Evaluation of some Hematological and Biochemical Parameters in the Grass Carp (Ctenopharyngodon idellus) Exposed to Sub-Lethal Concentrations of Benzene

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ABSTRACT
The current study aimed to investigate the impact of long-term exposure to sub-lethal concentrations of benzene on the biochemical and hematological parameters of grass carp Ctenopharyngodon idellus. Eighty healthy fish were randomly divided into four treatment groups: T1 (control group), T2, T3, and T4, with each exposed to different levels of benzene concentrations (0, 0.25, 0.5, and 0.75ppm) for a period of 96h. The biochemical parameters including glucose and total protein were measured, as well as the hematological parameters, including erythrocytes and leukocyte counts, lymphocytes, neutrophils, hemoglobin (Hb) content, and packed cell volume (PCV). The results showed that there were significant decreases ($P \leq 0.05$) in the total number of erythrocytes and glucose levels in the treatments (T2, T3, and T4) compared to the control group (T1). It was also noted that there were significant decreases ($P \leq 0.05$) in the total number of leukocytes and lymphocytes between T3 and T4 groups with the T1 group. No significant differences ($P > 0.05$) were observed in PCV, total protein, and neutrophils among the treatments (T2, T3, and T4) when compared to the T1 group. As for blood Hb, significant decreases ($P \leq 0.05$) were found between T4 and T1 groups. It was deduced that exposure to sub-lethal concentrations of benzene can severely affect the biochemical and hematological parameters of grass carp, thereby affecting their physiological status.

INTRODUCTION
Benzene, a volatile organic compound commonly found in industrial and environmental surroundings, is known for its harmful effects on humans (D'Andrea & Reddy, 2014), as well as environment, plants, and animals including various aquatic organisms (Connell et al., 2023). For the impact of benzene on the environment, benzene can contaminate water sources and accumulate in aquatic ecosystems. This contamination can occur through industrial discharge, oil spills, and improper disposal of benzene-containing products (Chatterjee et al., 2023). The exposure to benzene has shown to have harmful effects on fish, including changes in their biochemistry and hematological parameters. These changes can have significant impacts on the overall health and well-being of fish populations since disruption to biochemical pathways and hematological
processes can impair key physiological functions such as metabolism, organ function, and immune response (Duman & Şahan, 2018). It is important to study the long-term effects of benzene exposure on grass carp (Ctenopharyngodon idellus) due to their significance in freshwater ecosystems and their global importance in aquaculture (Wildhaber et al., 2023). This is particularly evident as they inhabit marshes in Iraq, where their food is readily accessible. (Taher, 2018). Understanding the effects of a long-term exposure to sub-lethal concentrations of benzene on grass carp is crucial for assessing the potential risks associated with benzene pollution and for implementing effective management strategies to protect aquatic ecosystems and the health of fish populations (Solaiappan & Prakash, 2021).

Some studies have already shown that benzene exposure can lead to altered liver enzyme levels, kidney damage, lipid oxidation, oxidative stress, and changes in hematological parameters, such as packed cell volume (PCV) and hemoglobin (Hb) concentration (D’Andrea & Reddy, 2014; Sharique et al., 2021; Solaiappan & Prakash, 2021).

The present study aimed to investigate the impact of chronic exposure to low levels of benzene on the biochemical profile of grass carp Ctenopharyngodon idellus, assessing their hematological changes following prolonged exposure to sub-lethal concentrations of benzene, evaluating the potential risks and hazards of benzene pollution on the health populations, and determining the threshold level of benzene concentration that may cause significant alterations in biochemical and hematological parameters in grass carp. Hence, conducting this study would contribute to understand the specific biochemical and hematological alterations that occur in grass carp following exposure to benzene, providing important information for assessing the potential risks of benzene pollution on fish populations and implementing appropriate management strategies. Furthermore, the results of this study can also contribute to the field of environmental toxicology by providing insights into the sub-lethal effects of benzene on biochemical and hematological parameters in grass carp. Understanding these effects can help in the development of guidelines and regulations to mitigate the negative impacts of benzene pollution on aquatic ecosystems and protect fish populations.

**MATERIALS AND METHODS**

**Collection and maintenance**

Grass carp were obtained from Aquaculture unit, College of Agriculture, and kept for 3 days in a controlled laboratory environment for acclimatization prior to the experiment. About 80 fish (weight range 10.4- 19.8g and length range 10.3- 12.4cm) were selected for the study.
Fish were randomly divided into five groups: a control group (not exposed to benzene) and four experimental groups exposed to sub-lethal concentrations (0.25, 0.5, 0.75, and 1 ppm) of benzene.

Water quality parameters, such as water temperature (thermometer), dissolved oxygen, salinity, pH were monitored daily to ensure optimal conditions for fish (Table 1).

During the acclimation period, the fish were fed on a diet that was prepared using feed materials containing fishmeal, soybean meal, wheat, wheat bran, starch, and a mixture of vitamins and minerals (Table 1). However, they were not fed during the acute tests.

Table 1. Components of the feed (%)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fishmeal</td>
<td>25</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>22</td>
</tr>
<tr>
<td>Wheat meal</td>
<td>29</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>20</td>
</tr>
<tr>
<td>Starch</td>
<td>2</td>
</tr>
<tr>
<td>Vitamins and minerals premix</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
</tr>
</tbody>
</table>

Preparing of water-soluble fraction of benzene

The water soluble fraction (WSF) of benzene was prepared by diluting 394 mg of benzene in one liter of distilled water in a glass container and slowly stirred for 20 h at room temperature (20 ± 2°C). After mixing, it was allowed to be separated for 1 to 6 h and the upper layer was siphoned off, leaving the residual solution composed of saturated benzene represented the stock solution. Subsequently, it was stored in a closed glass container at 10°C to prevent evaporation and contamination (Anderson et al., 1974; NAS, 1975).

Calculating concentration the median-lethal of benzene

The acclimated fish were distributed into five 30 L covered glass aquaria, each contained 5 animals, with three replicates (n=5 fish/treatment). Aqueous static renewal tests were conducted to estimate four days median lethal concentration (LC$_{0,5}$) values concentrations for benzene under standard laboratory conditions.

Grass carp in the experimental group were exposed to various concentrations of benzene (0.25, 0.5, 0.75, and 1 ppm) according to the following treatments (T2, T3, T4 and T5), respectively, while the control group (T1) was kept in the same condition without exposure to benzene.

The chemical analysis ratios were performed using a rapid content analyzer. The proximate analysis gave the chemical composition of the experimental diets which
included 25.22% protein, 3.7% lipids, 12.63% ash, 2.06% fiber, 45.26% nitrogen free extract (NFE), and 11.26% moisture.

**Blood standard**

Following the exposure period (96h), blood samples were collected from each fish heart using 2ml glass sterile syringe and transferred into a test tube with an anticoagulant for hematological analysis using a Mindray bc-30s equipment, manufactured in China.

The hematological analysis included measuring parameters, such as the number of leukocytes and erythrocytes, lymphocytes (%), neutrophils (%), Hb content, and PCV. The glucose and total protein levels were also determined in blood plasma. The kits from the Chinese company “Mindray” were used to analyze the blood samples for these parameters.

**Statistical analysis**

The hematological analysis data were statistically analyzed using a complete random design (CRD) in order to examine the impact of various treatments on the parameters being studied. To determine significant differences between the means, the analysis of variance (ANOVA) was employed, and the LSD test was used at a significance level of 0.05. The statistical analysis was performed using the SPSS program (Version 26).

**RESULTS**

In the acute toxicity test, the mortality rate increased with increasing benzene concentrations after 96h of exposure in grass carp. The mortality rate for grass carp exposed to benzene concentrations of 0, 0.25, 0.50, 0.75, and 1ppm were 0, 11.1, 38.87, 44.4, and 100%, respectively (Table 3). The results showed that LC50 of benzene was 0.619ppm for grass carp (Fig. 2).

In biochemical and hematological parameters *C. idellus* were exposed to different concentrations (0, 0.25, 0.5 and 0.75 ppm) of sub-lethal concentrations of benzene according to the following treatments: T1 (control), T2, T3, and T4, respectively.

**Table 2.** Environmental factors measured in benzene test.

<table>
<thead>
<tr>
<th>Environmental factor</th>
<th>Value measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>20± 0.32</td>
</tr>
<tr>
<td>Salinity (psu)</td>
<td>2.3± 0.21</td>
</tr>
<tr>
<td>pH</td>
<td>7.8± 0.62</td>
</tr>
<tr>
<td>Dissolved oxygen (mg/L)</td>
<td>7± 0.34</td>
</tr>
</tbody>
</table>
It was shown that there were significant decreases \((P \leq 0.05)\) in the total number of erythrocytes and glucose levels in the treatments (T2, T3, and T4) compared to the control group (T1; Figs. 2, 8). It was also noted that, there were significant decreases \((P \leq 0.05)\) in the total number of leukocytes and lymphocytes (%) between the T3 & T4 groups with the T1 group (Figs. 3, 4). However, there was no significant differences \((P > 0.05)\) observed between the T2 and T1 groups. No significant differences \((P > 0.05)\) were observed in PCV, total protein level, and neutrophils (%) among the treatments (T2, T3, and T4) compared to the T1 group (Figs. 5, 6, 9). For blood Hb, a significant decrease difference \((P \leq 0.05)\) was found between T4 and T1, and no significant differences \((P > 0.05)\) were observed between the other treatments.

**Table 3.** Mortality percentages of *C. idellus* exposed to different concentrations after 96h of exposure to different concentrations of benzene.

<table>
<thead>
<tr>
<th>Treatment (ppm)</th>
<th>Mortality rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>0.25</td>
<td>11.1</td>
</tr>
<tr>
<td>0.5</td>
<td>38.87</td>
</tr>
<tr>
<td>0.75</td>
<td>44.4</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

**Fig. 1.** LC\(_{50}\) of benzene on *C. idellus* after 96h.
Fig. 2. Erythrocytes count (cell × 10^6 / mm^3) of *C. idellus* exposed to sub-lethal concentrations of benzene for a period of 96h. Different letters in one row indicate significant differences (P ≤ 0.05)

Fig. 3. Leukocytes count (cell × 10^3 / mm^3) of *C. idellus* exposed to sub-lethal concentrations of benzene for a period of 96h. Different letters in one row indicate significant differences (P ≤ 0.05)
Fig. 4. Lymphocytes (%) of *C. idellus* exposed to sub-lethal concentrations of benzene for a period of 96h. Different letters in one row indicate significant differences \( (P \leq 0.05) \)

Fig. 5. Neutrophils (%) of *C. idellus* exposed to sub-lethal concentrations of benzene for a period of 96h. Different letters in one row indicate significant differences \( (P \leq 0.05) \)

Fig. 6. Packed cell volume (PCV %) of *C. idellus* exposed to sub-lethal concentrations of benzene for a period of 96h. Different letters in one row indicate significant differences \( (P \leq 0.05) \)
Fig. 7. Hemoglobin (Hb) (g/100ml blood) of *C. idellus* exposed to sub-lethal concentrations of benzene for a period of 96h. Different letters in one row indicate significant differences ($P \leq 0.05$)

Fig. 8. Glucose level (mg/100ml blood) of *C. idellus* exposed to sub-lethal concentrations of benzene for a period of 96h. Different letters in one row indicate significant differences ($P \leq 0.05$)

Fig. 9. Total protein in blood plasma (mg/100ml blood) of *C. idellus* exposed to sub-lethal concentrations of benzene for a period of 96h. Different letters in one row indicate significant differences ($P \leq 0.05$)
DISCUSSION

Water pollution issues represent significant challenges to the ecosystem community (Lin et al., 2022; Pereira et al., 2011). Among several polluting compounds, there are three specific monocyclic aromatic hydrocarbons which play a prominent role, including benzene, toluene, and xylene. These substances are utilized in several household and industrial applications, either individually or in combination, as documented by the Environmental Protection Agency (EPA, 1980). The current study revealed that the mortality ratio of grass carp exposed to different concentrations of benzene, with an LC$_{50}$ of 0.619 ppm, increased as the concentration of benzene increased (Fig. 1).

The investigation of blood in various fish species has the attention of researchers since it offers valuable insights into the intricate connections among blood characteristics, phylogeny, physical activity, habitat and the ability to adapt to environmental conditions (Tavares-Dias & Moraes, 2004).

Changes in the hematological characteristics of fish can serve as a reflection of the alterations in the surroundings experienced by these organisms. As a result, these characteristics function as invaluable markers of the condition of aquatic creatures that are exposed to contaminants since they embody one of the primary observable stress reactions prompted by shifts in the environment (Davison et al., 1993).

Furthermore, hematology analysis is crucial for determining the condition of fish and its importance in the evaluation of physiological biomarkers in animals. Their significance lies in their ability to serve as indicators in assessing these changes (Maftuch et al., 2020).

In a previous study, fish exposed to alkyl benzene sulfonate at concentrations equivalent to 10 and 20% of the 96-hour LC$_{50}$ showed a slight decrease, though notable, in the levels of erythrocytes, Hb, and PCV. The concentrations of alkyl benzene sulfonate used were 0.006 and 0.012 mg/l, respectively. This decrease in hematological parameters could be due to the harmful effects of the surfactant on hematopoietic tissues. Similarly, lower levels of erythrocytes and Hb can result from erythrocyte destruction in blood-forming tissues, hemolysis, abnormal heme-synthesis, increased generation of free radicals, and insufficient oxygen supply by gills (Ghaffar, 2020).

It was observed in the present study that exposure to sub-lethal concentrations of benzene can cause significant or insignificant decrease in the chemical and biological parameters of grass carp. The decrease in PCV levels may be due to the lysis of erythrocytes or owing to the benzene-induced damage to the bone marrow, where red blood cells are produced. Therefore, the reduction in erythrocytes, Hb, and PCV levels is a clear indication of anemia (Ololade & Oginni, 2010). Moreover, the decrease in erythrocytes, Hb, and PCV values may be attributed to altered erythropoiesis activity (Ghaffar, 2020).

In another study, the exposure of fish to benzene for 96 hours resulted in significant decreases in the values of red blood cells, white blood cells, lymphocytes, and glucose
levels in the blood plasma (Maduenho & Martinez, 2008). Additionally, there was a slight decrease in neutrophil cells, PCV, Hb, and total protein although it was not statistically significant. Hydrocarbons in petroleum can seriously impair oxygen transport by altering the permeability of erythrocytes, leading to a strong hemolytic effect (Duarte et al., 2010).

The toxic effects of alkyl benzene sulfonate on Oreochromis mosambicus were assessed by exposing the fish to the concentrations of 10 and 20% of the 96-hour LC₅₀. The levels of erythrocytes, Hb, PCV, plasma protein, and plasma glucose exhibited a significant initial increase followed by a subsequent decrease in the exposed fish. This reduction in hematological parameters indicates the negative impact of alkyl benzene sulfonate on the fish (Ghosh et al., 2022).

Stress responses play a vital role in the survival of organisms. The glucose availability is important since it provides an energy substrate for tissues, such as the brain, muscles, and gills, enabling the animal to respond defensively to stressors. Increased plasma glucose levels have been observed in previous studies after exposure to polycyclic aromatic hydrocarbons (PAHs) (Vijayan et al., 1997; Pacheco & Santos, 2001).

The vulnerability of organisms' immune systems to xenobiotic stress is well documented, and there is a substantial evidence indicating that hydrocarbons can have diverse effects on the immune components and functions of fish (Reynaud & Deschaux, 2005). Chronic exposure to the Arabian crude oil resulted in a significant decrease in white blood cell counts, specifically lymphocytes, in sea bass. This decrease in lymphocytes does not indicate a loss of white blood cells due to their extravasation from the circulatory system toward a possible site of tissue damage. Instead, it suggests a disturbance in apoptosis or necrosis activity, which can be attributed to the cytotoxic effects of PAHs due to their water solubility and lipophilicity (Schirmer et al., 1998).

In the current study, an increase in neutrophil percentage and a decrease in lymphocyte percentage in T4 compared T1 were observed after exposure to the sub-lethal concentrations levels of benzene. Moreover, similar effects have been observed in mammals injected with substances derived from petroleum representing the activity of the first and second lines of defense against cellular damage (d'Azevedo et al., 1996). Studies in cultured fish Oreochromis aureus have also confirmed increases in monocytes and neutrophils, along with a decrease in lymphocytes, during different stressors. Neutrophils are believed to have phagocytic activity, which may explain their increased percentage during infectious situations (Silveira-Coffignya et al., 2004).

Blood Hb levels in fish are commonly used to identify abnormalities and negative signs of fish health (Casanovas et al., 2021). The exposure to sub-lethal concentrations of butachlor, for example, resulted in significant hematological alterations in fish. High values of erythrocytes count, Hb concentration, and PCV of the exposed fish indicate a stress-induced production of erythrocytes and Hb (Habiba, 2012). Moreover, the exposure to butachlor also led to a tremendous increase in the count of white blood cells
(WBC) due to immune system activation. Furthermore, the percentage of lymphocytes in the fish increased after butachlor exposure, indicating an immune response (Habiba, 2012).

CONCLUSION

In conclusion, exposure to the sub-lethal concentrations levels of benzene can have a deleterious impact on grass carp's physiological state, biochemical and hematological parameters, and overall health. It can also drastically lower the productivity of fish and other aquatic organisms in water bodies. Further investigations into the effects of benzene and hematological, as well as other biochemical markers need to be examined in different types of animals.

REFERENCES


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