MAGYAR AGRÁR- ÉS
ÉLETTUDOMÁNYI EGYETEM

COMPLEX EFFECT STUDY OF *BENDIOCARB* EXPOSURE IN THE EARLY LIFE STAGE OF ZEBRA DANIO (DANIO RERIO) AT SUBLETHAL CONCENTRATIONS

PhD Thesis

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1. Background and Objectives of the Work

The use of various pesticides has become part of our everyday lives. They assist in intensive farming, ensure increased food production, and guarantee food supply security. It is estimated that without the use of pesticides, one-third of the crop yield would be lost. The continuous and widespread use of pesticides can burden non-target organisms and the environment, and pesticide residues can appear in areas far from the application site due to their bioaccumulative and persistent properties. Consequently, they can be detected in various foods today. The long-term consumption of foods containing pesticide residues above the threshold limits can cause serious health consequences. The World Health Organization (WHO) has identified pesticide poisoning as a public health issue, which can lead to significant morbidity and mortality worldwide. Of the pesticides used, 29.5% are insecticides, and depending on the mechanism, there are several subgroups within this category.

Bendiocarb is a broad-spectrum insecticide belonging to the carbamate group. It is still one of the substances recommended by the WHO, which can be effectively used in the *IRS (Indoor Residual Spraying)* technique to help control mosquito populations and reduce the spread of malaria. Despite this, very limited data is available on the biological effects caused by *bendiocarb* exposure. Due to its widespread use, it has become detectable in various waters worldwide and in human air and blood samples due to domestic use. Once absorbed into the human body, it crosses the placenta and can appear in unborn infants. *Bendiocarb* exposure during embryonic development, like other xenobiotics, can have significant consequences for the organism, affecting both morbidity and mortality. Consequently, there is a need for more detailed exploratory work to provide a more comprehensive understanding of the biological consequences of embryonic *bendiocarb* exposure.

The zebra danio (*Danio rerio*), well known from aquaristics, is one of the most widely used vertebrate model organisms across various scientific fields. The extensive background knowledge available on this species enables the development of complex model systems, allowing for detailed and extrapolatable information on the effects of pollutants in different environments. In my thesis, I used the embryos of this species as a model.

Objectives

During my doctoral research, my aim was to map the effects of *bendiocarb* exposure during embryonic development using the zebra danio model organism. Within this scope, I set the following sub-goals

1. Determine the LC_{50} value of the compound and detect morphological alterations in embryos/larvae.
2. Conduct studies closely related to the primary mechanism of action of the substance:
    a. Examine acetylcholinesterase inhibition
    b. Investigate heart function
    c. Map the activity of enzymes involved in the antioxidant system
    d. Record behavioral changes
3. Uncover transcriptome-wide changes and identify the biological pathways significantly affected by *bendiocarb* exposure. Based on the results, I further investigated alterations in:
    a. Processes related to the larvae's visual perception,
    b. muscle tissue and muscular system,
    c. and the immune system.

2. Materials and Methods

2.1 The location of the experiments, the housing of the animals

The experiments were conducted at the former Szent István University, MKK-KTI, Department of Aquaculture (now the Hungarian University of Agriculture and Life Sciences, Institute of Aquaculture and Environmental Safety). In my studies, I used the AB wild type and transgenic lines (2.2*shh*:gfp ABC#15, Tg(*mpx*:EGFP)) of the zebra danio species. The fish were maintained under constant water quality parameters in a recirculating system (Zebtec, Tecniplast, Buguggiate, Italy) that ensured continuous water flow. A light program provided a 14-hour daylight period and a 10-hour night period. The fish were fed ZEBRAFEED (Sparos, 400–600 μm) twice daily and freshly hatched brine shrimp larvae (*Artemia* spp) (Ocean Nutrition >230000NPG) twice weekly.

The zebra danio embryos used in the studies were obtained from the spawning of adult individuals. After spawning, the eggs were collected and sorted under a stereomicroscope (Leica M205 FA, Wetzlar, Germany). Fertilized embryos showing normal cell division were selected according to the OECD 236 standard recommendations.

The conducted studies complied with the European Parliament and Council Directive 2010/63/EU on the protection of animals used for scientific purposes. During the doctoral research, zebra danio embryos and larvae in a non-feeding stage were used.

2.2 Acute embryo tests

I designed the 96-hour acute embryo toxicity tests based on the OECD 236 guidelines. After synchronized spawning of the parent fish, I collected and sorted the eggs, placing them into test vessels before the 8-cell stage. The test duration was 96 hours, with daily solution changes to maintain constant concentrations. The tests were conducted in 24-well tissue culture plates, with 20 embryos examined per concentration in three replicates. I repeated the tests twice independently. System water was used as a negative control, and a solvent control (0.026 v/v% DMSO dissolved in system water) group was also set up. For the acute embryo toxicity testing, I used a 200 mg/L stock solution and prepared the test concentrations by diluting this stock solution, ensuring a consistent DMSO concentration. During the 96-hour acute embryo toxicity test, I determined mortality and searched for embryonic developmental abnormalities according to the OECD 236 guidelines. I visually compared the number and size of melanocytes with the control groups. 72 hours post-fertilization, after the larvae hatched, I took digital images of the embryos in

lateral orientation using a Leica M205 FA stereomicroscope. As the test endpoint, I calculated the $LC/EC_{1,\ 10,\ 50,\ 90}$ values using probit analysis according to the OECD guidelines.

2.3 Embryonic studies aiming to map the harmful effects of sublethal bendiocarb exposure

Based on the results of the acute embryonic studies, I selected sublethal *bendiocarb* concentrations (3, 1.5, 0.75, 0.4, 0.07) to determine the sublethal consequences of *bendiocarb* exposure. System water was used as a negative control, and a solvent control (DMSO) group was also set up. The DMSO exposure was below the limit set by the OECD 236 guidelines (0.01 v/v%). The experiments were conducted in 10 cm Petri dishes with daily solution changes. A 3 mg/L stock solution was used for the sublethal studies, and the test concentrations were prepared by diluting this stock solution, ensuring a consistent DMSO concentration. Each test concentration was set up in triplicate, and the tests were repeated twice independently. Test and control solutions (50 ml) were placed in the Petri dishes. The eggs used in the tests were obtained from synchronized spawning of the parent fish and were sorted and placed in the Petri dishes before the 8-cell stage. The test duration was 96 hours, similar to the acute test, followed by sampling necessary for further investigations.

2.3.1 Morphological examinations

Based on the acute embryo toxicity results, I conducted morphological examinations to determine the effect of *bendiocarb* on total body length and the length of the notochord region. 96 hours post-fertilization, I took digital images of the embryos (2.2*shh*:gfp:ABC#15 transgenic line) in lateral orientation using a Leica M205 FA stereomicroscope. For each concentration, images were taken of 10 larvae per replicate. The images were analyzed using Image J software with a size scale for measurement.

2.3.2 Heart rate measurement

Heart rate measurement was conducted 48 hours post-fertilization. The heart function of 10 larvae was recorded for 20 seconds in each treatment replicate, in lateral orientation, using a Leica M205 FA microscope. The recordings were slowed down, and the heartbeats of the larvae were manually counted.

2.3.3 Enzyme activity assays in zebra danio embryos

Samples were taken from the treatments at 48, 72, and 96 hours post-fertilization. I placed 20 larvae into Eppendorf tubes (two subsamples per replicate) and homogenized them (TissueLyser LT, Qiagen, Germantown, MD, USA). Enzyme activity was evaluated in triplicate at 25 °C for each replicate. The protein concentration of the samples was determined in triplicate using the Bradford method (Bradford 1976), adapted for microplate assays.

The AChE activity was measured according to Ellman et al. 1961., adapted for microplate assays (Guilhermino et al. 1996). Catalase (CAT) enzyme activity was measured using the method of Aebi (1984). Glutathione-S-transferase (GST) activity was determined using the method of Habig et al. (1974), adapted for microplate assays. For glutathione peroxidase activity measurement, I used the method of Paglia & Valentine (1967) with modifications by Lawrence & Burk (1976), adapted to a 96-well tissue culture plate (Faria et al. 2009). Total superoxide dismutase (SOD) activity was measured in triplicate using the xanthine oxidase/cytochrome c method (Crapo et al. 1978).

Lipid peroxidation (LPO) in tissue homogenates was assessed based on malondialdehyde formation using the thiobarbituric acid method developed by Wills (1987 a-b). Results were reported in micromoles of thiobarbituric acid reactive substances (TBARS) per milligram of homogenized protein.

2.3.4 Behavioral assessments

Following 96 hours of *bendiocarb* exposure, I conducted behavioral assessments. During the behavioral tests, the larvae were placed in a flat-bottomed 96-well tissue culture plate. For each concentration, 10 larvae were examined per replicate (1 larva per well) using the Zebrabox system (ViewPoint Life Science, France, Figure 6). During the assessment, the larvae were subjected to a light cycle stimulation: 10 minutes of light (daylight), 20 minutes of darkness (nighttime), and 10 minutes of light.

2.3.5 RNA sequencing and transcriptome analysis

The total RNA sequencing and subsequent analysis were performed at the Department of Biochemistry and Molecular Biology, University of Debrecen. For the study, embryonic *bendiocarb* exposure was conducted with sublethal concentrations, including a lower concentration (0.4 mg/L) and the highest concentration (3 mg/L). At the end of the treatment, 30 larvae per replicate were placed in 200 µL Trizol reagent in microcentrifuge tubes, with a total of 4 replicates. After homogenization, the samples were supplemented to 400 µL and stored at -80 °C. RNA isolation was performed using chloroform separation, isopropanol precipitation, and ethanol washing. Then, the integrity

of the RNA was assessed using the Eukaryotic Total RNA Nano Kit and Agilent BioAnalyzer. RNA libraries were prepared using the Ultra II RNA Sample Prep Kit. First, mRNA with poly(A) tails was bound to a deoxythymidine sequence conjugated to magnetic beads, then eluted and fragmented. cDNAs were created using reverse transcription and random primers. Sequencing was carried out with an Illumina NextSeq500 device, following adapter ligation. Raw data were aligned to the zebra danio reference genome (GRCz11) using the HISAT2 algorithm. Differentially expressed genes were identified using StrandNGS software. Finally, the results were analyzed with *Gene Ontology Enrichment Analysis* (GOEA) and evaluated using Cytoscape/ClueGo software.

2.3.6 Determination of gene expression levels

Based on the results of transcriptome analysis and literature review, various marker genes were selected. Their expression levels were measured using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Following *bendiocarb* exposure, for each group, 30 larvae were placed in 200 µL Trizol reagent in microcentrifuge tubes (5 replicates per group), then homogenized (30 larvae per tube). The homogenates were supplemented to 400 µL and stored at -80 °C. RNA isolation was performed using chloroform separation, isopropanol precipitation, and ethanol washing, similar to previous methods. The quantity and quality of the nucleic acid-free isolated RNA were assessed using a NanoDrop One (Thermo Fisher Scientific, Madison, USA) spectrophotometer. Complementary DNAs (cDNAs) were generated using the High Capacity cDNA Reverse Transcription Kit. Reactions were performed using a LightCycler 480 II (Roche) device, using 5x HOT FIREPol EvaGreen qPCR Supermix. The obtained results were normalized to the expression of the *ef1a* housekeeping gene and expressed as normalized mRNA levels.

2.3.7 Histological examination

Following 96 hours of *bendiocarb* exposure, samples were collected for histological examination. The preparation and analysis of tissue sections were conducted at the Department of Pathology, University of Veterinary Medicine. The fishes, fixed in 8% buffered formaldehyde, were dehydrated through an ascending alcohol series and then in absolute ethanol, followed by impregnation with xylene at 56°C. The samples were then embedded in Paraplast, and sections were cut using a microtome. The sections were deparaffinized in xylene twice, followed by washing in a descending alcohol series (absolute ethanol, 96%, 90%, 80%, 70%, 50% ethanol) and then with distilled water. Finally, the sections were stained with hematoxylin and eosin for subsequent microscopic examination.

2.3.8 Measurement of nitric oxide production in vivo

Nitric oxide production within the larvae was assessed using the diaminofluorescein-FM diacetate (DAF-FM-DA) fluorescent assay, following the protocol by Lepiller et al. (2007). After 96 hours of *bendiocarb* treatment, larvae were transferred individually to a 96-well tissue culture plate. Each well of the microplate was pre-filled with 200 µL of a 5 µM DAF-FM-DA solution. Following incubation (25.5 °C, 1 hour, in the dark), the larvae were washed with system water and anesthetized by adding a tricaine methanesulfonate solution (MS222, 168 mg/L). After anesthesia, the larvae were positioned laterally and photographed under the Leica M205 FA stereomicroscope with a GFP filter. Fluorescence intensity in the notochord region of the larvae was measured using Image J software.

2.3.9 Determination of neutrophil granulocyte cell count and distribution

The study used offspring from the Tg(*mpx*:EGFP) transgenic zebra danio line. Neutrophil granulocyte cell counts were determined using fluorescence-activated cell sorting (FACS) at the Department of Immunology of the University of Debrecen. The samples came from two independent experiments, each group with three replicates. For each replicate, 15 larvae were placed into microcentrifuge tubes cooled with ice-cold water. The larvae were then washed in Ringer's solution and digested with 0.25% trypsin-EDTA. Digestion was halted by adding fetal bovine serum (FBS) and calcium chloride. Following centrifugation (400 G, 5 min), the samples were resuspended in 5% FBS/PBS and filtered through 40 µm pore size cell strainers. The frequency of EGFP+ cells was determined using a NovoCyte Flow Cytometer, for 10^5 cells.

To assess neutrophil granulocyte distribution within the larvae, images were captured with a GFP filter (Leica M205 FA) after *bendiocarb* treatment, using lateral orientation. Prior to imaging, the larvae were anesthetized with tricaine methanesulfonate (MS222, 168 mg/L). Neutrophil granulocyte distribution was evaluated based on the images, and EGFP+ cells in the notochord region were quantified using Image J software.

2.3.10 Tail fin injury-based alterations

Tail fin injuries were performed based on the method by Cheng et al. (2020) with minor modifications. After *bendiocarb* treatment, 84 hpf Tg(*mpx*:EGFP) larvae were anesthetized with tricaine methanesulfonate (MS222, 168 mg/L). An incision was made along a straight line between the notochord and the tail end using a sterile razor blade. The larvae were then placed in 24-well cell culture plates containing fresh system water. Neutrophil migration to the injury site was recorded using a GFP-filtered Leica M205 FA stereomicroscope, at 4

and 12 hours post-injury. The number of neutrophils around the wound was quantified using Image J software.

2.3.11 Statistical evaluation

The data obtained from acute embryo tests were analyzed using the Prism 6.0 (6.01 for Windows) statistical software package (GraphPad Software Inc.). Non-linear regression was applied during the analyses, and a 95% confidence interval was used.

For sublethal studies, normality testing was performed using Shapiro-Wilk and Kolmogorov-Smirnov tests. Statistical differences between groups were evaluated depending on the normality test results. Non-parametric one-way ANOVA (Kruskal-Wallis test and Dunn's post hoc test) or parametric one-way ANOVA and Dunnett's post hoc test was used. Results are presented as mean ± standard deviation.

The data from enzyme activity assays were analyzed using the OriginPro (2019 version, OriginLab Corporation, Northampton, MA, USA) software package. Non-linear regression was applied to determine the concentration-dependence of biochemical marker activities. Two-way analysis of variance (ANOVA) was used to examine the interactive effects of different *bendiocarb* concentrations and exposure times on biochemical markers. Time (t = 48; t = 72 and t = 96 hpf), treatment (control, DMSO, 3, 1.5, 0.75, 0.4, and 0.07 mg/L), and their interactions were categorical predictor factors, while the measured biomarkers were considered dependent variables. Prior to statistical analyses, raw data were tested for normal distribution and variance homogeneity using Kolmogorov-Smirnov and Levene's tests.

In transcriptomic analysis, corrected T-tests (Moderated T-tests) and Benjamini-Hochberg FDR corrections were applied.

Results were considered statistically significant if the p-value was less than 0.05.

3. Results

3.1 Results of the fish embryo toxicity test

Following the 96-hour *bendiocarb* treatment, I calculated the LC/ $EC_{1, 10, 50, 90}$ values. Based on the mortality data, the 96-hour half-lethal concentration of *bendiocarb* was 32.52 mg/L. During the assessment of *bendiocarb*'s effects on embryos, I observed various deformities, which showed a dose-response relationship. The EC_{50} value based on these deformities was 2.30 mg/L for a 96-hour exposure. The most characteristic deformities observed at concentrations above 3.12 mg/L were tail development abnormalities, and at concentrations above 0.39 mg/L, the larvae's body length was reduced. In addition to these, other developmental abnormalities were also noted during the 96-hour treatment period.

3.2 Results of embryonic studies on the harmful effects of sublethal bendiocarb exposure

3.2.1 Results of morphological investigations

Based on the results of the acute embryonic experiments, I continued the investigations with a narrower concentration range using a transgenic line (Tg(*shh*:GFP)). The results reflected my previous observations. Both the total body length and the notochord length were significantly reduced at concentrations of 0.75 mg/L and above, compared to the negative and solvent control groups ($p<0.05$; $N=60$). No other abnormalities were observed during the experiment.

3.2.2 Changes in acetylcholinesterase activity, heart rate, and *hspb 11* gene expression

AChE activity in the larvae showed a significant decrease during development under constant *bendiocarb* exposure, across all three testing phases (48 hpf, 72 hpf, 96 hpf). The observed inhibition proved to be concentration-dependent and time-dependent at the measured time points. *Bendiocarb* treatment led to a significant, concentration-dependent induction of the *hspb 11* gene within the 0.75-3 mg/L range. However, a significant increase in heart rate was observed in the 0.4-3 mg/L concentration range compared to the control groups.

3.2.3 Determination of enzyme activity in zebra danio larvae

During the enzyme activity studies, my measurements indicated that, for SOD, a significant concentration-dependent inhibition developed in the 48 hpf larvae exposed to *bendiocarb*. In contrast, at the 72 hpf phase, SOD activity significantly increased with 0.07, 1.5, and 3 mg/L *bendiocarb* treatment. CAT enzyme activity showed no significant change in larvae at 48 hpf and 72 hpf following *bendiocarb* treatment. A significant increase was observed only in the 0.4 mg/L treatment group at 96 hours post-fertilization, while a significant decrease was noted in larvae exposed to 3 mg/L *bendiocarb*. GST activity exhibited both concentration and time dependency during the 48 hpf and 72 hpf developmental stages. GST activity significantly increased at 48 hpf following 3 mg/L *bendiocarb* treatment, and a significant increase was also observed at 72 hpf from 0.4 mg/L *bendiocarb* treatments. Interestingly, CAT and GST activities showed the highest activity at 96 hpf following 0.4 mg/L *bendiocarb* treatment. Despite this trend, significant differences and concentration-dependency were detectable only for CAT. Both total glutathione peroxidase (GPxTOT) and selenium-dependent glutathione peroxidase (GPx-Se) activities followed a similar pattern. A significant increase was measured only for GPx-Se, in embryos treated with 0.75 mg/L *bendiocarb* at 48 hpf, and with 3 mg/L concentration at 72 hours. Both GPxTOT and GPx-Se activities gradually decreased at the 96-hour treatment phase, but this concentration-dependent trend was not supported by statistical analysis.

Lipid peroxidation (LPO) levels did not show significant changes at either 48 hpf or 72 hpf following *bendiocarb* exposure. However, a significant increase was observed at 96 hours post-fertilization in groups treated with 0.4 mg/L *bendiocarb*.

3.2.4 Effects of sublethal bendiocarb exposure on larval behavior

Minute-by-minute records of the larvae's movement indicated that *bendiocarb* exposure reduced movement-related behavior, especially at higher concentrations. During the dark phase, both the total distance traveled and the time spent moving in large movements showed a significant decrease from the 0.75 mg/L concentration upwards compared to the control group. During the dark cycle, the time spent on small movements and the distance traveled with these small movements followed a similar trend to the previous results, but concentration-dependent inhibition was already evident in the 0.4-3 mg/L range. Larval activity in response to light stimulus before and after its onset indicated that the animals respond to changes in photoperiod even at higher treatment concentrations. A significant decrease in both small and large

movements was measurable immediately after the onset of the dark phase compared to the control group.

3.2.5 Effects of sublethal bendiocarb exposure on the larval transcriptome

Using full RNA sequencing and transcriptome analysis, I observed that *bendiocarb* treatment significantly altered the mRNA expression of 2,694 genes. Among these differentially expressed genes, 1,806 showed increased expression, while 884 exhibited decreased expression in *bendiocarb*-treated larvae. With a 0.4 mg/L *bendiocarb* exposure, 10 genes were downregulated, whereas 64 genes were upregulated. At a concentration of 3 mg/L, the number of underexpressed genes was 857, and the number of induced genes was 1,644. After exposure to 0.4 mg/L and 3 mg/L *bendiocarb*, the number of induced genes was 98, and 17 genes showed decreased expression in both treatment groups. One gene showed decreased expression at 0.4 mg/L but was overrepresented at 3 mg/L. Conversely, three genes demonstrated increased expression at 0.4 mg/L but were underrepresented after 3 mg/L *bendiocarb* treatment.

In the gene ontology analysis, 62 genes related to visual perception and sensory development showed decreased expression due to 3 mg/L *bendiocarb* treatment. Among genes associated with the immune system, 100 genes were induced, and 132 genes related to the muscular system showed overrepression after 3 mg/L *bendiocarb* exposure.

3.2.6 Effects of sublethal bendiocarb exposure on larval visual perception

I examined the expression of two genes involved in light perception and phototransduction (*rho, opn1mw2*) using RT-qPCR. *Bendiocarb* treatment resulted in significant repression of these selected marker genes. Histopathological analyses, focusing on changes to the larvae's eyes, revealed differences in individuals treated with a 3 mg/L concentration. Specifically, vacuolization developed among the retinal layers of the larvae's eyes.

3.2.7 Effects of sublethal bendiocarb exposure on larval muscle tissue

The expression of two muscle-specific genes found in vertebrates (*desma, fn1b*) changed significantly following *bendiocarb* exposure. *Bendiocarb* treatment resulted in a significant, concentration-dependent induction of the *desma* gene in the 0.75-3 mg/L concentration range. *Bendiocarb* treatment led to a significant, concentration-dependent induction of the *fn1b* gene in the 1.5-3 mg/L concentration range. Histopathological examinations targeting muscle tissue revealed mild nuclear and sarcoplasmic alterations (edema,

vacuolization) at a concentration of 1.5 mg/L. At a concentration of 3 mg/L, these alterations were exacerbated, with discoid degeneration also observed.

3.2.8 Effects of sublethal bendiocarb exposure on the larval immune system

Firstly, I examined the expression of two immune response-associated genes (*cxcl18b, cxcl8b.1*) using RT-qPCR. Significant, concentration-dependent induction of these marker genes was observed at *bendiocarb* exposures of 1.5 mg/L and 3 mg/L. These genes are, among other things, neutrophil granulocyte chemoattractant factors, suggesting that *bendiocarb* exposure may modulate the distribution, quantity, and behavior of granulocytes. Imaging of 96-hour larvae (Tg(*mpx*:EGFP)) revealed that *bendiocarb* treatment alters the distribution of neutrophil granulocytes within the larvae, with increased accumulation along the lateral line. However, fluorescence-activated cell sorting indicated no significant difference in the frequency of $EGFP^+$ cells. I then assessed granulocyte response using a local, sterile inflammation model based on tail fin injury. A significant, concentration-dependent decrease was observed in *bendiocarb*-treated larvae at the 4-hour post-injury mark. Subsequently, I examined NO production and found, using a fluorescent probe, that NO production increased in the 96-hour larvae along the lateral line and the notochord region.

4. Conclusions and Suggestion

Numerous pesticides have been found to be associated with health and environmental problems. Due to its use, *bendiocarb* has been detectable in various water bodies worldwide and even in the blood of pregnant women, which poses a serious risk to unborn children. However, there is limited information available on the potential effects of *bendiocarb* exposure, and therefore not all details of its health impacts and mechanisms of action are known. This necessitates studies that contribute more detailed insights into the biological consequences of *bendiocarb* exposure.

In the studies conducted, I determined that the LC_{50} value of *bendiocarb*, 96 hours post-fertilization, is 32.52 mg/L in zebra danio embryos. This value proved to be significantly higher than previously reported for other fish species, but similar to LC_{50} values reported for the water flea (*Daphnia magna*) (32-150 mg/L).

Regarding the mechanism of action, *bendiocarb* belongs to the group of neurotoxins and, as expected, caused concentration- and time-dependent inhibition of AChE activity. *Bendiocarb* exposure led to significant, concentration-dependent induction of the *hspb 11* gene in the range of 0.75-3 mg/L. In 48-hour zebra danio embryos, *bendiocarb* exposure resulted in a significant increase in heart rate within the 0.4-3 mg/L range. The effects of carbamates on heart rate are varied, and *tachycardia* can be as common as the likely *bradycardia* based on the mechanism. My results suggest that the tested range of *bendiocarb* concentrations causes *tachycardia*. This conclusion is further supported by the increased expression of the *hspb 11* gene associated with nAChR activity, indicating that effects related to nicotine receptors are likely involved.

Carbamates have previously been shown to induce oxidative stress. Recent studies have reported that *bendiocarb* alters antioxidant and detoxification enzyme activities in mammals. At 48 hours post-fertilization, the SOD activity in larvae significantly decreased due to 3 mg/L *bendiocarb* treatment. Embryonic *bendiocarb* treatment exhibited a specific pattern in CAT activity during the 96 hpf exposure window. The highest significant CAT activity was measured in the 0.4 mg/L treatment group, which significantly decreased at higher concentration ranges. Previous studies have reported that *bendiocarb* treatment can increase GST activity in the liver and kidneys of rats. My results were similar, with GST enzyme activity increasing depending on concentration at 48 and 72 hours post-fertilization. Lipid peroxidation levels followed a similar trend to CAT enzyme activity, with the highest LPO levels detected at 0.4 mg/L, and decreasing at higher concentrations. Comparing with the literature, the results suggest that LPO exhibits a hormetic response to carbamates across different fish species.

Various xenobiotics entering the environment influence the natural circadian behavioral rhythm and physiology of organisms. Based on the studies in this doctoral work, it can be concluded that sublethal *bendiocarb* exposure reduces the movement intensity of developing larvae at higher concentration ranges. My findings are consistent with several previous studies on the effects of various insecticidal carbamates on behavior. Overall, it can be stated that *bendiocarb* disrupts behavior, likely due to the accumulation of ACh and excessive receptor stimulation causing neurotoxic symptoms.

Post-exposure to sublethal *bendiocarb*, significant differences were found at the transcriptomic level, affecting the expression of more than 2694 genes. Among the repressed genes, those involved in sensory development and visual perception were notably enriched. Pesticides, including insecticidal carbamates, can impact the development, function, and morphology of fish retinas. Embryonic *bendiocarb* exposure resulted in significant repression of two marker genes (*rho, opn1mw2*) involved in light perception and phototransduction. In the highest concentration-treated individuals, vacuolization was observed between the retina layers. It is likely that *bendiocarb*, similar to other insecticidal carbamates, affects retinal development. However, behavioral studies indicate that treated larvae can detect changes in the dark-light cycle.

In the transcriptomic analysis of embryonic *bendiocarb* exposure, genes related to muscle tissue and muscle system processes were notably among those with increased expression. Embryonic *bendiocarb* exposure induced the expression of two muscle-specific genes (*desma, fn1b*), and histopathological examinations revealed tissue alterations at the two highest concentrations. *Bendiocarb* exposure produces similar symptoms to those mentioned in previous studies on AChE inhibitors.

The analysis of the transcriptome concluded that gene groups involved in immune system-related processes also showed increased expression. This was associated with the concentration-dependent induction of two immune response-associated genes (*cxcl18b, cxcl8b.1*), altered distribution of neutrophil granulocytes, and increased nitrogen monoxide production, particularly intense along the lateral line. In a local inflammation model (tail fin injury), neutrophil granulocyte accumulation in the injury area was reduced. Based on these results, *bendiocarb* exposure may induce immunomodulatory effects and likely triggers a systemic inflammatory response in developing larvae. Carbamate mechanisms are based on AChE inhibition and react with the serine primary alcohol hydroxyl group. However, serine hydrolase activity is likely linked to several immune functions. Previous studies have shown that carbamate pesticides can alter lymphocyte cholinergic signaling and esterase activities related to immune cell membranes through AChE inhibition. Furthermore, inhibition may lead to structural and functional

changes in immune cells, modify signaling pathways, and either inhibit or stimulate immune cell activation, proliferation, and effector functions. It is also likely that free oxygen radicals generated by carbamates inhibit T-cell function through membrane lipid peroxidation, although specific effects of insecticidal carbamates on immune function are still poorly understood.

During prenatal development, vertebrate embryos are particularly sensitive to substances that affect cholinergic receptors or AChE function. Neurotransmitters play growth-regulating and morphogenetic roles in developing tissues. The developing neurotransmitter system is especially vulnerable to acute cholinergic toxicity of organophosphates and carbamate pesticides, designed to affect receptors. This doctoral work provides insights into symptoms caused by *bendiocarb* concentrations below LC_{10} in early life stages. The studies were conducted on zebra danio embryos, a model organism used in human disease modeling and preclinical drug testing. Complementary toxicological, morphological, enzyme activity, behavioral, transcriptomic, histopathological, and immunological studies provided results consistent with each other and the literature. Overall, embryonic *bendiocarb* exposure affects numerous biological processes through AChE inhibition, which can significantly impact the later life processes of the developing organism. However, caution is required when extrapolating the results to more advanced vertebrates, and further studies are necessary. Nevertheless, the results obtained from zebra danio embryos provide a solid foundation.

Suggestions

Based on the results of this doctoral work, the following investigations are recommended for the future:

1. Conduct chronic exposure studies to understand embryonic/adult, and even intergenerational effects, and to uncover long-term consequences
    - Investigate potential hormone-modulating effects.
    - Perform toxicological tests to assess reproductive capabilities.
2. In light of the obtained results, carry out additional cardiotoxicity studies, complemented by the use of heart regeneration models.
3. Further explore the immunomodulatory effects, potentially involving a macrophage-specific transgenic zebra danio line.
4. Conduct mixture studies.

5. New Scientific Results

1. I was the first to examine the sub-lethal and effective concentrations of *bendiocarb* on zebra danio larvae using acute toxicity tests over a 96-hour exposure period. The 96-hour LC_{50} value was found to be 32.52 mg/l. I determined that *bendiocarb* exposure significantly affects larval development at concentrations above 3.12 mg/l.

2. I was the first to demonstrate that sub-lethal concentrations of embryonic *bendiocarb* exposure lead to decreased heart rate and movement intensity, with reductions in AChE, SOD, CAT, GST, and GPxSe activities, and decreased LPO levels in 96-hour zebra danio larvae at concentrations ranging from 0.4 to 3 mg/l.

3. I was the first to describe the effects of sub-lethal concentrations of embryonic *bendiocarb* exposure on the total transcriptome based on full RNA sequencing in 96-hour zebra danio larvae. The 3 mg/l *bendiocarb* exposure induced genes related to muscle and immune system processes and decreased the expression of genes associated with visual perception, sensory development, and nervous system development.

4. I determined that *bendiocarb* negatively affects the visual perception of developing embryos and causes histopathological changes in the eye at a 3 mg/l concentration, but enhances muscle function in developing embryos and causes histopathological alterations within the tissue.

5. I was the first to report that sub-lethal concentrations of embryonic *bendiocarb* exposure alter the distribution of neutrophil granulocytes in zebra danio larvae and reduce their inflammatory response. I also found that *bendiocarb* increases nitrogen monoxide production along the lateral line of the larvae.

Publications Related to the Topic of the Thesis

Publications in international journals

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