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Toxicological Examinations Following Oral Administration of *Bacillus thuringiensis* var *kurstaki* Biological Insecticide in Wistar Rats

Reza Abbasi Larki[1], Ehsan Zayerzadeh[2*], Naser Harzandi[1], Ali Anissian[3]

[1]Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran
[2]Department of Biology, Faculty of Food Industry and Agriculture, Standard Research Institute, Karaj, Iran
[3]Department of Veterinary Medicine, Islamic Azad University, Abhar, Abhar Branch, Iran

Corresponding Author: Ehsan Zayerzadeh, PhD, Assistant Professor, Department of Biology, Faculty of Food Industry and Agriculture, Standard Research Institute, Karaj, Iran. Tel: +98-9129237820, Fax: +98-26-22867922, Email: zayerzadeh@standard.ac.ir

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Abstract

Introduction: *Bacillus thuringiensis* var *kurstaki* is one of the best known biological insecticide which is used extensively in the world. This biopesticide has been broadly used against important insect pests and vectors of animals and humans. However, the controversial data was published to date considering toxicological studies on non-target species are urgent to determine probable adverse effects and risk assessment of this biopesticide. In this research, histopathological changes, hematological and some biochemical factors were evaluated following the single dose oral administration of *B. thuringiensis* in rats.

Materials and Methods: Twelve Wistar rats were randomly divided into 2 groups including experimental and control groups. Animals were treated orally by gavage to single dose of sub-lethal dose (5000 mg/kg) of *B. thuringiensis* suspension. Finally, hematological factors (WBC, RBC, Hb, HCT, MCV, MCH, MCHC and PLT), some biochemical parameters (ALT, AST, ALP, BUN and Cr) and histopathological observations were evaluated.

Results: The results demonstrated that oral administration of high dose of *B. thuringiensis* biopesticide induced some pathological complications including congestion and inflammation in vital organs of rats such as liver, heart, lung and kidney. It also caused hematological abnormalities and biochemical alterations in the experimental animals group.

Conclusions: According to the findings, it can be concluded that high dose of *B. thuringiensis* biopesticide can induce toxicity in rats. Therefore, further investigations including subacute and chronic are recommended.

Keywords: *Bacillus Thuringiensis*, Biological Pesticide, Cry Toxin, Pathology, Hematology, Rats

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Introduction

Potential health adverse effects following exposure to pesticides have raised the consideration of alternative safer methods for pest control. Fifty years ago, biological insecticides such as microbial pesticidees have received specific attention as environmentally safer alternatives.[1] *Bacillus thuringiensis* is one of the well-known and the most widely used biological pesticides.[2] This biopesticides can produce crystalline, proteinaceous and delta-endotoxin, which are highly toxic for different types of agricultural pests.[3,4] *The B. thuringiensis* toxins have massive potential for the control of agricultural diseases.[5] They target a limited spectrum of pest insects that contain specific physiological properties (i.e., gut pH and toxin receptor sites in the midgut) and eventually have low risk to non-target species than broad-spectrum insecticides.[6-8] Cry toxins are mostly found in *B. thuringiensis* strains that mediate their toxic effects on a target organism.[9] Following ingestion by larval insects, the crystals were solubilized in the midgut and subsequently activated by proteases. The activated toxins bind to midgut epithelial cells, form pores and cause osmotic cell lysis eventually leading to insect death. Different *B. thuringiensis* toxins and *B. thuringiensis* toxin genes have been commercially produced for the control of Lepidoptera, Diptera, and other insect orders.[10-12] However, oral exposure of this microbial pesticide is one of the major routes of human and animal exposure due to extensive application of this product in agricultural crops in Iran. Of course, human exposure during the manufacturing of this product is inevitable. On the other hand, there are concerns regarding the probable health effects of Cry toxins on vertebrates, particularly *B. thuringiensis* toxins with inducing toxic responses. Thus, the controversial results that were published considering earlier toxicological studies of *B. thuringiensis*, spores, toxins and *B. thuringiensis* crops on mammals are urgent to determine adverse effects of this biopesticide ensuring human and environmental biosafety.[9]

To our knowledge, there is no animal study which evaluates toxicity of *B. thuringiensis* microbial pesticide formulated in Iran. Therefore, in the present research, the oral toxicity of *B. thuringiensis* microbial insecticide in rat model was studied according to the histopathological, hematological and biochemical analysis. The results of the present study will be a fundamental step for the risk assessment of *B. thuringiensis* microbial insecticide in Iran.

Materials and Methods

Test Materials

Spore-crystals in the lyophilized form of *Bacillus thuringiensis* var *kurstaki* strain PTCC1531 (Cry1A and Cry1C insecticidal toxins) were obtained from the market in Iran (wettable powder, stability: 2 years).

Animals

In the present study, 12 adult male Wistar rats weighing 220 ± 5 g were obtained from the Pasture Institute of Iran. All rats were housed in separate cages and were allowed to be adapted with a lab environment before the experiment. After seven days adaptation with the laboratory environment, rats were randomly allocated into 2 groups. They were kept under sterile and standard conditions (temperature of 22 ± 2 °C, humidity 55 ± 5, and a 12:12 light/dark cycle) with adequate standard laboratory food and tap water. All animals were kept in according to the recommendation of the animal care committee of the Tehran University based on the 'Guide for Care and Use of Laboratory Animals' (NIH US publication 86-23, revised 1985).

Treatment

Bacillus thuringiensis biopesticide were suspended in a sterile saline solution. Twelve rats were randomly divided into 2 groups (6 for each group). One group was chosen as the saline control (first group) and the second group was used as the dosing group. Single sublethal dose (5000 mg/kg BW) of *B. thuringiensis* biopesticide suspension was administered orally to the experimented group of animals. In the pilot experiments, exposures greater than 5000 mg/kg had caused signs of toxicity, so this concentration was considered the maximum tolerated. Finally, after 2 weeks, the animals were sacrificed by intraperitoneal injection of ketamine (30-50 mg/kg) and xylazine (3-5 mg/kg) following the single dose oral administration of this biological insecticide.

Hematological assessment

Hematological parameters were performed using a hemocytometer for veterinary use (ADVIA 120, Hematology system, Siemens, Germany) and was calibrated for rat, in microtubes containing EDTA as anticoagulant. The results represent the mean ± standard deviation (SD) per percentage of cell suspension.

Evaluation of Biomarkers

Blood samples were collected from ventricle into test tubes containing EDTA, kept for 30 minutes at lab temperature and centrifuged at 3000 rpm for 20 minutes. After that, serum samples were separated and the levels of blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were measured using the auto analyzer (BT3000, Italy) and (Biosystems kits, Spain) according to the manufacturer's instructions.

Histological Analysis

The heart, lungs, kidney and liver were isolated and immersed in 10% buffered formalin for 48 hours at room temperature and then sectioned transversely in 3–4 µm slices. Samples were dehydrated in a graded series of alcohol and xylene and embedded in paraffin. For histological processing, the sectioned tissues were stained with Hematoxylin-Eosin and examined for morphological and histological parameters by light microscope (Labomed Lx 400, USA).

Statistical Analysis

All data were expressed as mean ± SD. The mean of all the parameter between the two groups were compared using the Student's *t* test. Data were analyzed using the SPSS software (version 19) and $P<0.05$ was considered statistically significant.

Results

Hematological Assessment

The means of hematological findings were demonstrated in Table 1. The means of parameters including white blood cell (WBC), red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular hemoglobin (MCV) and platelet (PLT) in the treatment group were significantly raised. On the other hand, the means of other hematological parameters including MCH and MCHC in this group did not show any significant alterations compared to the control group.

Biomarkers Assessment

Serum ALT, AST and ALP activity were significantly increased in the experimented group compared to the control group. However, serum BUN and creatinine level were not significantly changed in this group (Table 2).

Table 1. Hematology Parameters Changes Following Oral Administration of *Bacillus thuringiensis* var *kurstaki* Biopesticide (5000 mg/kg) in Rats

Parameters	Groups	
	First group	Second group
WBC (10^3/µL)	8.63±1.32	15.08±3.97*
RBC (10^6/µL)	6.70±0.97	7.86±0.13*
Hb (g/dL)	12.05±1.51	15.3±.643*
HCT (%)	33.6±5.80	44.0±1.32*
MCV (FL)	50.56±1.50	55.91±1.62*
MCH (pg)	18.01±0.972	19.55±0.683
MCHC (g/dL)	34.18±1.16	34.43±1.18
PLT (10^5/µL)	578.83±87.01	781.83± 89.82*

Mean values (standard deviation) are shown for the 6 animals in each group.
*Significant difference ($P<0.05$).
First group: control group. Second group: *B. thuringiensis* treatment group.

Table 2. AST, ALT, ALP, BUN and Creatinine Activity Following Oral Administration of *Bacillus thuringiensis* var *kurstaki* Biopesticide (5000 mg/kg) in Rats

Groups	Biomarkers				
	ALT (U/L)	AST (U/L)	ALP (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
First	48 ± 5.33	113.4 ± 12.20	441 ± 53.2	20 ± 4.91	0.8 ± 0.03
Second	49 ± 5.77	159.2 ± 11.18*	682 ± 59.8*	19 ± 3.24	0.68 ± 0.04

Mean values (standard deviation) are shown for the six animals in each group.

*Significant difference ($P<0.05$).

First group: control group. Second group: *B. thuringiensis* treatment group.

Histopathological Evaluation

Oral administration of *B. thuringiensis* biopesticide induced histopathological complications such as congestion in the liver and heart. It also caused inflammation and congestion in other vital organs of animals including lung and kidney in the experimented group (5000 mg/kg) (Figure 1).

Body Weight

Single dose administration of *B. thuringiensis* biopesticide induced a significant decline in the animal body weight mean in the experimental group (5000 mg/kg) in comparison to the control group (Table 3).

Discussion

The use of biological insecticides as substitutes for chemical pesticides is a safer alternative for insect control in agricultural crops.[13] Nevertheless, biological pesticides may induce adverse effects in human, animals, and the environment.[14]

This study is the first investigation to evaluate the potential toxic impacts of *B. thuringiensis* microbial pesticide in experimented animals in Iran. Previous studies have demonstrated the toxicity of Cry toxins to mammals would negligible due to absence known specific receptors in mammalian intestinal cells.[15,16] Nevertheless, the findings of our study demonstrated that these types of toxins could induce hematotoxicity in experimentally rats. In addition to our findings, other studies also demonstrated some hematological disturbances following *B. thuringiensis* biopesticides exposure in laboratory animals. Mezzomo et al reported the hematotoxicity and genotoxicity of four *B. thuringiensis* spore-crystals genetically modified (GM) to express individually Cry1Aa, Cry1Ab, Cry1Ac or Cry2A following single dose administration of 27 mg/kg, 136 mg/kg or 270 mg/kg in mice. A significant decrease in bone marrow cell proliferation presented cytotoxic but not genotoxic impacts.[17]

The findings of this study demonstrated that the Cry toxins of *B. thuringiensis* could induce hematotoxicity in mice. In this study, Cry 10Aa and Cry 1Ba6 significantly decreased the MCV values, while Cry1Ia increased the red cell distribution width above the reference values. Also, Cry1Ba6 and Cry1Ba6 significantly reduced the number of neutrophils and monocytes, and Cry1Ba6, which significantly decreased both.[18] High white blood cell count or leukocytosis can be resulted from some complications such as infection, inflammation, severe allergic reactions, a disease of bone marrow and immune system disorder.[19]

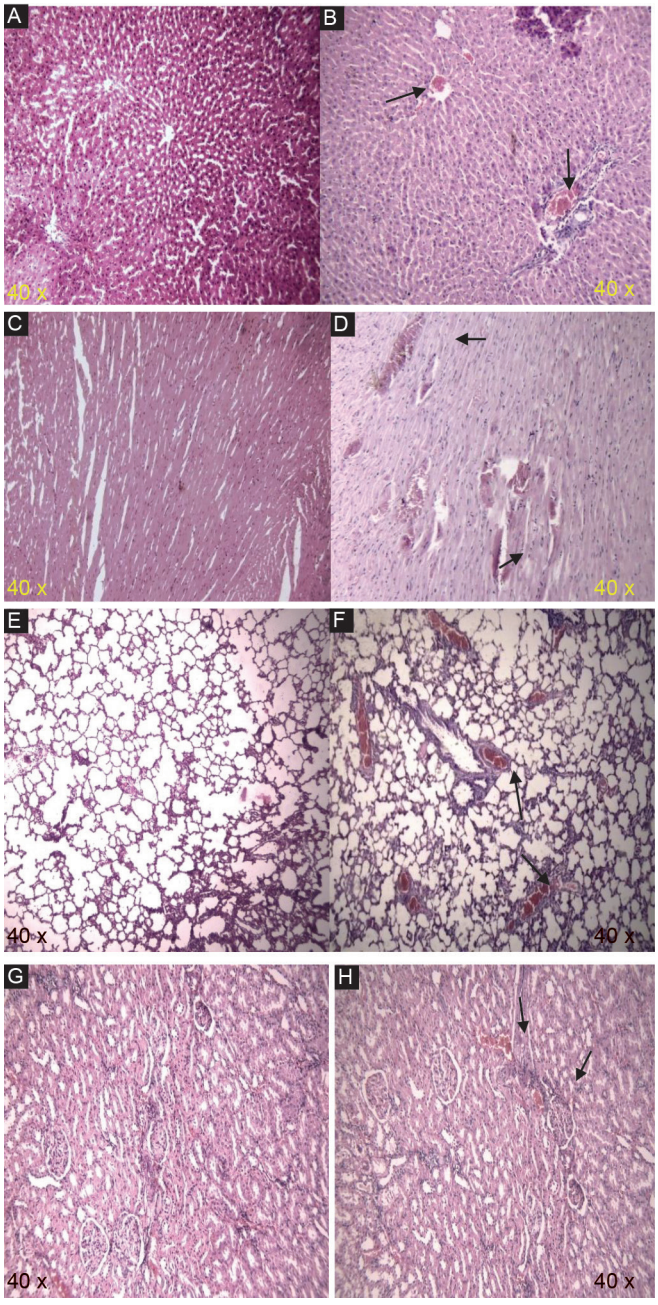

Figure 1. Photomicrographs Of Liver, Heart, Lung And Kidney, sections obtained from rats exposed *to Bacillus thuringiensis* var *kurstaki* Biopesticide (5000 mg/kg). (A): normal liver (H&E), (B): congestion in liver (H&E), (C): normal heart (H&E), (D): congestion in heart (H&E), (E): normal lung (H&E), (F): congestion and inflammation in lung (H&E), (G): normal kidney (H&E), (H): congestion and inflammation in kidney (H&E).

Table 3. Body Weight Following Oral Administration of *Bacillus thuringiensis* var *kurstaki* Biopesticide (5000 mg/kg) in Rats

Study Groups	Initial Body Weight (g)	Final Body Weight (g)	Body Weight Difference (g)
First	202 ± 20	253 ± 23	53
Second	198 ± 19	212 ± 24*	12

Mean values (standard deviation) are shown for the six animals in each group.

* Significant difference ($P<0.05$).

First group: control group. Second group: *B. thuringiensis* treatment group.

Platelets are acute-phase reactants, therefore, they are increased in response to various stimuli, including systemic infections, inflammatory conditions, bleeding, and tumors. Actually, this is called reactive or secondary thrombocytosis, which is a benign form of thrombocytosis. Abnormal blood clotting can be dangerous, as blood clots may block the flow of blood to the brain, liver, heart, and other vital organs.[20] Significant elevation in RBC, WBC and PLT counts was observed in this study. This rise was probably due to the harmful effects of Cry toxins on bone marrow, hematopoietic organs and hematopoietic progenitor cells. On the other hand, the results of this study revealed that oral administration of high dose of this biopesticide induced histopathological alterations such as congestion and inflammation in vital organs of the experimented rats. However, there was no report about pathological changes which can be induced with microbial biopesticides in animals. This is while, a few studies demonstrated inflammation, congestion, edema and hemorrhage in some organs following the administration of some chemical pesticides such as fipronil and chlorpyrifos in animals.[21]

Congestion or hyperemia represents the increase of blood in a territory, due to dilatation of small vessels. According to the mechanism, it may be active or passive. Active congestion is a result of arteriolar distension (e.g., skeletal muscle activity, inflammation, local neuro-vegetative reaction). Passive congestion also termed as stasis, is a consequence of an impaired venous drainage (heart failure, compression or obstruction of veins), that is followed by dilatation of venules and capillaries.[22-24] However, the mechanism of congestion that was induced by this biopesticide was not clear and needs further investigations. The changing of enzymes in the blood is usually used as a marker for the diagnosis of tissue damages. In the present study, the activity of hepatic biomarkers was significantly raised 2 weeks after oral administration of this biopesticide in animals, while the serum concentration of renal biomarkers did not significantly change. The ALT and AST are mainly found in the liver.[25] Also, the AST and ALT have been formerly called serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase, respectively.[26] The AST or ALT serum concentrations are an essential aid in the diagnosis of liver disorders in combination with other markers and histopathology findings.[27] The AST and ALT levels during liver damages can be elevated 10 to 20 times and up to 50 times compare to normal subjects, respectively.[28] On the other hand, the ratio of AST to ALT (AST/ALT) can occasionally help to determine whether the liver has been damaged or not. The ALP is an enzyme for the plasma membrane and endoplasmic reticulum. This biomarker is often used to evaluate the integrity of the plasma membrane.[29] Significant increment of the serum ALP level is usually noticed in liver damage and heart infections.[30,31] However, the findings of the present study demonstrated that high doses of this biopesticide might induce hepatoxicity respecting significant increases of ALT, AST and ALP activity in experimental rats. Creatinine and BUN are the final products of protein metabolism and their levels will be increased in renal failure.[32] Hence, respecting to the results, there is no evidence to nephrotoxicity. Arteaga et al demonstrated that new formulation of *Bti SH-14* is not acutely toxic via dermal route and has no dermal irritation or hypersensitivity but has low eye irritation in the experimented rabbits.[33] Wilcks et al performed an oral administration of *B. thuringiensis* in rats.[34] Finally, these bacteria were detected at high density in fecal and intestinal samples even 2 weeks following the last dosage.[34]

Peng et al reported an oral administration of GM *B. thuringiensis* with vegetative insecticidal protein gene in rats.[35] The results did not show significant differences between control and treated groups following analysis of some factors including body weight gain, food and water consumptions, clinical observations, hematology, serum biochemistry, organ weight ratios and histopathological findings.[35,36]

In the present study, the body weight of the *B. thuringiensis* treated group of the experimented animals was significantly raised in comparison to the control group. In another study, the acute oral toxicity of GM *B. thuringiensis* supplemented with an additional N-acyl homoserine lactones gene were studied in Wistar rats.[37] In contrast with the findings of this study, Meher et al reported that acute oral administration of *B. thuringiensis* in rats has no adverse effects.[37] Previous studies also demonstrated that different doses of *B. thuringiensis* did not have acute toxicity following the administration in rats.[38]

Conclusions

In conclusion, findings demonstrated that oral administration of high dose of *B. thuringiensis* var *kurstaki* biological insecticide can induce some biochemical, hematological and pathological abnormalities in vertebrates such as rats. Incorrect usage of this biological insecticide by farmers can raise the risk of human exposure to significant scale of this biopesticide and can finally cause consequent health problems. The findings propose that further in vivo investigations are needed to characterize the toxicological risks for human beings.

Authors' Contributions

RAL was involved to carry out experiments and preparation of the manuscript and finalized the manuscript. EZ participated in designing of the study and performed the statistical analysis and data interpretation. NH and AA were involved in the manuscript writing and data interpretation.

Conflict of Interest Disclosures

The authors declare that there are no conflicts of interest regarding the

publication of this manuscript.

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