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The Study of the Toxic Effect of Acetamiprid on Blood Factors, Protein Total, and Lactobacillus in the Intestine of Cyprinus Carpio

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ABSTRACT

Many pesticides enter aquatic ecosystem after use and act as environmental pollutants. Changes in the biochemical parameters of fish blood serum can be considered as a suitable factor for detecting the effects of toxicity and determining the physiological status of fish exposed to pesticides. Lactic acid bacteria are also the most common types of bacteria present in the digestive tract of fish. These bacteria are effective in treating rotavirus diarrhea, lowering blood sugar, blood pressure and cholesterol, and preventing large and small intestine and liver cancer, inflammatory bowel disease, infections, acute diarrhea, and the growth and proliferation of harmful bacteria. They also strengthen the immune system and help digestion and absorption of minerals and vitamins. In this study, Cyprinus Carpio were exposed to 17, 34, 56, 85 and 113 micrograms per liter of Acetamiprid pesticide for 35 days and its effect on protein, red blood cell, hemoglobin, hematocrit, white blood cell and lymphocyte were studied. The results of biochemical studies revealed that after 35 days that Cyprinus Carpio were exposed to Acetamiprid, the levels of red blood cells, hemoglobin, hematocrit, white blood cells and lymphocytes in fish blood decreased significantly; however, there was a significant increase in total blood protein compared to the control sample. Also, after separating the G^{+} bacilli from the intestine of the fish and examining its anti biogram with different doses of Acetamiprid, it was determined that in all doses we did not see the growth of lactose bacilli. Due to the effect of Acetamiprid on blood biochemical parameters and intestinal bacilli, it seems that the use of this pesticide and its subsequent application to running water can affect the survival of Cyprinus Carpio.

1. Introduction

The increasing development of agriculture in the recent years and the rapid growth of industrialization in our country, have led to a significant increase in the production and use of chemical fertilizers, pesticides, petrochemicals, detergents, and other synthetic chemicals which has posed a serious threat to the aquatic ecosystem and life (Ma et al., 2019).

Today, pesticides are used in agriculture and for many other purposes, such as for protecting human and animal health, pest control in forests and aquatic environments, and protecting buildings and other structures (Abedi et al., 2013).

Pesticides enter the water directly and indirectly, and fish usually receive them through

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gills and the surface of their body. The presence of pesticides in organs and adipose tissues causes severe changes in tissue biochemistry and fish histology (Muthukumaravel et al., 2013). Fish are considered as the most important and vital link in the ecosystem food chain and the domestic fishing industry is an important source of protein in a country's diet. So, a thorough understanding of the effects of pesticides on the intestine of fish is essential for fish conservation and fisheries development (Sabra & Mehana, 2015). Many pesticides cause deadly biochemical changes in fish, including Ion Consentration changes, organic compounds, enzyme activity, endocrine activity, and osmotic regulation (Kondera et al., 2017).

Acetamiprid is one of the most well-known insecticides which belong to the group of Neonicotinoids that affect the nervous system of insects. Acetamiprid, by disrupting the action of nicotinic acetylcholine neurotransmitters, has an effect on the synapses of the insect's central nervous system (Sminu et al., 2016).

This pesticide is trademarked by Mospilan SP20% which is a Neonicotinoid insecticide and has a contact and stomach action with very strong systemic properties and high local penetration. This compound is formulated in water-soluble powder (SP 20%) to fight against all sucking pests and larvae. Due to its high vapor pressure (V.P.: 1x10-3) Mospilan has a good cooling state and has a good effect on soil pests. It has high-speed insecticide properties but low toxicity to mammals. Acetamiprid degrades rapidly when exposed to the sun. The consumption dose of Mospilan is very low and with this dose, consumption on bees and other pollinating insects and predators is also of low risk (Hallaji Thani et al., 2011).

Insecticides are often washed from the surface of plants and soil after use through the drainage of agricultural fields or leaching of agricultural fields, following excessive irrigation or after seasonal rains and they are washed from the surface of plants and soil and enter the surface and groundwater (European Food Safety Authority, 2016). Very limited information is available on the toxicity of Acetamiprid to non-target organisms. Therefore, the use of animal models in monitoring environmental pollution and the biological effects of toxins on the health indicators of laboratory and animal models can be considered as one of the most common methods in toxicological studies (European Food Safety Authority, 2016).

Cyprinus Carpio are of the most important species of the carp family whose native breed is distributed in the Caspian Sea catchment area and is probably one of the few aquatic species common in Asia, most of Europe, and in some African and Latin American countries which is widely cultivated today. It has also been introduced in North America and Australia (Bahrami et al., 2015).

Blood characteristics and changes in blood biochemical profile are of the most important indices of general biology and indicate changes in metabolism and hemostasis of the fish body, which is mainly due to the effect of pollutants used in laboratory toxicology research (Edsall, 1999). As a fluid and accessible tissue, blood is one of the most important biological fluids in the body, and under the influence of various physiological conditions, its components fluctuate and change (Ma et al., 2019).

Proteins are important biological molecules that are involved in a wide range of cellular functions. A number of studies have reported a decrease in protein levels in various organs and tissues under the stress of various toxic chemicals. Protein reduction may occur because of proteolysis and increased metabolism under toxic stress, protein excretion by the kidneys due to kidney failure, and other factors (Banaee et al., 2019).

Toxic agents that act directly on ribosomes, RNAs, enzymes, or coenzymes may also have a significant effect on protein synthesis (Stanković et al., 2011). Decreased protein total concentration is common in many liver diseases and may be due to impaired synthesis, decreased absorption, or loss of protein. Therefore, protein total is used as an indicator of liver disorders in toxicological studies (Ghelichpour et al., 2017).

The intestines of most animals are made up of bacteria called lactobacilli. Lactobacilli are living microorganisms that, by settling in the intestinal tract, prevent the activity of useless microorganisms and pathogens (Andanil et al, 2012). Lactobacilli are gram-positive, still, without spores and catalase negative which convert different sugars into lactate and acetate (Guy et al., 2014). Lactobacilli also have good antagonistic effects on Staphylococcus Aureus, Escherichia Coli, and Aeromonas Hydrophila (Salehi, 2013).

The most important activity of lactobacilli in fish digestive tract is by improving food absorption through producing extracellular enzymes and vitamins. The results of various experiments have shown that growth, weight gain, specific growth rate, food consumption efficiency, protein efficiency ratio and protein increase were higher in fish fed with lactobacilli. Another important effect of lactobacilli is reducing the occurrence and prevalence of diseases, strengthening the immune system and antiviral activities (Behnsen et al., 2013).

Pesticides are a major threat to aquatic animals, especially fish, and are a major source of protein-rich foods for man (Fırat et al., 2011). In the present study, Cyprinus Carpio were tested for the effect of Acetamiprid on blood factors, protein total and lactobacilli in the intestines of the Cyprinus Carpio.

2. Materials and Methods

2.1. Fish Purchase and Maintenance

In this test, 360 Cyprinus Carpio with an average weight of 30 ± 0.4 g (mean ±SD) were purchased from a Fish Breeding Center and were transferred to 18 aquariums (180 liter tanks) for performing the test. 20 fish were added to each aquarium and distributed in 6 treatments and 3 replicates for each treatment. Of these aquariums, one aquarium was considered as a control in which compared to the others, both treatment and control were repeated three times. The fish were kept for 15 days to adapt to the laboratory conditions.

The physical and chemical characteristics of the water, including a pH of about 7 (Equipment Model PTR79 made by Zag Chemie Company), the total hardness of $CaCO_3$ 175 mg / l (measured using EPA equipment model HANNA H196735), the dissolved oxygen of more than 7 ppm (from Water Quality Meter Equipment, Model 8603) and the temperature of 25±2 C (using Mercury-in-glass thermometer) were controlled.

2.2. Fish Treatment

Acetamiprid was purchased from valid pesticide sales offices. The chronic toxicity test was performed on the Cyprinus Carpio for 35 days and in the form of a completely randomized design. Fish under Acetamiprid were treated with the concentrations of 17, 34, 56, 85, and 113 micrograms per liter, and one treatment was considered as a control group. The test was conducted to determine the lethal concentration based on the guideline OECD (1992) with static water conditions (physical and chemical parameters of water similar to the 15-day adaptation period). During the test period, and after each water change, the amount of pesticide was adjusted to match the changed water.

2.3. Measurement of the Blood Indices of Cyprinus Carpio under Acetamiprid Treatment

After 35 days and the end of the test period, to reduce the stress of the fish, their feeding was stopped 24 hours before collecting blood (Kalbasi et al., 2009). From the treatments, 24 fish (4 fish from each tank) were randomly selected. All fish were anesthetized after fishing to reduce the stress of fishing and blood collection in tubs containing 150 ml of clove extract (Mehrabi, 1999). Under anesthesia, blood samples were taken from the vessels of caudal peduncle at an angle of 45 degrees using 2-millimeter-liter syringes.

The blood of each fish entered the test tube and immediately a blood test was performed at the lab. The tube had an EDTA anticoagulant to prevent blood clots. The counting of white and red blood cells was performed by hemocytometry using Neobar Lams (Maqssod et al., 2009) and (Simmons, 1997). The differential counting of white blood cells was measured through the method proposed by Ameri Mahabadi (1999). Blood hematocrit (PCV) was measured using the method proposed by Mojabi and Heidarnezhad (2004) and hemoglobin (Hb) was measured using the method proposed by Shahsavani et al. (1999).

2.4. Biochemical Indices of Cyprinus Carpio under Acetamiprid Treatment

To determine the total protein content of plasma, a drop of plasma was placed on a special refractometer plate (Model: TMR 33-37), and after placing a special cap on the plasma drop, the device was placed in the direction of a light source (such as outdoor light) and it was looked inside the device through the lens. The grading area of the device showed the total amount of protein in the blood plasma based on grams per deciliter.

2.5. Measurement of Antibacterial Activity of Cyprinus Carpio under Acetamiprid Treatment

At this stage, the sampling of fish intestine was performed and grown in the MRS broth growth medium (Made by Quelab Company) and placed in an anaerobic jar with microaerophilic conditions. After the incubation period, the bacteria were examined for turbidity and were sampled again from the growth medium and grown in the agar MRS medium (Made by Quelab Company). After the incubation period, the desired colony was removed and went under Gram staining (Shah, 2000).

If the bacillus is gram-positive, the catalase test, oxidase test, and movement tests are performed and if it is negative, they are separated, and the separated lactobacilli types are counted through discs method in which the microbial lactobacilli suspension with the relative turbidity equal to half-McFarland tube (108× 1.5 CFU / ML), made from Barium Chloride and Sulfuric Acid, are counted and in turbidity assessment (Using Spectrophotometer, Model pd303s Apple) based on optical absorption is counted by standard bacterium and based on CFU / ML. They are cultured on the plate containing medium of Mueller Hinton Agar (Made by Quelab Company) and then, 10, 20, 30, 40, 50 microliters of Acetamiprid were added to the discs, respectively. Firstly, 10 microliters were added to all discs, another 10 microliters were added to the 20 microliter disc, and another 20 microliters were added to 30 microliter discs. Another 20 microliters were added to 40 and 50 microliters disc. Because the volume of a disk is 30 microliters, all discs must be placed in the hot air oven at 40 C for 15 minutes to dry. Then, 10 and 20 microliters were added to 40 and 50 microliter discs, respectively, and they were placed back in the hot air oven (Made by Pars Azma Company) to dry completely. Then they were placed at a certain distance from each other and from the edge. Next, the plates were placed in an incubator (Made by Pars Azma Company) for 24 hours at 37 C. The diameter of the growth inhibition created by Acetamiprid was measured in millimeters by a ruler (Campus et al., 2006).

2.6. Statistical Data Analysis

Before analyzing the data, the Shapiro-Wilk normality test was performed and if it was normal, the ANOVA test was used. To determine the significant differences between the groups, the Duncan test was used under SPSS 18.

3. Results

At the stage of examining the effects of toxicity, fish were exposed to concentrations of 17, 34, 56, 85, and 113 micrograms per liter of Acetamiprid. At this stage, behavioral changes and changes in blood indices and biochemical factors were studied during 35 days.

3.1. Behavior Change in Cyprinus Carpio under Acetamiprid Treatment

During the first 15 days of the test, the features of all aquariums were almost the same, but in the second half of the month, the appetite of aquariums 5 and 6, which had the highest concentration of pesticides, decreased sharply. Other symptoms include inactivity, poor swimming, accumulation of aerator, and slow breathing (opening and closing of the gills) compared to other aquariums. In the last two weeks, the effects of the pesticides became apparent and fish started to die. The highest losses were in aquariums 5 and 6 which had the highest concentrations of pesticides.

3.2. Change in the Blood Indices of Cyprinus Carpio under Acetamiprid Treatment

The process of change in the level of blood factors of fish under the treatment of Acetamiprid and the control group are presented in Table 1.

The lowercase English letters indicate the presence or absence of statistical differences. The English letters, like the control group, indicate the lack of significance and the rest indicate the significance.

Based on the results of analysis of variance, there was a significant difference between the levels of Red Blood Cells, White Blood Cells, Hemoglobin, Lymphocytes, and Hematocrit in the blood of fish treated with different concentrations of Acetamiprid ($p<0.05$). The highest levels of Red Blood Cells, White Blood Cells, Hematocrit, Lymphocytes, and Hemoglobin were observed in treatment two and the lowest levels were observed in treatment six ($p<0.05$).

According to the data mentioned in the table, increasing the concentration of Acetamiprid has led to almost a decrease in the amount of Red Blood Cells, White Blood Cells, Hemocrit, Lymphocytes and Hemoglobin. Therefore, there is a direct relationship between the concentration of Acetamiprid and the mentioned blood indicators.

3.3. Changes in Biochemical Factors of Cyprinus Carpio under Acetamiprid Treatment

The lowercase English letters indicate the presence or absence of statistical differences. The English letters, like the control group, indicate the lack of significance and the rest indicate the significance.

Based on the results obtained from the analysis of variance, there was a significant difference between the amount of red and white blood cells, hemoglobin, lymphocytes and hematocrit of fish treated with different concentrations of Acetamiprid ($p<0.05$). However, there was no significant difference between the total protein content of the experimental groups ($p>0.05$). The highest levels of red and white blood cells, hematocrit, lymphocytes, and hemoglobin were observed in treatment two, and the lowest rates were observed in treatment six ($p<0.05$).

According to the data in the table, increasing the concentration of Acetamiprid has caused an almost decreasing trend in the amount of red and white blood cells, hematocrit, lymphocytes and hemoglobin. Thus, there is a direct relationship between the concentration of Acetamiprid and the mentioned blood indices.

The amount of protein total in all treatments was significantly different from that of the control group ($p>0.05$). However, there was no significant difference between aquariums that received the pesticides ($p<0.05$).

3.4. The Impact of Different Concentrations of Acetamiprid Pesticide on Lactobacillus

In the disc method and Acetamiprid in discs of 10, 20, 30, 40, 50 microliters on the types of lactobacilli, growth inhibition was observed.

Table 1. Changes in the blood indices of Cyprinus Carpio exposed to Acetamiprid

Treatment	Control Group	Concentration of 17 micrograms per liter of Acetamiprid	Concentration of 34 micrograms per liter of Acetamiprid	Concentration of 56 micrograms per liter of Acetamiprid	Concentration of 85 micrograms per liter of Acetamiprid	Concentration of 113 micrograms per liter of Acetamiprid
RBC	2.04 ± 0.00^{c}	2.30 ± 0.63^{b}	2.21 ± 0.62^{a}	1.92 ± 0.06^{d}	1.98 ± 0.65^{a}	1.80 ± 0.06^{d}
WBC	15127.33 ± 378.50^{b}	18362.33 ± 363.50^{c}	$17147.66 \pm 00399.^{a}$	14790.00 ± 290.00^{d}	13230.00 ± 340.00^{a}	12670.00 ± 290.00^{d}
HCT	35.32 ± 0.58^{a}	34.74 ± 0.85^{b}	33.29 ± 0.65^{a}	32.30 ± 0.28^{c}	31.35 ± 0.28^{a}	29.33 ± 0.28^{c}
Hb	7.49 ± 0.22^{b}	7.70 ± 0.27^{b}	7.24 ± 0.32^{b}	7.12 ± 0.35^{b}	7.00 ± 0.36^{a}	6.78 ± 0.38^{a}
Lymphocyte	53 ± 0.57^{a}	60.00 ± 1.00^{c}	51.00 ± 1.00^{a}	48.00 ± 1.52^{b}	45.00 ± 1.42^{b}	33.00 ± 1.32^{b}

Table 2. Mean table of protein total content

Treatment	Control Group	Concentration of 17 micrograms per liter of Acetamiprid	Concentration of 34 micrograms per liter of Acetamiprid	Concentration of 56 micrograms per liter of Acetamiprid	Concentration of 85 micrograms per liter of Acetamiprid	Concentration of 113 micrograms per liter of Acetamiprid
Protein Total	3.0 ± 13.1^{a}	4.0 ± 72.35^{b}	4.0 ± 78.5^{b}	4.0 ± 75.16^{b}	4.0 ± 76.15^{b}	4.0 ± 73.14^{b}

Table 3. The diameter of the growth inhibition Lactobacillus sp. (mm) against different amounts of Acetamiprid using disc method

Acetamiprid µl mm	10	20	30	40	50
Lactobacillus.sp	9	12	14	18	25

4. Discussion

Changes in serum blood biochemical parameters can be considered as an appropriate factor to diagnose the toxicity effects on target organs and determine the physiological status of fish exposed to Acetamiprid (Raj and Joseph, 2015). Decrease in red blood cells, hemoglobin density, and Hematocrit levels are all signs of anemia due to intoxication with pesticides. This anemia is more likely to be due to the effect of pesticides on liver and to some extent the results are in line with the results of the study of Raj and Joseph (2015). Significant reduction ($P<0.05$) in the number of red blood cells (RBC) and Hematocrit (Hct) in fish exposed to Acetamiprid compared with fish in the control group are of the most important blood responses observed in this test. In other words, decrease in the number of red blood cells and Hematocrit leads to one of the indices of anemia in these animals. This anemia is caused by the effects of free radicals produced by pesticides on the spleen and kidney tissue, which are the hematopoietic centers in the fish. This will reduce the production of red blood cells. The pesticide also destroys red blood cells, which is in line with the result of the studies of Banaee (2019) and Edsall in (1999).

Significant decrease ($P<0.05$) in the number of white blood cells and lymphocytes in the fish exposed to Acetamiprid is quite evident compared to the control group. An increase in the number of white blood cells in the blood confirms the presence of an inappropriate external factor in animals. The decrease in blood counts in this study compared to the control group is a sign of a decrease in the immune system of fish. In response to stress in the aquatic environment, a decrease in the number of white blood cells can indicate suppression of immunity and an increase in their rate shows a respond to stress or infection (Campbell, 2013).

One of the major changes in the biochemical factors of Cyprinus Carpio blood following the exposure to Acetamiprid is a significant reduction in protein total of blood serum. The total concentration of plasma protein compared to the baseline range is used as a clinical index in measuring the health, stress, and physical status of aquatic organisms (Atamanalp et al., 2003). Change in protein synthesis is one of the most common responses to cell damage; therefore, by measuring the amount of protein, the rate of cell damage can be determined (Canli, 1996). Due to the fact that most proteins are synthesized in the liver, a decrease in protein of the blood plasma may be associated with fish liver defects in the exposed to pesticides, which are in line with with the results of the study of Muthukumaravel (2013). In this study, the lactic acid bacteria were separated from the MRS culture medium and the effect of different doses of Acetamiprid on Lactobacillus.sp caused a growth inhibition and the destruction of lactobacilli. This increased the dose of Acetamiprid, made this pesticide more effective, killed lactic acid bacteria and microbial flora, and resulted in the death of fish.

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