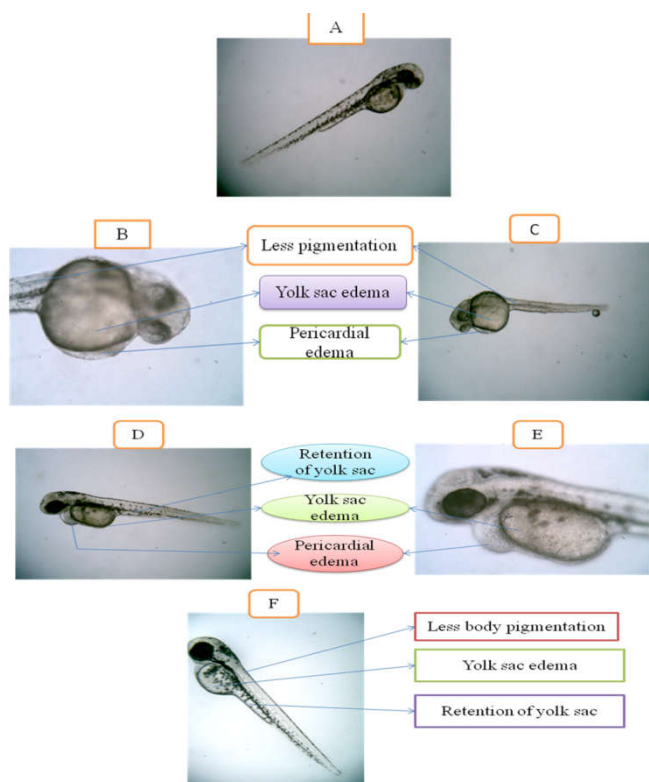

Fig. 5. Photo micrographs of embryos/larvae. 48hpf. A Control embryos. Experimental embryos, B & C (600µg/L), D & E (800µg/L), F (1000µg/L)
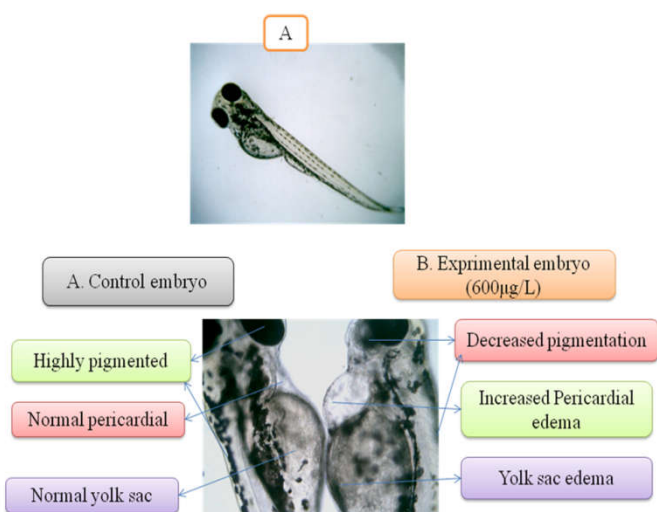

Fig. 6. Photo micrographs of embryos/larvae. 72hpf. A. Control embryo. Experimental embryos, B (600µg/L), C & D (800µg/L), E (1000µg/L)

DISCUSSION

From the results of our present investigation, we provide information which demonstrates that carbendazim in vivo interfered with normal development and functioning of Zebrafish. Observation of mortality has shown a dose dependent increase. Different degrees of mortality of Zebrafish embryos was reported earlier when exposed to azacel (Ahmad and Ansari, 2011), colloidal silver and gold nanoparticles (Ilan *et al.*, 2009), perfluorooctanesulfonate (Shi *et al.*, 2008), Hexabromocyclododecane (Deng *et al.*, 2009) and Arsenic (Li *et al.*, 2009). The differences in susceptibility can be ascribed to the difference in the ontogenetic stage of the eggs and permeability of different chemicals through chorion. Hatching, which is a critical stage of embryogenesis, has been critically used as endpoint in fish early life stage test. Fundamentally it is of two type's mechanical and enzymatic hatching. During normal hatching process, the chorion is digested by the hatching enzyme, chorionase secreted from the hatching gland cells of the embryo which in turn accumulated in the perivitelline space, reaches the chorion and induces its breakdown releasing the free-living larva. Delay in hatching especially at higher concentration of Carbendazim was clearly noticed indicating that Carbendazim could be inhibiting the release of chorionase/osmotic disturbances interfering with hatching enzyme activity.

It was shown by Fan and Shi (2002) that the structure and function of the protease might be destroyed by toxicants and might block pore canals of the chorions, resulting in the shortage of oxygen supply to the development of embryos. Another reason for delay or failure to hatch may be due to developmental abnormalities observed in the present study which may limit the developing embryos to mechanically break the outer chorion. Delay/Inhibition of hatching of zebrafish embryos was reported earlier due to SWCNTs (Cheng *et al.*, 2007), perfluorooctanesulfonate (Shi *et al.*, 2008), celastrol (Wang *et al.*, 2010), chitosan nanoparticals (Hu *et al.*, 2011), difenoconazole (Mu *et al.*, 2013), nanomaterials (nC60), emodin (He *et al.*, 2012) celastrol (Wang *et al.*, 2010b), β- diketone antibiotics (Wang *et al.*, 2013), genistein (Kim *et al.*, 2009), metals like copper, lead, mercury and nickel (Dave and Xiu, 1991), and this delay in hatching could also be a strategy employed to prolong the residence of the embryo within the egg in response to adverse environmental conditions. Exposure of fertilized eggs/embryos to Carbendazim resulted in dose dependent malformations during development. Development is a critically sensitive period where changes in environmental conditions can alter the normal programme of embryogenesis (Gilbert, 2001). Fish embryogenesis is sensitive to environmental factors including temperature, pH, nutrient levels, or chemicals such as pesticides. In our study also Zebrafish embryogenesis was found to be sensitive to Carbendazim and in this the most affected part by Carbendazim was yolk sac, pericardium. Edema of yolk sac was noticed from 24 hpf (Fig. IV-24 hpf D and E) and this yolk sac started to shrink after hatching making the pericardial cavity more conspicuous (Fig. V- 48hpf; Fig. VI- 72 hpf; Fig. VII 96 hpf). In fish eggs, the endogenous lipid reserves, mainly phospholipids and triglycerols are in the form of globules (Wiegand, 1996). Alteration of lipid synthesis and metabolism may cause yolk sac effect. Abnormalities relating to yolk in Zebrafish like yolk sac edema, delayed yolk sac absorption, yolk syncytium, pericardial edema and embryonic malobsorption syndrome were observed in Zebrafish exposed to variety of compounds namely bifenthrin (Jin et. al., 2009), chitosan nanoparticals (Hu *et al.*, 2011), difenoconazole (Mu *et al.*, 2013), emodin (He *et al.*, 2012), celastrol (Wang *et al.*, 2010b), genistein (Kim *et al.*, 2009), soxitocin (Lefebvre *et al.*, 2004), cadmium and methyl mercury (Cheng *et al.*, 2000;Yang, 2010).

The most notable malformation after hatching was dorsal curvature of the body with bending of the notochord, which is more pronounced at higher concentration of Carbendazim. The notochord is an axial structure common to the chordate phylum. In lower chordates and in larval stages of lower vertebrates it plays an important role as a structural element required for locomotion and coordinated movement. The notochord is also required for proper difference of

adjoining tissues like nueroectoderm, muscle and vertebral elements in all vertebrates. Therefore, the primary axial structure upon which many other tissues depend for their proper formation and differentiation.

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