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A laboratory Study: Effect of Cadmium Chloride on Biochemical Parameters and Histological Changes in Freshwater Mussel (*Unio tigridis*)

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Abstract

The present study investigated the toxicity of cadmium (Cd) to the freshwater mussel *Unio tigridis*. Mussels were treated with six doses of cadmium chloride ($CdCl_2$) control (0), 1, 2, 3, 4, and 5 ppm for up to 96 hours. The Cd accumulation and oxidative stress markers, including Acetylcholinesterase (AChE), malondialdehyde (MDA), reduced glutathione (GSH), and catalase (CAT) rather than histological changes were evaluated. Results showed that exposure to $CdCl_2$ induced a significant increase in MDA levels and CAT activity. Notably, a significant decrease was observed in the AChE and GSH levels in the groups exposed to $CdCl_2$, which was dependent on exposure time. The cadmium (Cd) concentrations in both aquarium water and the mussel's body have fluctuated, and these changes were influenced by the concentration and duration of the exposure. Histological investigation of the mussel gills showed alternation in gill structure after being exposed to different cadmium concentrations when compared to the control group.

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1. Introduction

Nowadays, metal contamination in aquatic ecosystems is a significant threat to the environment [1]. Many human activities, including the mining of heavy metal minerals, the burning of fossil fuels, and the use of organophosphorus pesticides, involve the usage of cadmium in modern industrial and agricultural production and increasing waste discharges into the environment [2]. Hence, these activities are thought to be the primary causes of Cd pollution in aquatic ecosystems. They affect marine species, probably mussels, as they are sessile mollusks and accumulate pollutants in their bodily tissues [3-5]. As a bioindicator species, mussels are commonly used to determine whether potentially toxic metals are present [1, 6]. Because biological indicators can tell us about the long-term effects of various pollutants in the environment, they are crucial for the global detection of contaminants [7]. A freshwater bivalve is essential to aquatic environments because it mixes the sediment with filtered water through burrowing mussels, which increases oxygen content and improves ecosystem health [8]. Recently, many researchers throughout the world have focused on using biomarkers for aquatic ecosystem biomonitoring of heavy metal pollution. Metal toxicity produces changes in biomarker levels, such as biochemical and histology of mussel tissues, and affects the organisms [9-13]. They, moreover, discovered that these changes are based on the concentrations and exposure times of pollutants [12, 14]. A study done by Li *et al*. (2022) [15] showed that Cd can cause tissue damage by inducing apoptosis in the mussels' gills. The current study aimed to assess the physicochemical properties of the Greater Zab River water and determine the toxic effects of heavy metal (cadmium) in freshwater animals by using mussels (*Unio tigridis*) as a vital indicator. For this purpose, oxidative stress parameters and histological changes were examined.

2. Research Method

2.1. Water sampling: In July 2024, river water samples were collected from the Greater Zab River/ Estrian village and analyzed immediately at the sampling station to determine key physical and chemical parameters. These included water

temperature (°C), pH (potential of hydrogen ions), electrical conductivity (EC, µS/cm), and total dissolved solids (TDS, mg/L). Dissolved oxygen concentrations (mg/L) were measured instantly upon the samples' arrival at the laboratory [16-18].

2.2. Mussel's sampling: Freshwater mussels of the species *Unio tigridis* were collected from the Greater Zab River in Erbil, Iraq (36°36'48"N 44°09'16"E), at an elevation of 365 meters above sea level (Figure 1). Approximately 180 individuals were sampled, cleaned, and transported in river water to the laboratory promptly. Upon arrival, the mussels were placed in aquariums with river water in an air-conditioned room maintained at 25±1°C with a 14-hour light/10-hour dark. The mussels acclimated to laboratory conditions for ten days in dechlorinated water, with the water being changed daily. During this period, the taxa were not fed. Identification was conducted using a common identification key [19].

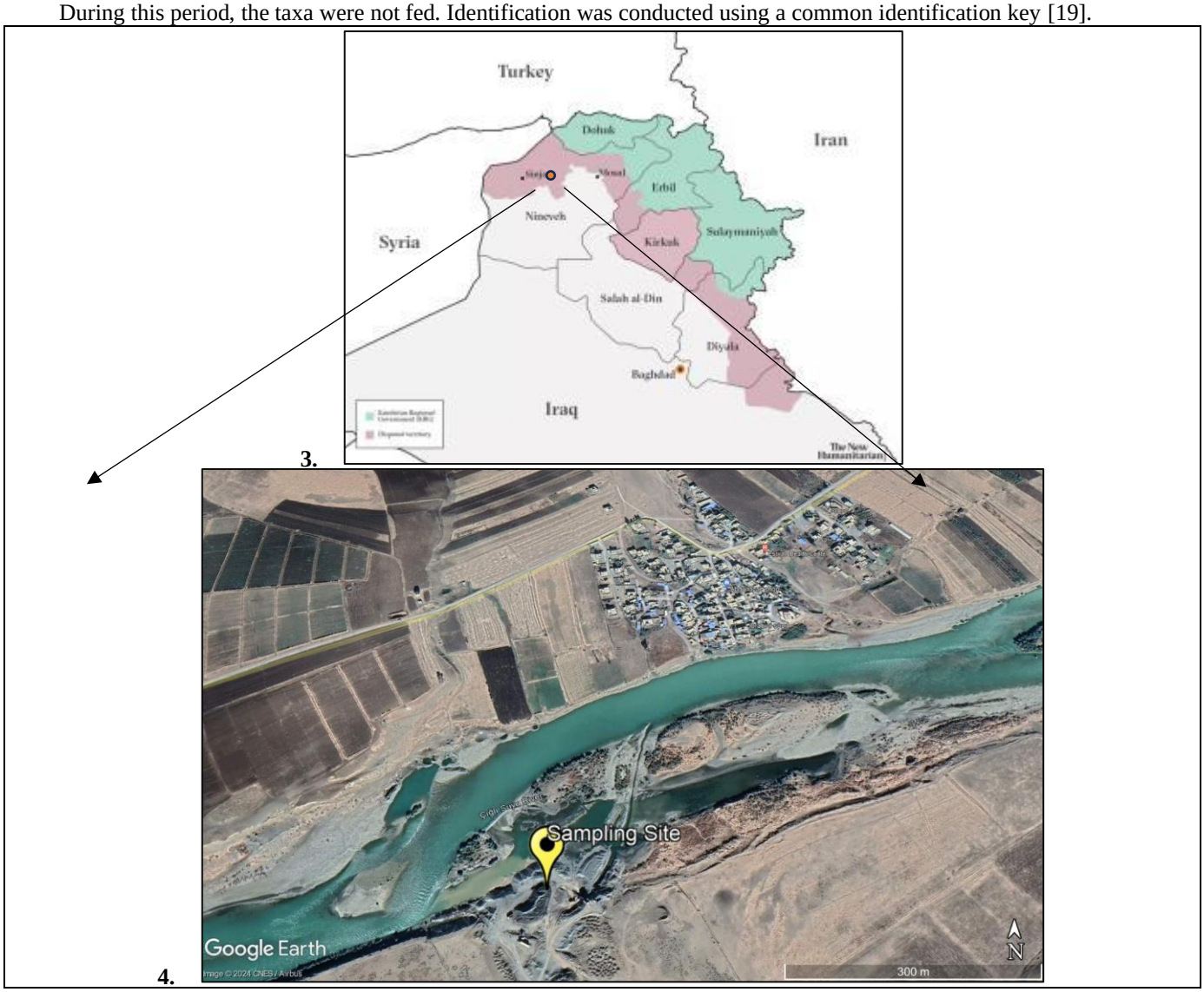

Figure 1: Map of the A. North part of Iraq, B. Sampling site (Google Earth 2024)

2.3. Experimental design: Before beginning the experiment, a detection test was achieved to ascertain the likely range of cadmium chloride ($CdCl_2$) concentrations. Approximately equal-sized taxa were selected, with 10 individuals allocated to both the control group and each of the $CdCl_2$ treatment concentrations, specifically 1, 2, 3, 4, and 5 ppm for 96 hours. Each aquarium had three replications. Throughout the test, no food was provided. Every day, any deceased individuals were collected. Immobility, excessive milky white mucus discharge, open valves, extended foot protrusion outside the shell, and a lack of response to mechanical stimuli indicated the endpoint [14].

2.4. Determination of Cadmium concentration: During the test period, Cd level was measured in the exposure medium at 24, 48, 72, and 96 hours. The procedure began with acid digestion for treated samples using nitric acid, followed by storage at 4°C

until analysis with a PerkinElmer USA 1100D atomic absorption spectrophotometer (AAS) [20]. The Cd standard solution, purchased from Merck in Germany, had a concentration of 1000 mg/L. In addition to site and aquarium water samples, Cd concentrations were assessed in the soft body and shell of mussels at each test period. The mussels were first washed with distilled water and briefly immersed in boiling water. The soft bodies were then separated from the shells, rinsed with deionized water, and dried at 70°C for 48 hours. The dried samples were powdered and stored in a desiccator for their metal analysis. The shells were also cleaned with deionized water, dried at 70°C for 48 hours, and then stored in a desiccator [21]. Approximately 0.5 grams of dried mussel tissues and shells were placed in digestion containers and treated with HNO_3. The samples were digested using a combination of HNO_3 and H_2O_2. Following digestion, the acid was removed, and the samples were diluted to 10 mL with 1% HNO_3. An atomic absorption spectrophotometer was then used to measure Cd concentrations, which were expressed in (ppm) [22].

2.5. Tissue preparation for biochemical analysis: For a biochemical assay, a gills tissue sample of 0.1 g from each individual was washed in an ice-cold saline solution. The tissue was homogenized using a glass homogenizer in a 20 mM phosphate buffer. The homogenates were then centrifuged at 4,000 rpm for 10 minutes at 4°C. The supernatant was taken and stored at -80°C for later analysis [5]. Each biomarker was measured in triplicate. The levels of acetylcholinesterase (AChE), malondialdehyde (MDA) as an indicator of lipid peroxidation, reduced glutathione (GSH), and catalase (CAT) activity were measured using Solarbio® LIFE SCIENCES [8].

2.6. Histological examination: Histological analysis was performed in the laboratory of the Biology Department at Salahaddin University's College of Science. Gills tissue samples were initially fixed in Bouin's fluid for 24 hours and then dehydrated utilizing a graded ethanol series (50%, 70%, 95%, and 100%). Post-dehydration, the samples were treated with xylene and embedded in paraffin wax. Thin tissue sections, each 5 micrometers thick, were cut using a rotary microtome. These sections were thus stained with hematoxylin and eosin (H&E) according to the method outlined by Suvarna *et al*. (2018) [23]. The paraffin-embedded sections were then examined using a light microscope provided with a camera and Image Analysis Software.

2.7. Data analysis: Each measurement was conducted in triplicate, and the data were analyzed using SPSS Version 25. A one-way ANOVA was done to assess the Cd concentration and biochemical data. Duncan's post hoc test was applied to detect significant differences among treatments, with a significance threshold set at a p-value of 0.01.

3. Results and Discussion

Many human activities, including the mining of heavy metal minerals, the burning of fossil fuels, and the use of organophosphorus pesticides, involve the usage of cadmium in modern industrial and agricultural production. In aquatic environments, these applications are thought to be the primary causes of cadmium pollution [10]. Recently, scientists found that utilizing freshwater mussels as a reliable bioindicator of metal contamination [6] as metals are essential to many different processes in living things. Despite the fact that some metals, such as iron, copper, lead, and cadmium, can be toxic to living organisms in high values, exposing mussels to substantially different metal doses can induce acute toxicity [14].

The study of the Greater Zab River involved measuring key water quality parameters: a temperature of 17 °C, a pH of 7.5, electrical conductivity of 599 µS/cm, total dissolved solids at 300.33 mg/L, and dissolved oxygen at 7.633 mg/L. These parameters are crucial as they can significantly impact the health of freshwater mollusks, which are sensitive to environmental changes [24]. To ensure accurate experimental conditions, these parameters were controlled and maintained within acceptable limits. In tests involving fish and mollusks, a survival rate of 90% or higher was targeted to confirm that the conditions were suitable. Continuous monitoring of these factors throughout the experiment was conducted to ensure reliable and meaningful results.

3.1. Determination of Cd concentration in the aquarium water and soft tissues

Cadmium is a toxic heavy metal that may accumulate in aquatic life, leading to adverse effects on their physiology. The study aimed to evaluate the uptake of Cd in mussels by exposing them to five different concentrations of $CdCl_2$ and measuring the Cd concentrations in the aquarium water, soft body, and shell of the mussels. In the control group, the concentration of Cd in the aquarium water ranged from 0.0003 to 0.001 ppm during the test periods. The statistical analysis showed no significant differences ($p > 0.01$) in Cd concentrations across treatments (Table 1). In the treated groups, the higher Cd concentration in aquarium water was recorded in individuals exposed to 3 ppm $CdCl_2$ over a 24-hours. Conversely, a lower concentration of 0.093 ppm was recorded in individuals exposed to 4 ppm $CdCl_2$ after 72 hours. The change in Cd concentrations in the aquarium water is influenced by the exposure duration. Mussels initially accumulate Cd rapidly, resulting in higher water concentrations at shorter exposure times, such as 24 hours with 3 ppm $CdCl_2$. Over longer periods, Cd is absorbed into the mussel body, leading to lower water concentrations, as seen with 4 ppm $CdCl_2$ after 72 hours. For the 5 ppm $CdCl_2$ exposure over 96 hours, the Cd concentration in the water was 0.283 ppm, reflecting an intermediate level of bioaccumulation. This pattern is consistent with findings from similar studies, where longer exposure leads to increased Cd accumulation in organisms and reduced water concentrations [25, 26]. There were no statistically significant differences ($p > 0.01$) in cadmium concentrations across the different test periods for the majority of the experimental media.

Table1. Concentrations in ppm of cadmium (Mean ± SE) in experimental medium across various test periods

con. ppm	24 h	48 h	72 h	96 h
0	0.001±0.000^{a}	0.001±0.000^{a}	0.0003±0.0003^{a}	0.0003±0.0003^{a}
1	0.190±0.015^{a}	0.170±0.017^{a}	0.170±0.011^{a}	0.140±0.028^{a}
2	0.250±0.005^{a}	0.233±0.014^{a}	0.190±0.005^{b}	0.133±0.003^{c}
3	0.316±0.024^{a}	0.256±0.020^{a}	0.116±0.037^{b}	0.133±0.003^{b}
4	0.226±0.008^{a}	0.176±0.018^{a}	0.093±0.008^{b}	0.166±0.014^{b}
5	0.230±0.005^{a}	0.133±0.003^{a}	0.116±0.003^{a}	0.283±0.208^{a}

Note: Values within each row marked with different letters indicate significant differences, while values marked with the same letters do not show substantial differences.

In the control group, cadmium levels in the mussel shells remained relatively consistent, ranging from 5.300 to 5.246 ppm. In contrast, treated groups showed significant changes in cadmium levels over time in most aquaria ($p < 0.01$). The minimum concentration, 0.093 ppm, was found in mussels exposed to 1 ppm $CdCl_2$ after just 24 hours, while the highest concentration, 21.303 ppm, was observed in mussels exposed to 5 ppm $CdCl_2$ after 96 hours (Table 2).

These results highlight significant Cd accumulation in mussel shells, which varies with exposure concentration and duration. In the control group, stable Cd levels indicate a baseline concentration. However, in treated groups, longer exposure times and higher $CdCl_2$ concentrations correlated with increased Cd levels. This pattern is consistent with findings from other studies demonstrating that bioaccumulation of heavy metals in aquatic biota increases with time and with higher exposure levels [27].

Table 2. Concentrations in ppm of cadmium (Mean ± SE) in the shell of mussel across various test periods

con. ppm	24 h	48 h	72 h	96 h
0	5.236±0.012^{a}	5.246±0.018^{a}	5.240±0.015^{a}	5.300±0.005^{a}
1	5.643±0.021^{d}	6.146±0.012^{c}	6.413±0.008^{a}	6.313±0.003^{b}
2	7.720±0.011^{c}	8.013±0.008^{b}	7.543±0.089^{c}	10.522±0.008^{a}
3	8.076±0.037^{c}	8.676±0.006^{b}	8.330±0.026^{c}	9.206±0.104^{a}
4	8.423±0.014b^{c}	9.820±0.561^{b}	7.800±0.142^{c}	13.733±0.391^{a}
5	15.480±1.261^{b}	15.093±0.014^{b}	13.406±0.092^{b}	21.303±0.083^{a}

Note: Values within each row marked with different letters indicate significant differences, while values marked with the same letters do not show significant differences.

In the control group, Cd levels in the soft body of mussels remained relatively constant, ranging from 3.293 to 3.320 ppm, with no significant differences observed across the test periods ($p > 0.01$) (Table 3). However, in the exposed groups, Cd concentrations fluctuated. The lowest concentration, 4.566 ppm, was detected in the soft body of mussels exposed to 1 ppm $CdCl_2$ after 24 hours, while the highest concentration, 32.760 ppm, was recorded in mussels treated with 5 ppm $CdCl_2$ after 96 hours. Significant differences in Cd levels were observed between different exposure times for most of the treatments ($p < 0.01$). This variation suggests a substantial uptake and retention of Cd, influenced by both the concentration and duration of exposure. The relatively stable levels in the control group indicate a baseline concentration that is unaffected by additional Cd sources. In contrast, the treated groups' significant Cd accumulation highlights the biota's response to increased environmental Cd. [28].

Table 3. Concentrations in ppm of cadmium (Mean ± SE) in the soft body of mussel across various test periods

con. ppm	24 h	48 h	72 h	96 h
0	3.300±0.005[a]	3.310±0.005[a]	3.320±0.005[a]	3.293±0.008[a]
1	4.566±0.014[c]	13.486±0.008[b]	13.723±0.008[b]	24.093±0.428[a]
2	7.393±0.043[d]	15.713±0.014[c]	18.513±0.014[b]	25.083±0.031[a]
3	9.356±0.321[d]	11.870±0.364[c]	20.820±0.325[b]	24.090±0.157[a]
4	17.04±0.569[c]	17.850±0.104[c]	22.723±0.581[b]	26.006±0.367[a]
5	20.166±0.118[c]	21.213±0.072[c]	29.546±0.461[b]	32.760±0.500[a]

Note: Values within each row marked with different letters indicate significant differences, while values marked with the same letters do not show significant differences.

3.2. Effect of Cd on the oxidative status of the freshwater bivalve *Unio tigridis*

Traditional ecotoxicological research has mainly focused on evaluating the acute and chronic sublethal effects of pollutants. The findings showed fluctuations in the levels of these biomarkers over time. The AChE levels decreased significantly $p \leq 0.01$ with prolonged exposure; specifically, at 24 hours, the AChE level was higher and diminished with increased exposure duration. The lowest AChE level recorded was 155.173 U/mg protein in the group exposed to 5 ppm $CdCl_2$. In contrast, the highest level, 190.030 U/mg protein, was observed in the control group, remaining relatively constant throughout the study period (Figure 2). The significant $p \leq 0.01$ decrease in AChE levels in the gill tissues of mussels exposed to $CdCl_2$ indicates a clear impact of Cd on enzyme activity. This reduction suggests cadmium's inhibitory effect on AChE, potentially disrupting cholinergic functions and overall physiological health in mussels [29].

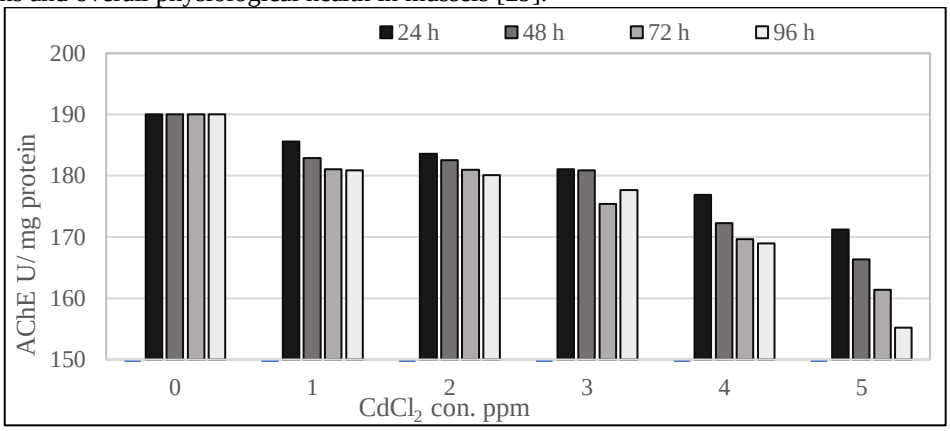

Figure 2. Acetylcholinesterase (AChE) in the gills of *Unio tigridis* exposed to $CdCl_2$ across 24, 48, 72, and 96 hours.

Throughout the experiment, the malondialdehyde (MDA) levels were measured in both control and treated *Unio tigridis* groups. In the control group, MDA levels remained relatively constant, averaging 0.123 nmol/mg protein. In contrast, the treated groups exhibited a significant $p \leq 0.01$ increase in MDA levels with longer exposure times. The highest MDA concentration, reaching 1.653 nmol/mg protein, was recorded in individuals exposed to 5 ppm $CdCl_2$ over 96 hours (Figure 3). The increase in malondialdehyde (MDA) levels with exposure to $CdCl_2$ can be attributed to the enhanced oxidative stress induced by the metal. $CdCl_2$ exposure leads to the generation of reactive oxygen species (ROS) in biological tissues. These ROS can damage cellular components, including lipids, leading to lipid peroxidation. MDA is a byproduct of this lipid peroxidation process and serves as a biomarker for oxidative stress. As exposure time increases, the accumulation of ROS and subsequent lipid peroxidation intensifies, resulting in higher MDA levels. This indicates a heightened state of oxidative damage in the tissues [30].

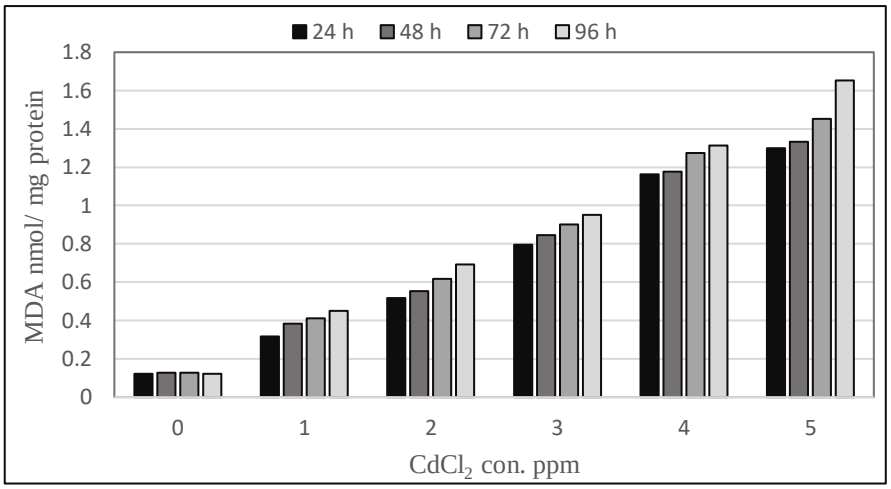

Figure 3. Malondialdehyde (MDA) levels in the gills of *Unio tigridis* following $CdCl_2$ exposure over 24, 48, 72, and 96 hours

In the control group, the concentration of reduced glutathione (GSH) in gill tissue remained relatively stable across all time points, averaging approximately 79.383 nmol/mg protein. In contrast, the GSH levels in the groups exposed to $CdCl_2$ showed a significant $p \leq 0.01$ decline. The most pronounced reduction was observed in individuals treated with 5 ppm $CdCl_2$, where GSH levels decreased to as low as 15.226 nmol/mg protein (Figure 4). The specific reason for the reduction in GSH levels due to $CdCl_2$ exposure is that cadmium induces the production of reactive oxygen species (ROS). These ROS cause oxidative damage to cellular components and deplete GSH, which is a primary antioxidant responsible for neutralizing ROS. Cadmium also interferes with the synthesis and regeneration of GSH, further exacerbating its depletion. This mechanism is more pronounced at higher concentrations of $CdCl_2$, leading to the observed severe reduction in GSH levels [31].

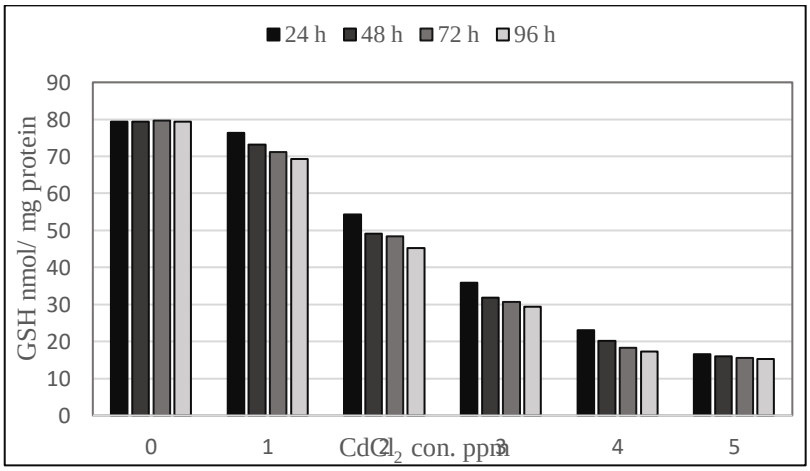

Figure 4. Reduced glutathione GSH levels in the gills of *Unio tigridis* after exposure to $CdCl_2$ for 24, 48, 72, and 96 hours

In the control group, the catalase (CAT) activity remained constant at a minimum level of 14.193 U/mg protein throughout the duration of the experiment. Conversely, in the groups exposed to $CdCl_2$, CAT activity increased significantly ($p \leq 0.01$). The highest activity was recorded in individuals exposed to 5 ppm $CdCl_2$, reaching a maximum value of 18.603 U/mg protein after 96 hours of exposure (Figure 5). The increase in catalase (CAT) activity in mussels exposed to $CdCl_2$ is primarily due to the oxidative stress induced by cadmium exposure. Cadmium generates reactive oxygen species (ROS) in the cells, leading to oxidative damage. In response to this oxidative stress, mussels upregulate antioxidant enzymes like catalase to counteract the increased ROS levels and protect cellular structures from damage. Catalase helps to decompose hydrogen peroxide, a common ROS, thereby reducing oxidative damage. This adaptive response results in elevated CAT activity, particularly at higher concentrations of $CdCl_2$ and extended exposure periods [32].

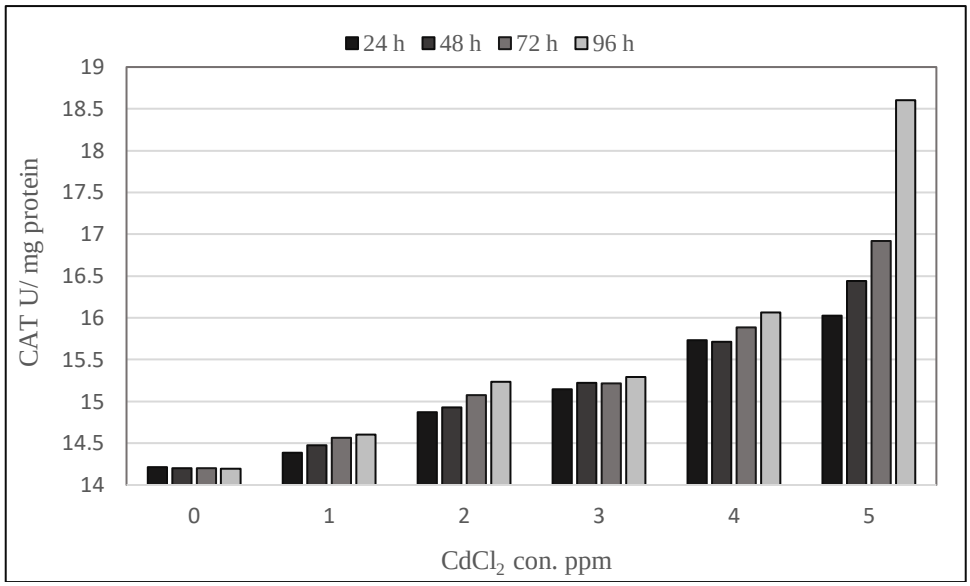

Figure 5. Catalase (CAT) activity in the gills of *Unio tigridis* following exposure to $CdCl_2$ over 24, 48, 72, and 96 hours

3.3. **Histopathological alterations in the gills of freshwater mussels *Unio tigridis***

Histopathological investigation revealed the normal architecture of gills in the control animals. As shown in fig. (6, A), the bivalve ctenidium is made up of water canals and filaments. The gill filaments consist of well-organized epithelial cells with lateral and frontal cilia and central core tissue. They are supported by two plates (chitinous rods) that lie beneath the epithelium [5]. Gills exposed to different cadmium concentrations showed alternation in gills structure. The 1 ppm cadmium exposed group resulted in a change in gill lamellae length, damaging chitinous rods and detaching of lateral cilia. In addition, the cellular structure of the connective tissue and epithelium cells has been lost (Fig.6, B). After exposure to 2 ppm cadmium chloride ($CdCl_2$), frontal cilia of gill filaments were detached, elongated gill filaments with an enlarged lumen, and the gill filaments showed evidence of hemocytic infiltration (fig.6, C). Following exposure to 3 ppm of $CdCl_2$ for 96 hours, the gill filaments were shortened, chitinous rods were damaged, and the frontal and lateral cilia shed off. In addition, the lumen showed a higher number of haematocytes as compared to normal gills (Fig. 6, D). The gill filaments' structure revealed elongation of filaments, and the frontal and lateral cilia had shed. Also, clumping of blood cells was present in the top of the filaments after they were subjected to 4 ppm of $CdCl_2$ for four days (fig.6, E). Following exposure to 5 ppm of $CdCl_2$ there was destruction of the gill structure, as gill filaments were completely disorganized and detaching in covering cilia.

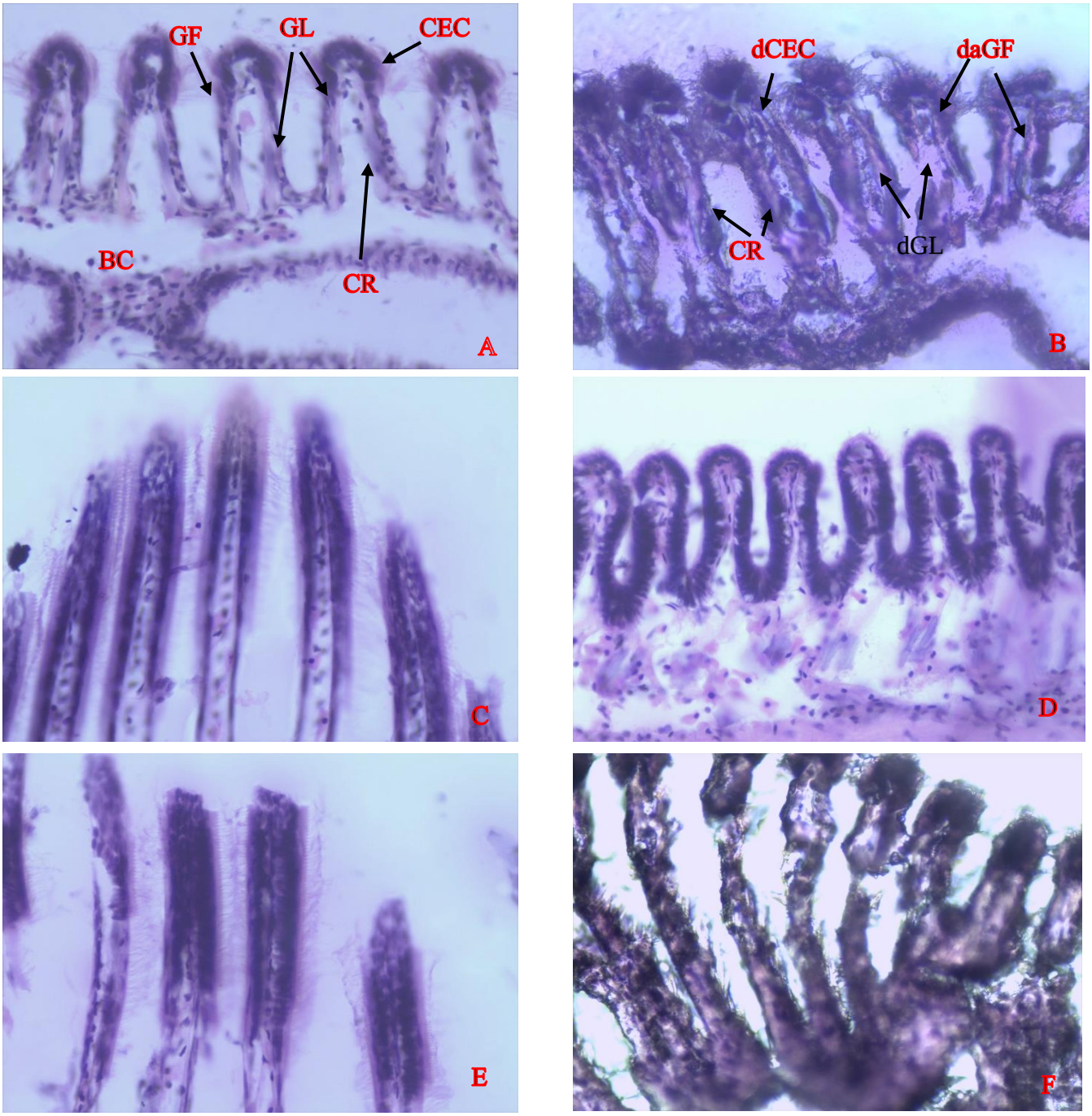

Fig. 1. Light micrographs of gills of *Unio tigridis* exposed to cadmium chloride ($CdCl_{2)}$ concentrations (40X H&E). (A)

Control group; (B) 1 ppm, (C) 2 ppm, (D) 3 ppm, (E) 4 ppm & (F) 5ppm. ***GF*** *gill* filament, **GL** Gill lamella, ***BC*** *blood cells*, ***CEC*** *ciliated columnar epithelial cells*, ***CR*** *chitinous rods*, ***dCEC*** *detached ciliated columnar epithelial*, ***dGL*** *degenertated Gill lamellae*, ***daGF*** *damaged gill* filament.

Moreover, the epithelium became vacuolated, necrotic, and oedematic and severely lost (Figs. 6, F). Overall, the organisms that were exposed to cadmium concentrations displayed damaged interlamellar connections and complete loss of gill structures as tissue rupture, and the breakdown of normal cell structure were seen in connective tissue. Also, the breakdown of the respiratory epithelium was seen in the cytoplasm, this might occur because of necrosis [15]. These results come in accordance

with results obtained by [9-11, 33]. Also, recently, [15, 34] in their study documented histopathological findings may be used as reliable indicators in programs that monitor aquatic ecosystems for heavy metal pollution.

4. Conclusion

In conclusion, the results indicate that Cd concentrations affect freshwater mussels. The study emphasizes the potential for heavy metals to bioaccumulate in freshwater mussel tissues and demonstrates how heavy metals induce changes in the histology of the mussels' gills. This indicated that *Unio tigridis* was a sensitive bioindicator and could be used to monitor the pollution of water in the past and the present day. Thus, the study elaborates on the vulnerability of aquatic ecosystems to low levels of disturbances, such as heavy metal pollution.

5. Acknowledgments

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دراسة مختبرية: تأثير كلوريد الكادميوم على المعايير الكيميائية الحيوية والتغيرات النسيجية للمحار العذب (*Unio tigridis*)

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المستخلص:

أجريت الدراسة الحالية للتحقق من سمية الكادميوم (Cd) على المحار المياه العذب *Unio tigridis*. تم تعرض المحار الى ست جرعات مختلفة من كلوريد الكادميوم ($CdCl_2$) السيطرة (0)و 1 و 2 و 3 و 4 و 5 جزء في المليون لمدة 96 ساعة. تم تقييم تراكم الكادميوم وعلامات الإجهاد التأكسدي بما في ذلك أسيتيل كولين استريز (AChE) ومالونديالدهيد (MDA) والجلوتاثيون المختزل (GSH) والكاتالاز (CAT) بالاضافة الى التغيرات النسيجية. أظهرت النتائج أن التعرض لـ $CdCl_2$ يؤدي إلى زيادة كبيرة في مستويات MDA ونشاط CAT. والجدير بالذكر أنه تم ملاحظة انخفاض كبير في مستويات AChE و GSH في الكائنات المعرضة لـ CdCl2 وكان هذا يعتمد على وقت التعرض. كانت تراكيز الكادميوم في كل من مياه الحوض وجسم المحار متقلبة و تدرجت شدة هذه التغييرات حسب تركيز التعرض ومدته. وأظهر الفحص النسيجي للخياشيم تناوبًا في بنية الخياشيم بعد التعرض لتركيزات مختلفة من الكادميوم عند مقارنتها بمجموعة التحكم.

الكلمات المفاتحية: المحار ، سمية الكادميوم ، تغيرات نسيجية، مؤشرات كيموحيوية.