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ASSESSMENT OF LC_{50} INCLUDING HEPATO-INTESTINAL ABNORMALITIES IN *CIRRHINUS MRIGALA* INDUCED WITH GRAPHENE NANO SHEETS

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Abstract

This study investigates the hepato-intestinal abnormalities induced with graphenenano sheets in *Cirrhinus mrigala*.The study was initially started with the aim to investigating the LC50 of GNS by the formation of groups and named as C, GNS-1, GNS-II, GNS-III, GNS-IV, GNS-V and GNS-VI.The fish (n=70) in these groups were administrated with 0, 250, 500, 750, 1000, 1250, and 1500 mg/L of CNS in water for 96-hrs. The mortality within 96-hrs was recorded as 0, 0, 1, 2, 3, 5 and 7 in Nos. The LC50 calculated in this study was 121.37 mg/L after applying probit analysis. In this study various histological changes in intestine are necrosis in the villi of the intestine, destroyed intestinal integrity, inflammation in the intestine, disintegration of the epithelial layer, vacoulation and degenerated goblet cell and liver was reported with histological alterations such as central vein injury, melanomacrophage center, pyknotic nuclei, dilation of sinusoids, vacuolation, necrosis of hepatocytes and congestion. In conclusion, this study reveals significant hepato-intestinal abnormalities in *Cirrhinus mrigala* induced by graphenenano sheets, with observed mortality patterns and histological changes, underscoring the potential impact of graphene exposure on the health of aquatic organisms.

Keywords: Hepato-intestinal abnormalities, Graphenenano sheets, *Cirrhinus mrigala*, LC_{50}, Mortality, Histological changes

Introduction

Graphenenano-sheets are a two-dimensional allotrope that falls under the domains of nanotechnology and materials sciences. The tightly interwoven honeycomb-like structure, with a one-carbon atomic structure, makes it a thin and robust material (Du et al., 2010; Tang & Zhou, 2013) To use graphenenano-sheets in fields such as energy, materials, and medicine, its atomic structure and chemical composition needs to be understood (Weiss et al., 2012; Krishnamoorthy et al., 2013). Graphenenanosheets act an important role in nanotechnology and materials science advancement (Morales &Merkoçii, 2014).

Due to its unique 2D structure, graphenenano-sheets have a greater range of applications, including storage, rapid charging supercapacitors, and extending the lifespan of batteries (Memisoglu et al., 2023; Alwarappan et al., 2009; Guo& Dong, 2011). Despite the fact that graphenenanosheets are not harmful in nature, the mechanism and route of exposure are not insignificant because structural modifications have a variety of effects on biological systems (Ganguly et al., 2018). The detrimental effects of graphenenanosheets vary depending on the particular biological system being studied, their size, shape, and surface chemistry (Sanchez et al., 2012).

Cirrhinus mrigala, a freshwater fish, is a well-known model organism for toxicological research that demonstrates how pollutants affect aquatic life. Numerous studies have been reported a range of toxicity-related issues in *C. mrigala*, from acute toxicity to chronic toxicity (Yadav et al., 2010). Recent studies in toxicology have shown that nitrite toxicity and organophosphorus insecticides have an impact on the histopathological characteristics of *C. mrigala*. These findings show how toxicants that impact tissues have an impact on fish behavior. This result adds significantly to the most recent toxicological research by elucidating the intricate link between pollutants, histological changes, and behavioural responses in C. mrigala. (Baskar 2014; Hossain et al., 2000; Velmurugan et al., 2007).

Materials and Methods:

Experimental Design:

In this studyhepato-intestinal abnormalities induced by Graphene Nano Sheets (GNS) in *Cirrhinus mrigala* were assessed. The research was started with the estimation of the lethal concentration at which 50% of the experimental organisms perish (LC_{50}). This study consist of Seven experimental groups named as follows: Control (C), GNS-1, GNS-II, GNS-III, GNS-IV, GNS-V, and GNS-VI.

Test Organism:

Test specimens of *Cirrhinus mrigala* (n=70) were used for the study. The fish were sourced from Chashma fish hatchary, Mianwali, Punjab Pakistan and acclimatized in controlled laboratory conditions prior to experimentation.

Exposure Protocol:

The test animals were subjected to varying concentrations of Graphene Nano Sheets (GNS) in water for a duration of 96 hours. The applied concentrations were 0 mg/L (Control), 250 mg/L (GNS-1), 500 mg/L (GNS-II), 750 mg/L (GNS-III), 1000 mg/L (GNS-IV), 1250 mg/L (GNS-V), and 1500 mg/L (GNS-VI).

Mortality Assessment:

Mortality rates were recorded at the end of the 96-hour exposure period. The number of deceased fish in each experimental group was documented as follows: Control (0), GNS-1 (0), GNS-II (1), GNS-III (2), GNS-IV (3), GNS-V (5), and GNS-VI (7).

LC50 Calculation:

The lethal concentration at which 50% of the fish succumbed to GNS exposure was calculated using probit analysis. The LC_{50} value obtained from this analysis was 121.37 mg/L.

Statistical Analysis:

Data obtained from mortality assessments were subjected to statistical analysis through Probit test by using SPSS version 20

Histopathological Examination:

Liver and intestinal tissues were collected, processed, and examined to identify any abnormalities induced by GNS exposure.

Ethical Considerations:

All experimental procedures were conducted in compliance with ethical guidelines for the use of laboratory animals and approved by the Departmental Committee for Animal Experimentation

Data Presentation:

Results obtained from the study, including mortality rates, LC50 values, and histopathological findings, were presented graphically and descriptively.

Results and Discussion

Environmental consequences of the increasing use of GNMs are needed to address because this is an important and unresolved question. The reportings related to use of graphene based nano materials GNMs include differences in analytical methodologies for quantification, no standardized test guidelines and differences in morphology of GNMs (Markovic et al., 2018) According to the previous study of Markovic et al.(2018)The most sensitive organism was found to be the freshwater shrimp *Palaemonpandaliformis*. The derived guideline values for 99, 95, 90, and 80% species protection were 350, 600, 830, and 1300 μg/L, respectively.Another study by Katsumiti et al. (2017) reported that Graphene-based wastes are expected to end up in the marine environment and assess the toxic effects of GO and rGO on marine organisms, (*Mytilusgallo provincialis*) hemocyte. The results showed that GO, GO-PVP, and rGO-PVP induced low and concentration-dependent cytotoxicity.

Graphene oxide nano sheets induce diverse histopathological changes in various organs, suggesting potential harm to the life (El-Yamany et al. 2017).In this study liver was reported with histological alterations such as central vein injury, melanomacrophage center (mmc), pyknotic nuclei, dilation of sinusoids, vacuolation, necrosis of hepatocytes and congestion.Several studies have investigated the histological changes induced by graphene in the liver. A previous study of Sasidharan et al. (2015) reported that intravenously injected graphene reached the liver, raising concerns about potential tissue injury in Swiss albino mice. Another study by Amrollahi-Sharifabadi et al. (2018) on investigating in vivo toxicological effects of graphene oxide nanoplatelets, with a focus on clinical applications revealed histopathological changes in liverinclude mild sinusoidal injury and hyperemia at lower doses, progressing to granulomatous reactions, giant cell formation, perivascular infiltrations, and massive particle accumulation at higher doses. These findings suggest a dose-dependent relationship between GON exposure and liver damage, with higher doses leading to more severe histological alterations.Nirmal et al. (2021) investigated the hepatotoxicity of graphene oxide in Wistar rats. Histological features in GO-treated rats include damaged portal veins, dilated sinusoids, inflammatory cell infiltration, edema-filled spaces, vacuolations, alterations in hepatocyte morphology, and pyknotic nuclei. The pathology score suggests a statistically significant difference in liver histology between the control and GO-treated groups, indicating the potential adverse effects of graphene oxide exposure on

hepatic tissue.Fernandes et al. (2017) found that exposure of graphenenano sheets through the diet in marine shrimp *Litopenaeus vannamei* induced oxidative stress and histological changes in the shrimp's liver. These studies collectively suggest that graphene, especially in the form of graphene oxide nanosheets, can induce histological changes in the liver and other organs, raising concerns about its potential toxicity and impact on biological systems. It is important to note that further research is needed to fully understand the mechanisms and potential long-term effects of graphene exposure on liver health.These studies, including the current *Cirrhinus mrigala* study, emphasize the potential toxicity of graphene-based nanomaterials and their impact on the histology of liver.
In this study various histological changes in intestine are necrosis in the villi of the intestine, destroyed intestinal integrity, inflammation in the intestine, disintegration of the epithelial layer, vacoulation and degenerated goblet cell. The study of Jia et al. (2019) reported abnormalities in the ultra-micro structure and width of villi in the anterior intestines of zebrafish, caused by chronic exposure to GO. Injuries, represented by red ellipses, appeared in the villi of the anterior intestine. Average measurements of villi height and width were significantly decreased in GO-exposed zebrafish compared to the control group. In the mid intestines, the height of villi increased significantly in GO-exposed group. Control zebrafish showed a normal structure of anterior intestines, while GO-exposed zebrafish exhibited injuries in villi structure and depletion of goblet cells. The number of goblet cells per villus decreased in the anterior intestine but increased in the mid-intestine of GO-exposed zebrafish. The study suggests that GO exposure caused structural damage and reduced integrity in the anterior intestines of zebrafish. Zheng et al. (2019) reported alterations in intestinal morphology and antioxidant enzyme activities induced by exposure to graphene-family materials. Vacuolations (empty spaces or cavities) were observed in all GFM groups, with the GR group showing more vacuolations than the GO and rGO groups. The number of goblet cells (cells that produce mucus) increased in all GFM groups. Disintegration of cell boundaries was observed in the intestines of zebrafish exposed to rGO.Souza et al. (2017) reported that Graphene oxide induced structural damage, particularly in the intestinal mucus membrane of zebrafish. Fernandes et al. (2018) reported no changes in intestine when the fish is exposed to graphene toxicity. The study by Jia et al. (2019), Zheng et al. (2019), Souza et al. (2017), Fernandes et al. (2018) highlight the detrimental effects of graphene oxide (GO) and other graphene-family materials (GFM) on the intestinal structure and found in accordance to the present study to validate our findings.

Conclusion

In conclusion, this study demonstrates pronounced hepato-intestinal abnormalities in *Cirrhinus mrigala* induced by graphenenano sheets (GNS). The investigation, which initially aimed to determine the LC50 of GNS, involved the administration of varying concentrations to different groups. The calculated LC50 was 121.37 mg/L after a 96-hour exposure, as determined through probit analysis. Histological examinations revealed significant changes in the intestine, including necrosis in the villi, disrupted intestinal integrity, inflammation, epithelial layer disintegration, vacuolation, and degenerated goblet cells. Concurrently, the liver exhibited notable histological alterations such as central vein injury, melanomacrophage center, pyknotic nuclei, sinusoidal dilation, vacuolation, hepatocyte necrosis, and congestion. The observed mortality patterns further underscore the potential adverse effects of graphene exposure on the health of aquatic organisms, emphasizing the need for continued research in understanding and mitigating the environmental impact of graphenenano sheets.

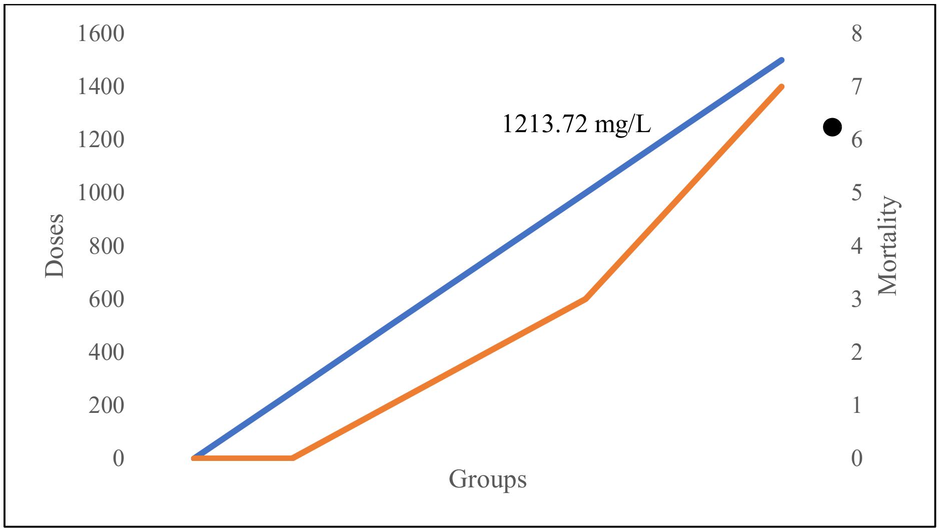

Figure 1:Probit analysis of graphenenano-sheets administrated to fish for the calculation of LC_{50}.

Table 1: Scoring histological changes in liver induced by graphenenano-sheets in fish *Cirrhinusmrigala*

Liver Histological Abnormalities	**Control C**	**Treated GNS-I to GNS-VI**
Melanomacrophage center	-	+
Pyknotic nuclei	-	+
Dilation of sinusoids	-	++
Vacuolation	-	+
Necrosis of hepatocytes	-	+
Congestion	-	+

(-); No change (+); Detected (++); Severely Detected

Table 2: Scoring histological changes in intestine induced by graphenenano-sheets in fish *Cirrhinus mrigala*

Intestinal Histological Abnormalities	**Control C**	**Treated GNS-I to GNS-VI**
Destroyed intestinal integrity	-	+
Inflammation in the intestine	-	+
Disintegration of the epithelial layer	-	+
Vacoulation	-	+
Degenerated Goblet cell	-	+

(-); No change (+); Detected (++); Severely Detected

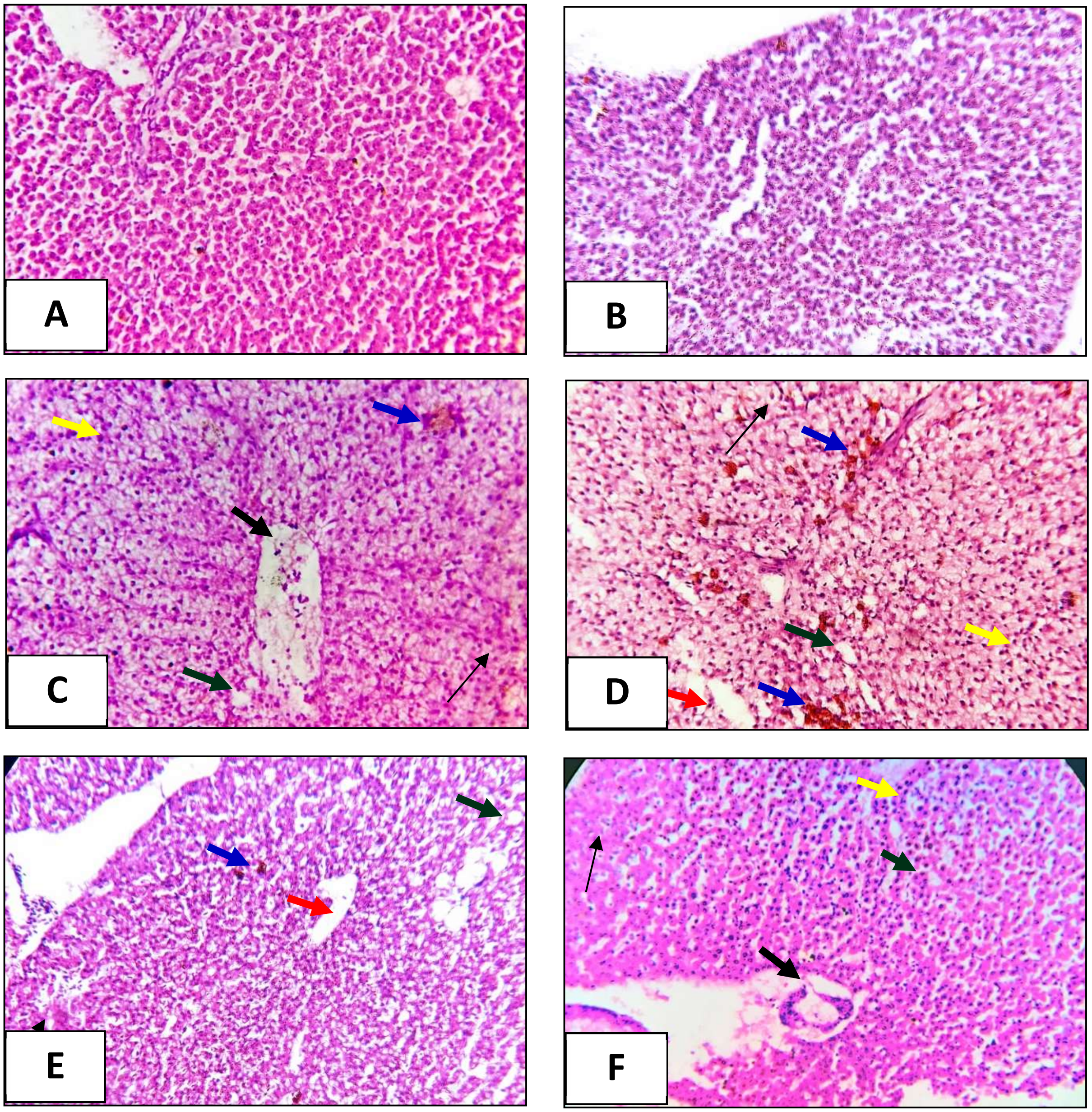

Figure 2: Liver micrographs (400x); A-B, Control; C-F, Treated; Black Arrow: Central vein injury Blue arrow: Melanomacrophage center (MMC), Yellow Arrow: Pyknotic nuclei; Red Arrow: Dilation of sinusoids; Green Arrow: Vacuolation; Thin Black Arrow: Necrosis of hepatocytesBlack Circle: Congestion

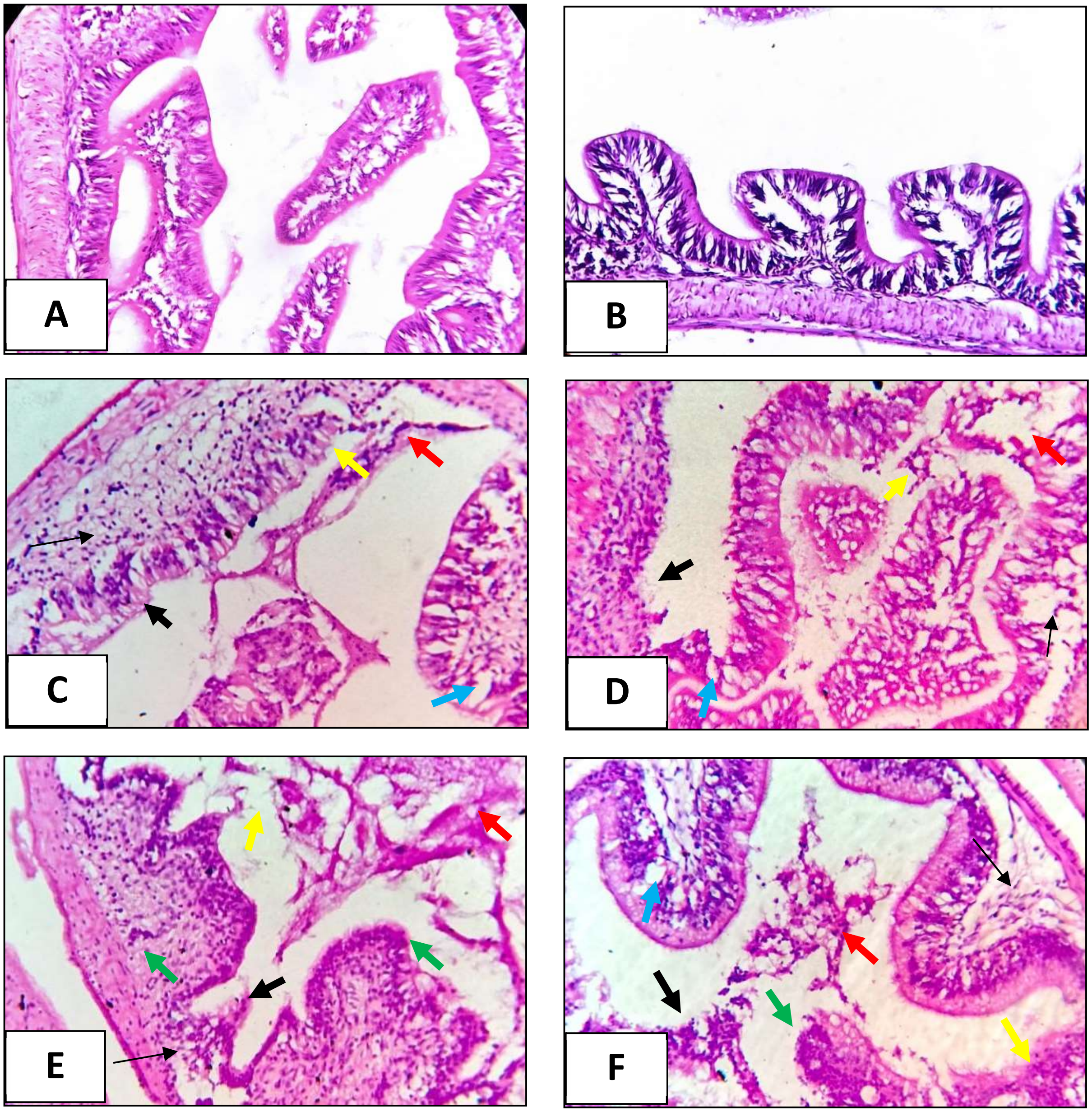

Figure 3: Intestine micrographs (400x); A-B, Control; C-F, Treated; Black Arrow: Necrosis in the villi of the intestine; Yellow Arrow: Destroyed intestinal integrity ; Red Arrow: Inflammation in the intestine; Green Arrow: Disintegration of the epithelial layer; Blue Arrow: Vacoulation ; Thin Black Arrow: Degenerated Goblet cell.

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