International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

ISSN: 2454-3055

Assessment of Toxicity of Coragen, an Insecticide, on Aquatic Ecosystem Using Hydra as a Model

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Received: 1st March, 2021

Accepted: 2nd April, 2021

Published online: 4th April, 2021

<https://doi.org/10.33745/ijzi.2021.v07i01.012>

Abstract: Overuse of insecticides, without analyzing their harmful effects, can cause serious environmental issues. Coragen is a commonly used insecticide due to its broad range activity. In spite of enormous use, there are hardly any reports on the effects of coragen on non-target aquatic species and thus the environmental risk of coragen to aquatic ecosystem is underestimated. The present study was carried out to evaluate the potential risk of coragen on the water bodies and the life therein using hydra, a fresh water simple diploblastic invertebrate, as the model system. Coragen was found to reduce the life expectancy of hydra along with the reduced ability to regenerate at various tried doses. The present study thus suggests precocious use of coragen due to its harmful effects on aquatic animals at very low concentrations also. The continued overuse of coragen may disturb the aquatic ecosystems at a large scale in long run.

Keywords: Coragen, Hydra, Lethal concentration, Regeneration, Toxicity

Citation: Borgave Seema Sachin: Assessment of toxicity of coragen, an insecticide, on aquatic ecosystem using hydra as a model. Intern. J. Zool. Invest. 7 (1): 159-168, 2021.

<https://doi.org/10.33745/ijzi.2021.v07i01.012>

Introduction

Persistent, though slow, release of chemical pesticides and fertilizers may accumulate in the nearby water bodies, thus change its quality and may turn it inappropriate for the life present therein (Staley *et al.*, 2015; Solis *et al.*, 2018). Coragen, (chlorantraniliprole), is one of the most widely used insecticides due

to its broad spectrum of activity. It is effective against immature and adult stages of many key pests like European corn borers, armyworm, black cutworm, Colorado potato beetle etc. Coragen protects more than 100 vegetable crops by controlling infestation of variety of insect pests, mainly Lepidopteran,

probably by affecting nervous system and interrupting feeding of these pests immediately after coragen exposure. Coragen activates ryanodine receptors by the stimulation of calcium, causing paralysis and death of the sensitive species (Mishra *et al.*, 2016). In spite of being a widely applied insecticide with potential long term effects on environment, there are hardly any reports describing toxicological aspects of coragen on non-target aquatic species.

Hydra, a freshwater cnidarian, has been explored as one of the most suitable model for studying the aquatic toxicology (Karntanut and Pascoe, 2002). It has an unparalleled ability to regenerate, allowing the assessment of teratogenic compounds and the impact of contaminants on stem cells as well. The most important feature of hydra is that they are pervasive in freshwater bodies and are comparatively easy to culture in laboratory. Thus, hydra has emerged as an aquatic environmental indicator and for small scale bioassays (Quinn *et al.*, 2012; Riedl *et al.*, 2018). In the context to enormous use of coragen in India, the present work was undertaken to investigate the possible ill-effects, if any, of coragen on non-target aquatic species using hydra as a model system. The study evaluated effects of coragen on whole hydra, regenerating hydra and estimated lethal concentration 50% of coragen for the test animal.

Materials and Methods

Culture of hydra: A clonal culture of *Chlorohydra viridissima* (green hydra) was maintained at room temperature in the laboratory in clean glass beakers filled with double filtered and autoclaved pond water.

Zooplanktons (*Paramoecium*, *Daphnia*, *Cyclops* etc.) were used for feeding hydra on alternate days. The leftover food and dead debris were cleaned 5 h post-feeding.

Preparation of treatment doses of coragen: Coragen was procured from a local shop and serially diluted (1:10, 1:100, 1:1000, 1:10000 and 1:100000 dilution) using filtered sterile pond water to prepare the treatment solutions with various potencies ranging from 18400, 1840, 184, 18.4 and 1.84 ppm of coragen, respectively.

Treatment of whole hydra with coragen: Fully grown healthy hydra were selected and randomly distributed in equal numbers into the beakers containing equal volume of filtered sterile pond water with or without known concentration of coragen. Hydra, kept in pond water alone served as controls and rest were used for treatment with various concentrations, ranging from 18400 to 1.84 ppm of coragen. The death rate, overall cellular morphology and contractibility of hydra from all the beakers was monitored over a period of 72 h post-treatment. Detailed cellular morphology was observed under Leica DM 1000 microscope (DST-FIST instrument) and the observations were recorded using Future WinJoe camera. The experiment was repeated for 5 times with 10 hydra in each beaker every time.

Treatment of regenerating hydra with coragen: Full-grown hydra were selected and starved for 2 days for the experiments. Hydra were cut transversely in the middle of the body column using tungsten needles. The two body column pieces generated were with either hypostome or basal disc. These pieces were separated on the basis of hypostome and basal disc, and randomly distributed into

separate beakers for treatment with or without coragen. One beaker was kept as a control and the other was treated with 1:100000 dilution (1.84 ppm) of coragen. In this way, the experimental design had 4 beakers; one beaker with regenerating hypostome pieces without coragen, second beaker with regenerating hypostome pieces with coragen, third beaker with regenerating basal pieces without coragen and the last beaker with regenerating basal disc pieces with coragen. The regeneration in all the groups was monitored on the basis of morphological and functional criteria for next 72 h and the observations were recorded. The hydra were not fed during this period. All the experiments were repeated minimum 5 times with minimum 10 pieces in each control and treatment beakers every time.

Results

The effects of dilutions of coragen on whole hydra and regenerating hypostome or basal disc pieces were followed for 72 h post-treatment. Observations were recorded at the end of every 24 h.

Effects of coragen on whole hydra: Hydra from the control group exhibited normal morphology and contractility throughout the experiment. Lethal effects of coragen, at all the tried doses, were recorded as depicted in Table 1. Coragen (1.84 ppm) was found to kill approximately 50 per cent of the treated hydra by 72 h. In this way, the LC_{50} of coragen on whole hydra for 72 h was estimated to be 1:100000 dilution i.e. 1.84 ppm (Table 1).

The overall morphology of the body column, tentacles, attachment and feeding pattern of hydra from the control group was normal throughout the experiment (Figs. 1 A, 2 A, 3 A). Coragen, at 18400 ppm

concentration, completely disintegrated the hydra whereas hydra treated with 1840 ppm were immotile with partial loss of the tentacles and disintegrated body column cells within 24 h (Fig. 1 B). They were considered dead due to absence of reflex action. At the 184 and 18.4 ppm concentrations of coragen, the cells of the body column and tentacles appeared to loosen up by 24 h with impaired movement of tentacles (Figs. 1 C, D). The morphology of body column cells and tentacles was found to deteriorate as compared to respective controls when the treatment period was extended beyond 24 h for these concentrations (Figs. 2 A, C, D, 3 A, C, D). The morphology of hydra in 1.84 ppm coragen treated group was comparable to the respective controls for initial 48 h (Figs. 1; A, E, 2 A, E). By the end of 72 h, hydra treated with 1.84 ppm concentration of coragen also showed indications of cellular disintegration with death of 50% of the treated hydra (Fig. 3 E). Coragen treatment led to deterioration of the contractility and relaxation pattern of hydra as compared to the respective controls within 72 h.

Effects of coragen on regenerating hydra: Hydra were cut transversely by taking a sharp cut along the body axis as depicted in Figure 4. The regeneration of basal disc in the pieces with hypostome, and hypostome in the pieces with basal disc was monitored every 24 h. During regeneration, the blastema formation begins immediately within 24 h and the rearrangement of body column cells to reform the lost parts is achieved within 72 h. Since 1.84 ppm concentration of coragen was detected as LC_{50} for whole hydra (72 h), the same dose was used for treatment of regenerating body pieces of hydra. The overall regeneration was achieved with

Table 1: Effects of coragen at various concentrations on whole hydra

Treatment window	Control	18400 ppm	1840 ppm	184 ppm	18.4 ppm	1.84 ppm
				ppm	ppm	
24 h	100 %	0%	0%	11.11 %	50 %	88.88 %
48 h	100%	0%	0%	11.11 %	50%	61.11 %
72 h	100 %	0%	0%	5.55 %	33.33 %	50 %

The percentage given here depicts the overall per cent of live hydra in each category of coragen concentration at the end of the treatment window. Note no live hydra at higher doses of coragen i.e. 18400 ppm and 1840 ppm within 24 h. LC₅₀ of coragen for hydra for 72 h was observed to be 1.84 ppm.

Table 2: Effects of coragen (1.84 ppm) on regeneration of hydra

Treatment window	After 24 h		After 48 h		After 72 h	
	Hypostome	Basal Disc	Hypostome	Basal Disc	Hypostome	Basal Disc
Control	100%	100 %	97.61 %	100%	97.61%	100 %
Coragen	95.23 %	95.23 %	80.95%	85.71%	76.19%	66.15 %

The percentage is calculated on the basis of regeneration shown in the hypostome and basal disc pieces of live hydra in each category. Note reduction in percentage of regeneration in hypostome pieces and basal disc pieces after coragen treatment as compared to the respective controls after 72 h regeneration.

formation of normal structures in the control groups whereas the regeneration of the hypostome and basal disc pieces, both were hampered due to coragen (Table 2).

The body column pieces with hypostome, started regeneration of the lost basal discs in the control group within 24 h (Fig. 5 A). Hypostome exhibited formation of functional basal discs by 72 h (Figs. 5 B, C). The hydra could attach to the glass surface with the regenerated basal discs. On the other hand, hypostome pieces treated with coragen showed signs of blastema formation and

beginning of basal disc regeneration after 24 h (Fig. 5 D). However, later on the body cells showed disintegration to some extent (Figs. 5 E, F). In the control group, the basal disc pieces started hypostome regeneration by the end of 24 h (Fig. 6 A). The regenerated hypostome and tentacles exhibited normal morphology in control group (Figs. 6 B, C). On the contrary, coragen treated basal disc pieces showed regeneration of hypostome with miniature tentacles with disturbed morphology (Figs. 6 D, E). The regenerated hydra exhibited no or low movements in

Figure 1: Effects of various concentrations of coragen on whole hydra (24 h): (A) Control hydra; note control hydra with normal morphology of body column and tentacles, (B-E) Coragen treated hydra - (B) 1840 ppm; observe disintegration of tentacles and distorted body column, (C) 184 dilution and (D) 18.4 ppm; note loosening of cells in tentacles, (E) 1.84 ppm; observe hydra morphology comparable with control as seen in A.

Figure 2: Effects of various concentrations of coragen on whole hydra (48 h): (A) Control hydra with normal cellular morphology, (B-E) Coragen treated hydra - (B) 1840 ppm; note complete loss of tentacles and immobile body column, (C) 184 ppm; observe abnormal tentacles and body column morphology, (D) 18.4 ppm; observe loosening of cells of tentacles and basal disc region, (E) 1.84 ppm; beginning of distortion in cell morphology in tentacles and body column cells was evident.

Figure 3: Effects of various concentrations of coragen on whole hydra (72 h): (A) Control hydra exhibiting normal cellular morphology all over body, (B-E) Coragen treated hydra – (B) 1840 ppm; though the hydra were dead within 24 h the body did not disintegrate completely by 72 h (C) 184 ppm and (D) 18.4 ppm; note disturbed cellular morphology of body column and tentacles, (E) 1.84 ppm; observe advancement in loosening of cells as compared to 48 h time point.

Figure 4: Diagrammatic representation of cutting of whole hydra into 2 pieces that were used for regeneration experiments. Out of the two pieces generated this way, one was the regenerating hypostome piece with tentacles while the other piece was the regenerating basal disc. The regenerating hypostome piece would reform the lost basal disc while the regenerating basal disc would regenerate the lost hypostome region with tentacles in due course of time.

Figure 5: Effect of 1.84 ppm of coragen on regenerating hypostome pieces: (A) control hypostome after 24 h, (B) control hypostome after 48 h, (C) control hypostome after 72 h, (D) coragen treated hypostome after 24 h, (E) coragen treated hypostome after 48 h, (F) coragen treated hypostome after 72 h; note formation of functional basal disc in controls (C) by 72 h whereas the formation of basal disc was delayed in treated hydra. Also, the tentacles were damaged by 72 h in these cases (F).

Figure 6: Effects of 1.84 ppm of coragen on regenerating basal disc pieces: (A) control basal disc after 24 h, (B) control basal disc after 48 h, (C) control basal disc after 72 h; observe the length and compact arrangement of cells in the regenerated tentacles, (D) coragen treated basal disc after 24 h (E) coragen treated basal disc after 48 h; note miniature tentacles as compared to respective control (B), (F) coragen treated basal disc after 72 h; complete disintegration of the regenerating tentacles was observed in about 30% of the pieces as shown here while in most of the cases the cells of the tentacles were not compactly arranged as compared to C.

coragen treated groups as compared to the respective controls. Coragen, thus was found to interfere with the regeneration process at the tried dose in the present study.

Discussion

Hydra is the most widely used model animal for aquatic toxicity investigations as it acts as one of the crucial members of the food chain operating in stagnant fresh water bodies (Quinn *et al.*, 2012; Blaise *et al.*, 2018; McKinley *et al.*, 2019). Most of chemicals used as agricultural aids enter into nearby water bodies as runoff water. This may pollute the water bodies making them unsuitable for the biotic components of the ecosystem. In this scenario, the present study was undertaken to address the toxic effects of the most commonly used insecticide, coragen on aquatic ecosystem using hydra as a model.

Ryanodine receptors, located in the sarcoplasmic reticulum, function in calcium induced calcium release (CICR) in excitable tissues like muscles (Bouchard *et al.*, 2003). Activation of ryanodine receptors by coragen (active ingredient - chlorantraniliprole), resulting in paralysis and ultimately death of the susceptible species has been demonstrated (Dutta *et al.*, 2014; Mishra *et al.*, 2016). Coragen causes cardiac problems and other metabolic abnormalities related to magnesium and calcium also (Mishra *et al.*, 2016). In the present study, the overall movement and contractibility of the hydra were reduced after coragen treatment. Hydra possess a primitive neuromuscular system. Recently, using calcium imaging technique, scientists have demonstrated activation of ectodermal and endodermal epitheliomuscular tissue that participate in dynamic kinetics of hydra (Szymanski and Yuste, 2019).

The reduced motility observed in coragen treated hydra might be due to effects of coragen on the epithelial muscular tissue. It would be interesting to investigate the mechanism further.

Higher doses of coragen led to immediate death and disintegration in 100% of the treated hydra whereas longer exposures to low concentration of coragen were necessary to exhibit the toxic effects in the present study. The lethal effects of chlorantraniliprole on non-target species like crayfish in rice-crayfish cropping system has been reported (Barbee *et al.*, 2010). Coragen was found to hamper the regeneration also, which is in accordance with the earlier observations that pesticides and insecticides may affect crucial life processes of animals. The reproduction capability of worker bumblebees is shown to be suppressed by dietary intake of chlorantraniliprole, an active ingredient of coragen (Smagghe *et al.*, 2013). Chlorantraniliprole exposure is shown to reduce overall growth and formation of cocoons in *Bombyx mori* through accumulation of reactive oxygen species (ROS), down-regulation of genes related to oxidative phosphorylation pathway and antioxidant defense system in midgut of the animal (Hu *et al.*, 2019). *Coccinella septempunctata* is a commonly used insect predator for controlling of many arthropod pests in integrated pest management. A recent study has proved significant reduction in the hatchability of eggs, pupation rate, emergence of adults of *Coccinella septempunctata* due to treatment with chlorantraniliprole, eventually resulting in low population levels of the beneficial species (He *et al.*, 2019). Similar study by another group on the multicolored Asian lady beetle *Harmonia axyridis*, a non-specific predator, showed impaired population

growth due to sublethal concentrations chlorantraniliprole (Nawaz *et al.*, 2017). Chlorantraniliprole (in a range of 5 to 100 ppm) is shown to be highly toxic to eastern subterranean termites resulting in 100% mortality within 14 days post-exposure (Buczowski *et al.*, 2012). Exposure to chlorantraniliprole for more than 14 days led to reduction in weight and increased ROS levels in earthworms (Liu *et al.*, 2018). Chlorantraniliprole is shown to interfere with the vertebrate embryogenesis process also (Borgave *et al.*, 2020).

The present study thus demonstrates toxic effects of coragen on non-target aquatic species. It further emphasize precocious use of coragen in agricultural fields as it interferes with biological phenomenon like regeneration and leads to lethal effects in a supposedly immortal organism like hydra. There are hints of elevation in ROS and impairment of antioxidant mechanism due to chlorantraniliprole (Liu *et al.*, 2018; Hu *et al.*, 2019). Impact of ROS in planarian regeneration has been demonstrated by Pirotte Hu *et al.* (2015). In light of these studies, it would be interesting to elucidate the mechanism by which coragen interferes with the regeneration process in hydra.

Acknowledgement

The author acknowledges Department of Science and Technology-FIST for the funding for DM 1000 microscope and Department of Biotechnology for the grants under 'Star college scheme' through which the Future WinJoe camera was purchased.

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