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Histological and Biochemical Evidence of Zinc Toxicity in White Leg Shrimp, *Litopenaeus vannamei* Boone

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***Abstract*:** In an in-vitro study conducted during June to October 2021, the effect of zinc sulphate on the biochemical and histological parameters of *Litopenaeus vannamei* was evaluated at six different doses: 0.5, 1, 2, 4, 6, and 8 mg/L. The higher doses of zinc sulphate were toxic to *L. vannamei* and there were significant changes in the biochemical and histological parameters of vannamei exposed to increasing concentration of zinc sulphate. At higher concentration (8mg/L), a decrease in R-cells, F-cells, B cells, and E-cells, low hemocyte count, high hemolytic infiltration, abnormal lumen, necrosis, and melanization were observed in hepatopancreas. In gills, gill lamella was found to be fused, pillar cells became interspersed, and show large deformities in comparison to control. In the intestine, maximum height of the epithelial cell was seen in control which continuously decreases with the increasing concentration of zinc sulphate. The increasing doses of zinc sulphate showed significant differences in total hemocyte count (THC) of shrimp. The total hemocyte count (THC), total granulocyte counts (TGC), and total agranulocyte counts (TAC) of shrimp were significantly lower in $ZnSO_4$ 8mg/L (14.45, 10.51 and 3.94 in comparison to control (25.18, 17.50and 7.83). Total hemocyte counts is an indicative of health marker for shrimp which showed gradual decrease at higher concentrations.

***Keywords*:** Biochemical, Haemocytic infiltration, Histological changes, *Litopenaeus vannamei*, Necrosis

Heavy metals are well known for their significant contribution in environmental pollution which has gained prominence in the recent years (Chen et al 2020). Many of these metals exist naturally in the environment and are required in trace amount for metabolism of aquatic species. These substances enter the environment through manmade and natural sources, where mostly they form complex with other substances that harm the ecosystem (Häder et al 2020). However, the industrial and agricultural pollutants have aided in increasing the natural levels of such metals in the aquatic environment in. Metals in the marine/estuarine aquatic environment are of ecotoxicological significance because they can disrupt physiological processes such as osmoregulation, respiration, and growth. The hepatopancreas, analogous to liver performs many functions of liver, intestine and pancreas in vertebrates as well as various metabolic processes in crustaceans (Vogt 2019). According to a previous work on the hepatopancreas at different levels *viz*. anatomy, physiology, metabolism, biochemistry, and development, it also performs a variety of other functions such as absorption, digesting, storage, and secretion (Satgurunathan et al 2022). Although, mostly the detoxification processes in crustaceans is performed by hepatopancreas, some xenobiotics, such as pesticides and aflatoxin, are likely to impair the structure and functioning of this organ in aquatic crustaceans (Campbell et al 2022). These compounds have been reported and proven to cause toxicity, resulting in histological alterations in hepatopancreas, gills, and intestines of the investigated species. The primary goal of this study was to investigate effect of zinc on biochemical and histological alterations in the hepatopancreas, gills, and intestine of *L. vannamei*.

MATERIAL AND METHODS

Experimental design: The experiment was conducted for four months, from June to October 2021, at Chaudhary Charan Singh Haryana Agricultural University Hisar, India. *L. vannamei* juveniles were taken from a commercial shrimp farm in Hisar and brought to the laboratory in live condition. After three days of acclimatization, *L. vannamei* juveniles were transferred to experimental aquaria (100 liters each) at a stocking density of five juveniles per tank filled with saline water (20 ppt). To keep the temperature stable (27±0.5°C), each tank was outfitted with a filter, submerged air diffuser, and submersible heater. Due to its prevalence in shrimp tissue, zinc was chosen for the experiment. $ZnSO_4$ salt was used to prepare the stock solution. Six different doses *viz*. T1-T6 of $ZnSO_4$ (0.5, 1, 2, 4, 6 and 8 mg/L) were prepared in triplicates from stock solution and were determined by Atomic Absorption Spectrum (AAS).

Morphological and behavioral changes: The morphological and behavioral alterations in *L. vannamei* were detected during the experiment. The exoskeleton, gills, appendages, telson, and uropod were examined and any

color changes was observed for morphological modifications. The behavioral alterations were recorded throughout the study by observing their movement patterns and swimming habits.

Histological examination: During the experiment moribund shrimp from a different aquarium were collected and injected with Davidson's fixative in hepatopancreas and posterior abdominal regions. Post injection, the shrimps were preserved in the same fixative for 24 hours. Shrimp was dissected and longitudinal body section of different body parts were allowed to dehydrates in 70, 80, 96, and 100% ethanol, then cleared with xylene, embedded in paraffin waxes. Thin sections of tissue were cut at size of 5µ were using a manual microtome. Each sample was rehydrated and stained with hematoxylin and eosin dye.

Total hemocyte count: Shrimp were chosen at random from each treatment. 0.8 ml of blood was collected using the 1 ml hypodermic syringe (26-gauge) from the ventral sinus of shrimp in the first abdominal segment. Each syringe had 0.2 ml of anticoagulant (pH 7.6, 250 mM sucrose, 10 mM Tris-HCl, 100 mM sodium citrate) prefilled. To achieve an equivalent volume ratio of hemolymph to the anticoagulant, more anticoagulant was injected. Anticoagulated hemolymph of about 50 µl volume were kept frozen for 30 minutes in an equivalent volume of neutral buffered formalin (10% NBF) to determine the total hemocyte count (THC) and differential hemocyte counts (DHC). The remaining anticoagulated hemolymph was centrifuged at 300g in 4°C for 10 min to separate the hemocytes from plasma. Phosphate buffer saline (PBS) (pH 7.2, 20 mM) was used to dilute fixed hemolymph 2, 4, 8, 16, and 32 times. Fixed hemolymph was spread on a slide and stained with 10% Giemsa solution, kept for 10 minutes. Tsing et al (1989) were then used to characterize the differential hemocytes.

Total hemocyte count (THC): For total hemocyte counting, a drop of anticoagulant mixed hemolymph (1:1) was transferred to a Neubauer hemocytometer kept under the light microscope in100x magnification (Wootton and Pipe 2003). THC was calculated as total hemocyte cells per ml of hemolymph.

$$\text{THC ml}^{-1} = \frac{\text{Average haemocyte cell count per square} \times \text{dilution factor}}{\text{Number of square counted} \times 10^{-6}}$$

Differential hemocyte count (DHC): After counting total hemocytes, they were differentiated into granulocytes and agranulocytes (hyaline cells) based on granular content and expressed as total granulocyte cells per ml (TGC ml^{-1}) and total agranulocyte cells count per ml (TAC ml^{-1}) using the formulas below.

$$\text{TGC ml}^{-1} = \frac{\text{Average granulocyte cell count per square} \times \text{dilution factor}}{\text{Number of square counted} \times 10^{-6}}$$

$$\text{TAC ml}^{-1} = \frac{\text{Average agranulocyte cell count per square} \times \text{dilution factor}}{\text{Number of square counted} \times 10^{-6}}$$

RESULTS AND DISCUSSION

Morphological and behavioral: Shrimp exposed to higher doses of zinc sulphate, developed black spots on their body, telson, and carapace region. Slow and sluggish movement of the pleopods, less feeding, and erratic movement were recorded during the experiment.

Effect on hepatopancreas: The hepatopancreas of vannamei as observed in control has a well-organized glandular tubular structure**.** The hepatopancreas consists of hepatopancreas tubules and four types of cells: E- cells (embryonic or “embryonalzellen”), F-cells (“fibrillenzellen” or fibrous), R-cells (“restzellen”), and B-cells (“blasenzellen”). The cells exhibited normal structural differentiation into E-cells at the distal end of the tubule, young R -cells and F -cells at a short distance from the distal region, and B- cells in the middle region and proximal end of the tubules. The B-cells have large sized apical secretory granules, the R-cells were loaded with large amounts of rough endoplasmic reticulum (RER) and small lipid droplets, while the F-cells were non-vacuolated and well stained. The lumen of tubules has a normal “star-like” appearance. Tubules were lined by a single layer of epithelial cells and have normal interstitial sinus between them. The structure of hepatopancreas in treatments from T1-T6 (Fig. 1) (0.5mg\L to 8mg/L) was not normal. Hemocyte infiltration (HI), abnormal lumen (ALU) in the interstitial sinus (IS) was observed in the hepatopancreas of vannamei exposed to T2 (1mg/L) and T3 (2mg/L) treatments. In treatment T3-T4 (4mg/L-6mg/L), distinct structural alteration was seen whereas in treatment T1(0.5mg/L) and T2 (1mg/L) observed alterations include., necrotic tubules (NT), melanization (MZ), separation of necrotic tubules of hepatopancreas (NCH), tissue debris in lumen (TD), coagulation in thickened basal laminae (CO) and walling of tubules by hematocytes around basal laminae. Vacuolation was observed in hepatopancreas tissue in all treatments. The exposure of shrimp to very low levels of zinc in the environment resulted in various physiological, biochemical, and histological changes in vital organs and tissues (Frías-Espericueta et al 2008). Findings of Liang et al (2022) also suggest that exposure to zinc enhanced Zn buildup in the hepatopancreas, changed immunological, antioxidant, and detoxifying responses via controlling the gene expression of associated genes, and ultimately could cause apoptosis. Due to zinc toxicity cellular differentiation of hepatopancreas altered resulting in large deformities. The B-

cells which is responsible for the formation of digestive enzymes are reduced in stress condition. The R-cells act as nutrient reserve in the hepatopancreas (Ruiz et al 2022). Hence, upon reduction of R-cells, it may be due to a large fluctuation in demand for osmoregulation (Li et al 2022). This suggested that exposure to zinc has an impact on physiology and resulted in histological alterations in shrimp.

Histological effect on gills: The cellular differentiation in gills was found to be normal in control and T1 (0.5mg/L). The primary gill filaments were branched from the central axis and the secondary gill lamellae showed a finger-like appearance. The secondary lamellae were attached to primary lamellae. The cellular structure of gills in T2-T3 (Fig. 2) (1 mg/L-2 mg/L) exhibited a fusion of gill lamella and showed large vacuolation. In T4 (4mg/L), gill lamella was found to be fused which created a large void in cellular space. In T5 and T6 (6mg/L- 8mg/L) pillar cells became interspersed and show large deformities in comparison to control. Gills are more prone to environmental pollution. The cellular structure of vannamei in T2-T3 (1mg/L-2mg/L) exhibited a fusion of gill lamella and large vacuolation. In T4 (4mg/L) treatment gill lamella was found to be fused which created a large void in cellular space. In T5 (6mg/L) and T6 (8mg/L) pillar cells became interspersed and exhibited more deformities in comparison to other treatments. The zinc disrupts the normal organization of the gill and reduces the oxygen consumption of shrimp. At higher concentrations, it was difficult to distinguish the cellular structure of gills and resulted in 100%

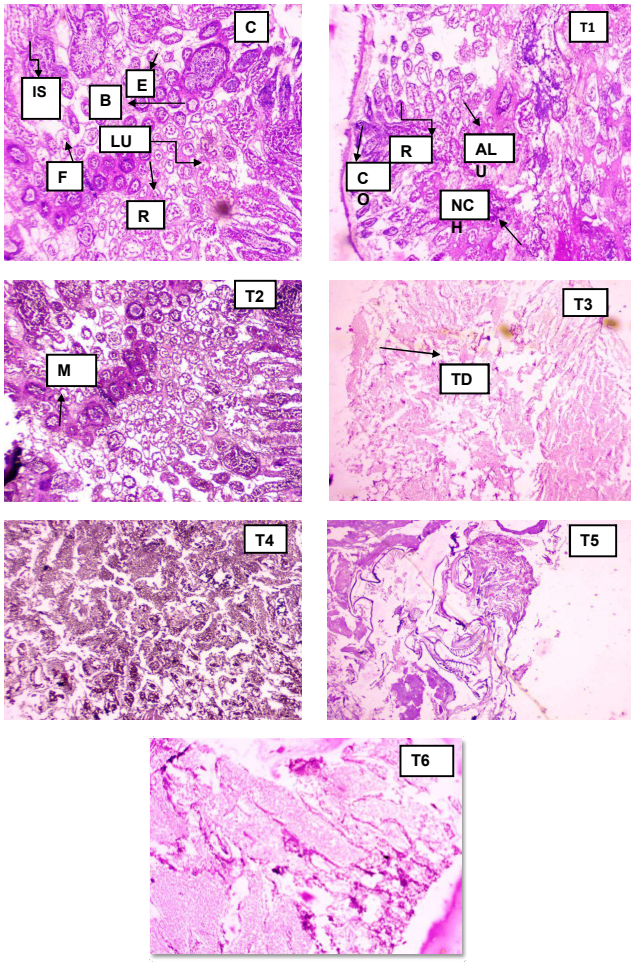

Fig. 1. Typical organization of juvenile *L. vannamei* hepatopancreas of control (C) and all treatments i.e., T1, T2, T3, T4, T5, and T6 at 10X magnification. ALU= Abnormal Lumen; E=Embryonic Cells, B= B-cells (Blasenzellen cells) with their large apical secretary vacuoles, IS= Interstitial sinuses, LU= Lumen; R= R-cells (Restzellen cells), Coagulation (CO), Melanization (M); F=F-cells (Fibrillenzellen cell)

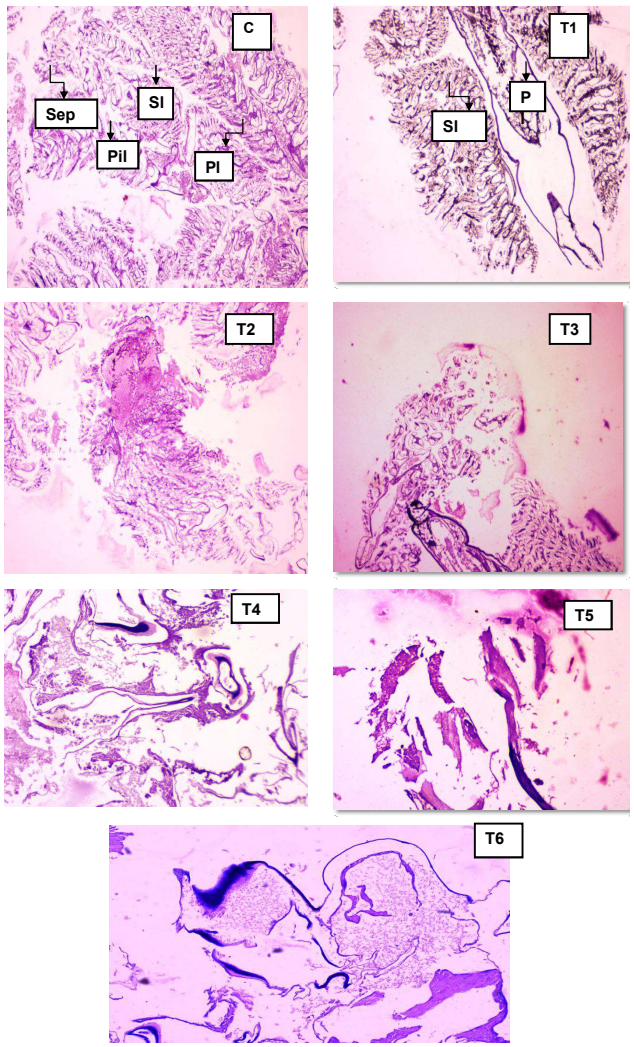

Fig. 2. Typical organization of juvenile *L. vannamei* gills in control (C) and all treatments i.e., T1, T2, T3, T4, T5, and T6 at 10X magnification. PL- Primary lamellae, SL- Secondary lamella, Pil- Epithelial pillar cell processes, Nec- Cell necrosis, Sep- septum

mortality as seen in treatment T4-T6. Due to prolonged exposure to zinc secondary lamella fused and appears as an undifferentiated mass and ladder-like arrangements of pillar-like cells become collapsed (Amoah et al 2022).

Effect on the intestine: The intestine in control has a normal structure with epithelial cells and columnar villi, intestinal crypts. In treatment T1 (0.5 mg/L) there was a minor alteration but in T2-T3 (1 mg/L-2mg/L) it was observed disruption of epithelial cells and large deformities in crypts. In treatment T4 (4mg/L), T5 (6mg/L), and T6 (8mg/L) intestine was completely damaged and there was no cellular differentiation in epithelial cells, villi, and crypts (Fig. 3). It was assessed that zinc was highly toxic for shrimp and resulted in large deformities of intestinal tissue. Maximum height of epithelial cells continuously got decrease with increased concentration of zinc sulphate. Zinc is highly toxic for shrimp and resulted in deformities of intestinal tissue. It observed that there was maximum height of epithelial cells which continuously decreases with an increase in the concentration of zinc (Nagarjuna et al 2019, Khushbu et al 2022). To consider a crustacean as "healthy", a sufficient number of circulating hemocyte cells must be present as hemolymph helps in maintaining homeostatic integrity.

Total haemocyte count (x 10^6 cells ml^{-1}): The effect of zinc sulphate on the total haemocyte counts (THC), total granulocyte counts (TGC), and total agranulocyte counts (TAC) of *L. vannamei* juveniles at the end of the study are (Table 1 and Fig 4). The increasing level of zinc sulphate showed significant differences in (THC) of shrimp. The total haemocyte count (THC), total granulocyte counts (TGC), and total agranulocyte counts (TAC) of shrimp were significantly lower in $ZnSO_4$ 8mg\L (14.45, 10.51 and 3.94) than in control (25.18, 17.50 and 7.83). Haemocyte number monitoring has

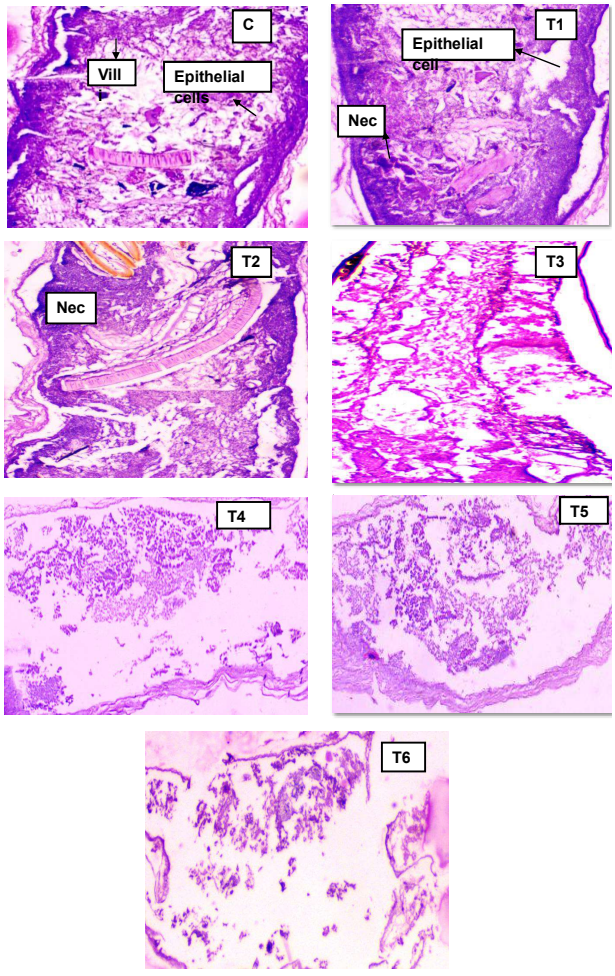

Fig. 3. Typical organization of juvenile *L. vannamei* intestine in control (C) and all treatments i.e., T1, T2, T3, T4, T5, and T6 at 10X magnification

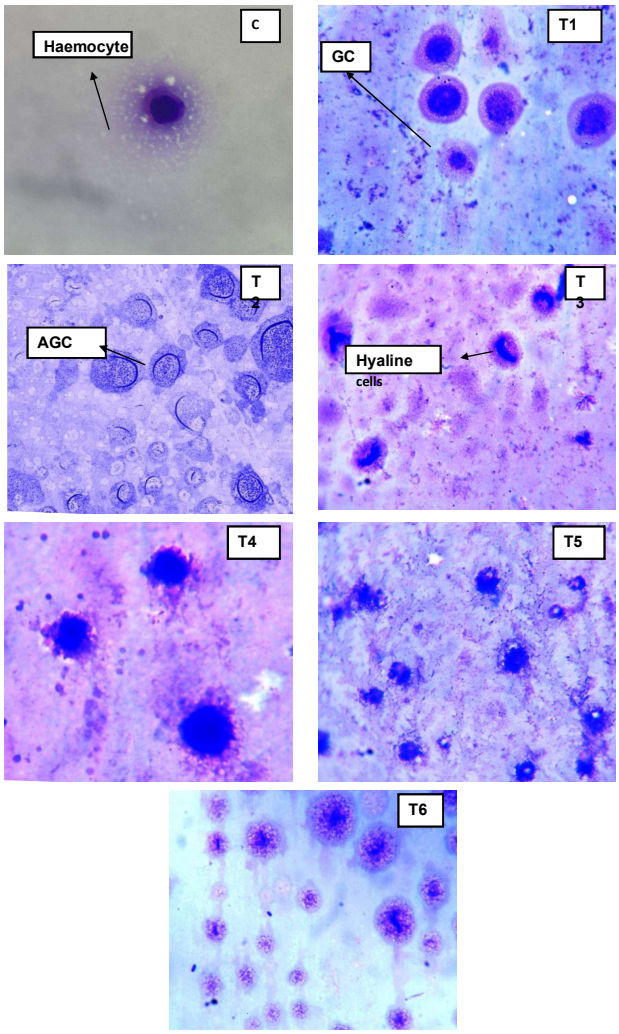

Fig. 4. Typical organization of the Haemocytes of control (C) and all treatments i.e., T1, T2, T3, T4, T5, and T6 has done at 100X magnification of juvenile *L. vannamei*, Granulocyte count (GC), agranulocyte count (AGC)

Table 1. Effect of zinc toxicity on the hematological parameters of *L. vannamei* (Mean±SE) recorded at the at end of study period

Treatments (mg/L)	THC (x 10^6 cells ml^{-1})	TGC (x 10^6 cells ml^{-1})	TAC (x 10^6 cells ml^{-1})
Control	25.183±0.533	17.500±0.433	7.833±0.120
$ZnSO_4$ 0.5	24.283±0.429	16.583±0.303	7.400±0.153
$ZnSO_4$ 1	21.720±0.711	14.820±0.930	6.900±0.306
$ZnSO_4$ 2	19.083±0.480	13.170±0.793	6.100±0.265
ZnSO4 4	17.860±0.175	12.260±0.525	5.600±0.351
ZnSO4 6	16.163±0.276	11.350±0.578	4.880±0.386
ZnSO4 8	14.457±0.609	10.513±0.399	3.943±0.228
CD (p=0.05)	1.386	1.845	0.839

been proposed by Duarte and Caçador (2019) as a measure of stress and/or physiological state in crustaceans, but their predictive significance in the evaluation of health status and immunological competence has not been adequately examined. Most importantly, it is to be known the sufficient amount of hemocyte number. Sabu and Gopal (2016) observed that total and differential number of hemocytes in crustaceans may vary significantly, and these variations are assumed to be correlated with life stage, moult cycle, level of nutrition, disease, and physiological or environmental conditions. In the current work, the mean number of hemolytic cells in control shrimp served as a baseline for comparison with the number of shrimps exposed to various harmful metal doses. The results demonstrated that hemocyte numbers showed reduction at all concentrations of exposure to zinc sulphate. A further research needs to be done on hemocyte counts as a biomarker of 'health' in crustaceans.

CONCLUSION

There were significant changes in the biochemical and histological parameters of vannamei exposed to increasing concentrations of zinc sulphate. At higher concentrations (8mg/L), a decrease in R-cells, F-cells, B cells, and E-cells, low haemocyte count, high haemocytic infiltration, abnormal lumen, necrosis, and Melanization in hepatopancreas were recorded. In gills, gill lamella was found to be fused, pillar cells became interspersed, and show large deformities in comparison to control. In the intestine, there was a maximum height of the epithelial cell in control which continuously got decrease with increasing concentration of zinc sulphate. Total haemocyte count was used as a health marker for shrimp which gradually decrease at high concentrations. Additionally, it is important to take into account and will be further researched how heavy metals affect the capacity and effectiveness of the hepatopancreas, gill, and gut to bio transform other environmental contaminants.

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