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"EFFECTS OF HEXAVALENT CHROMIUM ON THE DEVELOPMENT OF ZEBRAFISH *(DANIO RERIO)*"

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INTRODUCTION

1. The urgency of the thesis

In studies regarding the toxicity of the metal Chromium, numerous research projects have been conducted using various animal models such as mice, rabbits, and other higher-order mammals. However, to date, no comprehensive study has been undertaken employing the zebrafish model *(Danio rerio)* to comprehensively investigate the effects of different concentrations of Chromium (VI) on the development of this species. Specifically, Chromium (VI) toxicity impacts the expression of genes associated with apoptosis and oxidative stress in zebrafish. The scarcity of information regarding the influence of Chromium (VI) on zebrafish development may create a knowledge gap in the understanding of heavy metal toxicity and its implications for human health in the future.

Therefore, the study "Assessing the impact of chromium on the development of zebrafish *(Danio rerio)*" employed the zebrafish model to assess how different concentrations of Chromium (VI) affect zebrafish development at different stages, including the embryonic, larval, and adult stages. The analysis in this study encompassed the observation and evaluation of morphology, tissue structure, survival rates, heart rate, as well as the assessment of changes in the expression of genes associated with apoptosis and oxidative stress.

2. Research objectives of the thesis

The objective of this study is to utilize the zebrafish model *(Danio rerio)* to assess the impact of Chromium (VI) on the developmental process of zebrafish at different stages, including the embryonic, larval, and adult stages. The specific objectives are as follows:

Evaluate the influence of Chromium (VI) on the development of zebrafish by examining survival rates, heart rate, and the length of zebrafish larvae.

Determine changes in the expression of genes associated with oxidative stress response and apoptosis, as well as alterations in the internal structure of certain organs in zebrafish when exposed to Chromium (VI).

3. The main research contents of the thesis

To assess the impact of Chromium (VI) on the development of zebrafish at various stages, including the embryonic and larval stages, and to determine the accumulation of Chromium (VI) during the developmental process of zebrafish.

To evaluate changes in the expression of stress response genes and damage control genes in zebrafish when exposed to Chromium (VI).

To assess the influence of Chromium (VI) on the structure of internal organs (intestine, liver, and ovaries) in zebrafish.

CHAPTER 1. OVERVIEW

1.1. Overview of Chromium (VI)

Introduction to Chromium's Properties and Applications in Human Production Activities and Listing Research Directions on Chromium Toxicity in Experimental Animals.

This section provides an overview of chromium's properties and its applications in human production activities. It also enumerates research directions concerning the toxicity of chromium on experimental animals.

1.2. Overview of Zebrafish *(Danio rerio)*

This section introduces the origin and development of zebrafish.

1.3. Studies on the Effects of Heavy Metals on Zebrafish

This section compiles studies conducted domestically and internationally that utilize zebrafish as a toxicity testing model.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Zebrafish (Danio rerio)

Zebrafish, bred at the Institute of Tropical Biology, are employed in experiments. The fish are maintained on a 14-hour light and 10-hour dark cycle, with water temperature consistently held at 28 ± 0.5°C. They are fed twice daily, with artemia provided in the morning and commercial pellet feed in the afternoon.

2.1.2. Equipment and Necessary Tools

The equipment and tools used in this research project include a PCR machine, Realtime RT-PCR machine, analytical balance, pH meter, Western blotting system, among others.

2.1.3. Environment and Chemicals Used

Chemicals used in the research project comprise alcohol, Western blot antibodies, Potassium Dichromate, 2x qPCR SyGreen 1-Step Go Hi-ROX kit, and others.

2.2. Methods

2.2.1. Identification of zebrafish

2.2.1.1. Morphological characteristics

In this study, the observational method of recording data in research combined with the method of comparison and contrast with published documents was used. Supporting tools and equipment: Camera, ruler (10cm).

2.2.1.2. Molecular biology methods

This method helps determine the similarities or differences between the gene sequences of zebrafish (Danio rerio) and other fish groups on Genbank.

2.2.2 Breeding and embryo collection

The adult zebrafish (Danio rerio) were kept in an aquarium recirculation system under a 14/10 h light/dark cycle throughout the experiments. The water temperature was maintained at 28 ± 0.5ºC. Males and females were chosen based on secondary sexual traits and placed in breeding tanks with mesh at the bottom in the previous night before spawning. In the next day, after 30 minutes of light turning up, the zebrafish embryos were collected from the breeding tanks.

2.2.3. Chromium exposure

The zebrafish embryos were collected and raised at 28 °C in a 10% E3 solution over a 14:10 h light/dark cycle. One hour after fertilization, zebrafish embryos were divided into treatment groups and exposed to solutions containing chromium at different concentration (0.1, 1, 3.125, 6.25, 12.5, 50, and 100 µg/mL). 50 zebrafish embryos were selected and transferred to 100 mm petridish. At 24-hour intervals, the solutions were changed. To keep surviving embryos from being contaminated, dead embryos were taken out of the exposure chambers.

2.2.4. Evaluation of the effects of Chromium (VI) on the development of zebrafish embryos and larvae

2.2.4.1. Effect of chromium on zebrafish embryo development

To estimate the embryo development, the number of live embryos were determined from day 1 to day 7. The zebrafish embryos were checked under stereomicroscope to discard the deformed embryos, the degenerated and dead embryos, and the embryos with morphological abnormalities. The survival rate of treatment group and control group were recorded.

2.2.4.2. Heart rate and body length evaluation

Embryos at day 3 were brought to room temperature and left to stable for 30 minutes in order to assess the heart rate. Ten embryos were randomly chosen for each group, and under a stereomicroscope, their heart rates were

recorded four times for a total of 15 seconds. Ten embryos were chosen at random at day 3 to determine the body length. Using a digital camera mounted on a microscope, the length of the body was measured along the body axis. Image J.

2.2.5. Quantitative real-time RT-PCR

The embryos were collected and the total RNA was extracted using a Ribospin Total RNA Purification Kit. The NanoVue Plus spectrophotometer was used to evaluate the RNA sample's amount and quality. The PCR reaction was conducted: 1 μL of total RNA, 2 μL of primers (including forward and reverse primers), 10 μL Mix Ro-Lox, 1 μL RTAse, in a total volume of 20 μL in each reaction. The PCR reaction were performed by one cycle of 45^{o}C for 15 min; one cycle of 95^{o}C for 2 min; 40 cycles of 95^{o}C for 10 sec, 62^{o}C for 15 sec; and 71 cycles of 60^{o}C for 30 sec. Primers were as follows: bcl2-F: 5'- GGA TGA CTG ACT ACC TGA ACG G -3' and bcl2-R: 5'- GTA TGA AAA CGG GTG GAA CAC A -3'; bax-F: TGC CTT TTA TTA GAA AGA CCT GCA T -3' and bax-R: TCC AGC AAG GAA AAC TCC AAC T -3'; caspase 3-F: 5'- ATG AAC GGA GAC TGT GTG GA -3' and caspase 3-R: 5'- GTA TCT GAA GGC ATG GGA TTG A -3' (Castro et al., 2021), cdk4-F: 5'- GTA TGA GCC AGT AGC AGA GAT CG -3' and cdk4-R: 5'- AGT TGT GGT GGG AAA GAG TGA C -3'; cdk6-F: 5'- GTA CAA GGC TCG GGA TTT G-3' and cdk6-R: 5'- CTC TGG GGC TCG ATA CCA TA -3'; cdk21-F: 5'- CTG AAG CCT GAC AAT GTG CT -3' and cdk21-R: 5'- GCA AGC CAA TTA CCT CAA AGA -3' (Webster et al., 2018); and elongation factor 1 alpha (EF1α): EF1α -F: 5'- GTA CTA CTC TTC TTG ATG CCC -3' and EF1α -R: 5'- GTA CAG TTC CAA TAC CTC CA -3' (Papasani et al., 2006). The $2^{-\Delta\Delta Ct}$ method was applied for Ct value analysis (Livak and Schmittgen, 2001).

Western blot

The embryos were collected and treated with Optiblot LDS Sample Buffer (ab119196, Abcam, United States). Protein was added to each well of the Precast Gel SDS-PAGE 4-12% (ab139596, Abcam, United States) in an equal proportion. The gel was run in Optiblot SDS Run Buffer (ab119197, Abcam, United States) for 2 h at 50 V. The protein was transferred to a PVDF membrane (ab133411, Abcam, United States), and the membrane was blocked overnight at 4°C with blocking buffer (ab126587, Abcam, United States). The membrane was incubated with primary antibodies in blocking buffer overnight at 4°C.

Anti-caspase 3 antibody (ab44976, Abcam, Cambridge, MA, USA), anti-Bax antibody (ab53154, Abcam, Cambridge, MA, USA), anti-bcl-2 antibody (ab196495, Abcam, Cambridge, MA, USA) were employed at the 1/5000 dilution. anti-GAPDH antibody (ab181602, Abcam, United States) were used at a 1:10,000 dilution.

The membrane was washed three times with TBST for 10 min each. The membrane was incubated with secondary antibody in blocking buffer at room temperature for 1 h. Goat anti-mouse IgG (HRP) (ab6789, Abcam, United States) and goat anti-rabbit IgG (HRP) (ab6721, Abcam, United States) were used to detect the beta-actin antibody and other primary antibodies, respectively. The blots were visualized using the ECL Western Blotting Substrate Kit (ab65623, Abcam, United States). Imaging was carried out with an X-ray film.

2.2.6. Assessing the Accumulation of Chromium (VI) in the Body of Zebrafish (Danio rerio)

Adult zebrafish were raised in a Chromium (VI) contaminated environment with respective concentrations of LC_{50} and $LC_{6.25}$. Each concentration group consisted of 300 adult zebrafish. On days 5, 10, 15, 20, and 30, 50 adult zebrafish from each concentration group were selected for

analysis. The control group consisted of adult zebrafish that were not exposed to Chromium (VI). Zebrafish samples were sent to Viet Tin Analytical Testing Company for analysis.

The levels of Chromium (VI) accumulation in the bodies of zebrafish were measured on days 5, 10, 15, 20, and 30, and compared with the control group, which consisted of zebrafish not exposed to Chromium (VI).

2.2.7. The impact of Chrom(VI) on the internal organ structure (intestines, liver, and ovaries) of zebrafish (Danio rerio)

To assess the impact of Chrom(VI) on the structure of certain internal organs, zebrafish in the experimental groups exposed to Chrom(VI) at LC_{50} and $LC_{6.25}$ concentrations at different time points (day 5, day 10, day 20, and day 30) and the control group were collected and fixed in paraformaldehyde. These tissue samples were sent to the University of Medicine and Pharmacy in Ho Chi Minh City for sectioning. The internal organ samples of the fish were stained with Hematoxylin and Eosin (HE) and examined under a light microscope at various magnifications.

Changes or alterations in the structure of the samples, such as shape, size, cellular organization, and other structural changes, can be observed and assessed during this examination.

2.2.8. Statistical methods

The data were analyzed for statistical significance by one-way ANOVA where $P < 0.05$ was considered statistically significant.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Identification of zebrafish

Zebrafish have five longitudinal stripes running along their bodies from the head to the end of the tail fin. When observing the body shape of the fish, differences between males and females are evident. Males typically have a slimmer body, whereas females tend to have a more robust body, especially during pregnancy when the female's abdomen becomes noticeably swollen due to egg retention.

Figure 3.1. Description of Zebrafish Morphology - Male and Female
(A: Male Zebrafish, B: Female Zebrafish)

The results of the PCR electrophoresis showed that the amplified products for the cytochrome b and cytochrome c genes had sizes of 1141 bp and 712 bp, respectively. When compared with the cytochrome b sequence of the zebrafish used in the experiment to the sequence of the zebrafish in the gene bank (NC002333), the similarity level exceeded 99%. Additionally, the similarity analysis results showed that the zebrafish in this study and the zebrafish in the gene bank had a similarity level exceeding 99% for the cytochrome c gene.

3.2.1. Survival

The embryos treated with 0.1 µg/L and 1 µg/L chromium showed the same survival percentage from day 1 to day 7 (98.8% and 96.4%, respectively). The survival percentage decreased at day 2 in embryos treated with the higher concentration of chromium (3.125 µg/L, 6.25 µg/L, and 12.5 µg/L). The survival percentage of embryos treated with 25 µg/L chromium decreased to 47.2% on day 2 and continued to reduce to 30.8% on the day 7.

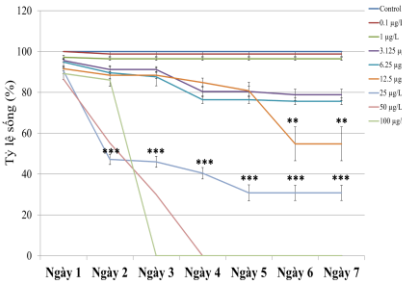


Figure 3.2. Survial rate of zebrafish embryos and larvae under chromium treatment

3.2.2. Body Length

The degree of larvae development was estimated by the measurment of body lengths (Figure 3.3). Because of the developmental blocks in embryos treated with 50 µg/L and 100 µg/L chromium, the body length was assessed in larvae treated with a range of chromium concentrations (0.1 µg/L to 25 µg/L). As seen in the Figure 2, The high concentration of chromium gave rise to the reduction of larvae body length. The body length of larvae from control group increase from 2982 µm to 3461 µm during time-dependent maner (day 3 to day 7). The larvae in groups treated with chromium (0.1

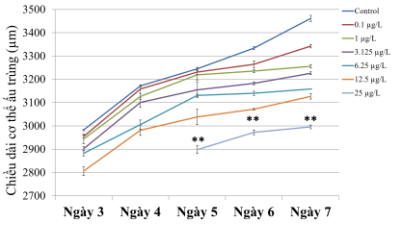


Figure 3.3. The body length of zebrafish larvae under chromium treatment.

µg/L to 12.5 µg/L) showed a lower body length than the control group during the development. The hatching delay of embryo treated with 25 µg/L chromium lead to the low growth of larvae from day 5 to day 7 (2898 µm to 2996 µm, respectively).

3.2.3. Heart rate

On the third day, an increase in heart rate was observed when zebrafish larvae were exposed to Chromium (VI) at various concentrations compared to the control group. The heart rate increased from 2 to 89 beats per minute at Chromium (VI) concentrations ranging from 0.1 µg/L to 25 µg/L, and this difference was statistically significant ($p < 0.05$).

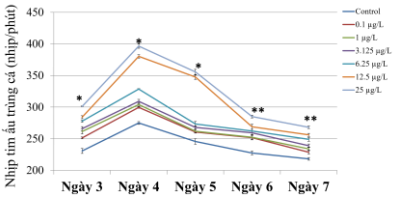


Figure 3.4. Effect of Chromium (VI) on the Heart Rate of Zebrafish Larvae

From the third day to the seventh day, the average heart rate of zebrafish larvae continued to increase with Chromium (VI) concentration, similar to the observations on the third day. The heart rate of zebrafish larvae increased from 33 beats per minute to 193.6 beats per minute at Chromium (VI) concentrations ranging from 0.1 µg/L to 25 µg/L compared to the control group, and this difference was statistically significant ($p < 0.05$).

3.3. Chromium (VI) influences changes in the expression of responsive genes and genes controlling damage in the development of zebrafish *(Danio rerio)*

3.3.1. Gadd45a and gadd45g genes

The results of the Real-time RT-PCR analysis (Figure 3.5 A) show that there is no significant difference in the mRNA expression of the gadd45a gene between the experimental groups and the control group. This indicates that, prior to exposure to Crom (VI), the expression level of the gadd45a gene is stable and not affected by Crom (VI) at this time point..

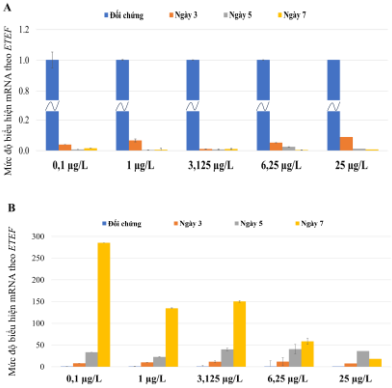


Figure 3.5. mRNA Expression Levels of gadd45a and gadd45g Genes Over Time (A: gadd45a gene, B: gadd45g

The research results show that higher concentrations of Crom (VI) had a detrimental effect on the expression of the gadd45a gene, leading to reduced gene expression. This reduction in expression is attributed to the influence of Crom (VI) on cellular gene regulation processes, resulting in decreased cellular responses to gene damage and repair capabilities in response to the damage caused by Crom (VI).

The Real-time RT-PCR analysis results (Figure 3.5 B) show that there is no significant difference in the mRNA expression of the gadd45g gene between the experimental groups and the control group. This indicates that, prior to exposure to Crom (VI), the expression level of the gadd45g gene is stable and not affected by Crom (VI) at this time point.

On the 5th day, the expression of the gadd45g gene continued to increase compared to day 3 in all experimental groups, but there were no significant differences between the experimental groups. This suggests that the effect of Crom (VI) continued to cause damage, and the gadd45g gene remained activated to counteract the damage caused by Crom (VI).

However, the differences in mRNA expression of the gadd45g gene between the experimental groups and the control group became evident on the 7th day. On the 7th day, the mRNA expression of this gene decreased compared to day 5 in the experimental groups, while the experimental groups treated with Crom (VI) showed a significant increase in the expression of this gene.

3.3.2. Sod1 và sod2 genes

The results of Real-time RT-PCR analysis (Figure 3.6 A) show that the mRNA expression of the sod1 gene did not differ significantly between the experimental groups and the control group. This indicates that before exposure to Crom (VI), the expression level of the sod1 gene was stable and not influenced by Crom (VI) at this time.

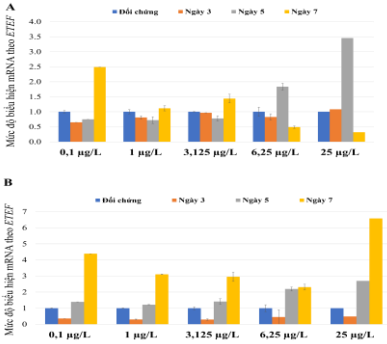


Figure 3.6. Effects of Chromium (VI) on the Transcriptional Expression of sod1 (A) and sod2 (B) Genes in Zebrafish Embryos

However, the sharp increase in mRNA expression of the sod1 gene at Crom (VI) concentrations of 6.25 µg/L and 25 µg/L on the 5th day was followed by a significant decrease on the 7th day, whereas the other groups continued to exhibit increased gene expression on the 7th day

These results indicate that Crom (VI) affects the expression of the sod1 gene at different concentrations and time points. The expression of the sod1 gene can either increase or decrease depending on the concentration of Crom (VI) and the duration of exposure.

Similarly, the results of the Real-time RT-PCR analysis (Figure 3.6 B) show that in the control group, there was no significant difference in the expression of the sod2 gene among the experimental groups. This suggests that before exposure to Crom (VI), the expression level of the sod2 gene was stable and not influenced by Crom (VI) at that time.

The differences in mRNA expression of the sod2 gene among the experimental groups and the control group became more pronounced on the 7th day. On the 7th day, the mRNA expression of the sod2 gene increased compared to the 5th day in most experimental groups except for the Crom (VI) concentration of 6.25 µg/L.

These results demonstrate that Crom (VI) affects the mRNA expression of the sod2 gene at various concentrations and time points. The mRNA expression of the sod2 gene can increase at certain Crom (VI) concentrations and decrease at others, and this may be related to how Crom (VI) influences the regulatory processes of this gene within the cells.

This study highlights the impact of Crom (VI) on the development of horsehair worm larvae, directly affecting the expression of the sod1 and sod2 genes. These two genes play crucial roles in the antioxidant defense processes within the horsehair worm larvae's body.

On the 3rd day, a decrease in the mRNA expression levels of both the sod1 and sod2 genes was observed in the horsehair worm larvae when exposed to Crom (VI) in the experimental groups. This indicates that Crom (VI) led to a reduction in the expression of these two genes, affecting the

larvae's ability to eliminate free radicals and engage in antioxidative processes.

However, only the expression of the sod2 gene remained low, signifying a prolonged or impaired recovery of the antioxidative system due to Crom (VI) exposure. In contrast, the sod1 gene showed signs of recovery, with increased expression on the 7th day. The increased expression of the sod1 gene indicates a recovery response in the horsehair worm larvae's body after exposure to Crom (VI) over 7 days.

These findings suggest that Crom (VI) significantly influenced the antioxidative defense mechanisms of the horsehair worm larvae, altering the expression of the sod1 and sod2 genes. This impact resulted in a weakened antioxidative defense capacity within the horsehair worm larvae's body.

3.3.3. Gene mt2

The RT-PCR analysis results (Figure 3.7) show the mRNA expression of the mt2 gene. There was no significant difference in the mRNA expression of the mt2 gene between the experimental groups and the control group. The expression of this gene remained unchanged in all experimental groups. This indicates that, before exposure to Crom (VI), the expression level of the mt2 gene was stable and not affected by Crom (VI) at that time.

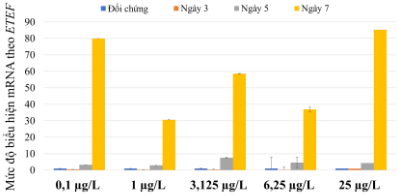


Figure 3.7. Effect of Chromium (VI) on the Transcriptional Expression of the mt2 Gene in Zebrafish Embryos

On the 3rd day, after exposure to Crom (VI), the mRNA expression of the mt2 gene remained unchanged in all experimental groups. However, there was no significant difference in the expression of this gene among the

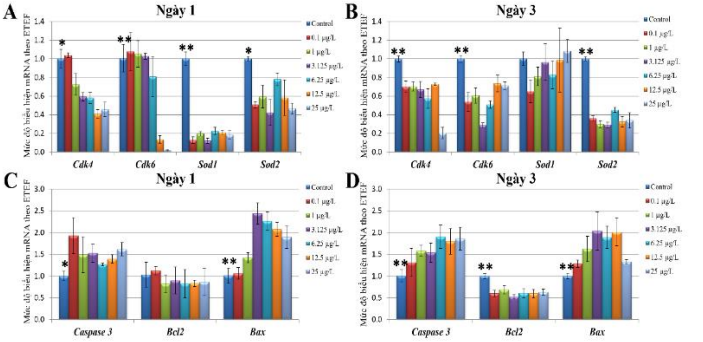
groups. This suggests that Crom (VI) did not significantly affect the mRNA expression of the mt2 gene at this time.

On the 5th day, the mRNA expression of the mt2 gene increased in all experimental groups, but there was no significant difference in the expression of this gene among the experimental groups. This increase in expression may be related to a self-protective response of the zebrafish larvae to counteract the effects of Crom (VI) that had entered their bodies since the 3rd day. The mt2 gene was activated to participate in the process of detoxifying or controlling the toxicity of Crom (VI) in the zebrafish larvae's bodies.

On the 7th day, the mRNA expression of the mt2 gene increased compared to the 5th day, and there was a significant difference in the expression of the mt2 gene among the experimental groups and the control group. This indicates that Crom (VI) had an impact on the mRNA expression of the mt2 gene after a period of exposure, and this increased expression may play an important role in the body's self-defense mechanism against the toxicity of Crom (VI).

These results show that the mt2 gene plays a crucial role in protecting the zebrafish larvae's bodies against the toxicity of Crom (VI), and its expression increases over time under the influence of Crom (VI) concentration. The mt2 gene plays a significant role in protecting the zebrafish larvae's bodies against the harmful effects of Crom (VI) and shields the body from the influence of this heavy metal.

3.3.4. The expression of genes related to the cell cycle, oxidative stress inhibition, and the apoptosis process



Hình 3.8. The transcript expression of cell cycle related genes (cdk4, cdk6), antioxidant related genes (sod1, sod2), and apoptosis related genes (caspase 3, bcl2, and bax).

The experimental groups exposed to Chrom(VI) showed an increase in the expression level of sod1 on day 3 after initially decreasing on day 1. The expression of sod2 mRNA in the experimental groups exposed to Chrom(VI), on the other hand, remained lower than that of the control group on days 1 and 3.

Real-time RT-PCR technique also revealed that the mRNA expression of caspase 3 and bax increased in the zebrafish embryos exposed to Chrom(VI) on day 1 (Figure 3.18 C). However, there was no significant difference in the expression of bcl2 between the control group and the groups exposed to Chrom(VI). The upregulation of caspase 3 and bax in zebrafish embryos exposed to Chrom(VI) for 3 days and the downregulation of bcl2 expression were also observed in these embryos (Figure 3.18 D)

3.3.5. Western blot

The Western blot method was utilized to analyze the expression of the Bcl2 protein among different experimental groups. The analysis results

showed that zebrafish embryos on the 1st day (Figure 3.9 A) in the 0.1 µg/L Chromium (VI) concentration group exhibited increased Bcl2 expression compared to the control group. This elevated expression continued to be observed in the 1 µg/L Chromium (VI) concentration group, the 3.125 µg/L Chromium (VI) concentration group, and the 6.25 µg/L Chromium (VI) concentration group. There was no statistically significant difference in the expression of this protein between the 1 µg/L Chromium (VI) concentration group and the 3.125 µg/L Chromium (VI) concentration group. The expression of the Bcl2 protein in the zebrafish embryos at the 6.25 µg/L concentration tended to decrease compared to the other experimental groups but remained higher than the control group. Furthermore, on the 1st day, the expression of several proteins related to the apoptosis process was also assessed using the Western blot method.

The Western blot method was employed to analyze the expression of the Caspase 3 protein among different experimental groups. The analysis results showed that zebrafish embryos on the 1st day (Figure 3.9 A) in the 0.1 µg/L Chromium (VI) concentration group exhibited increased Caspase 3 expression compared to the control group. This elevated expression continued to be observed in the 1 µg/L Chromium (VI) concentration group and the 3.125 µg/L Chromium (VI) concentration group. There was no statistically significant difference in the expression of this protein among the 0.1 µg/L, 1 µg/L, and 3.125 µg/L Chromium (VI) concentration groups. The expression of Caspase 3 in the zebrafish embryos at the 6.25 µg/L concentration tended to decrease compared to the other experimental groups but remained higher than the control group. Furthermore, on the 1st day, the

expression of several proteins related to the programmed cell death process was also assessed using the Western blot method.

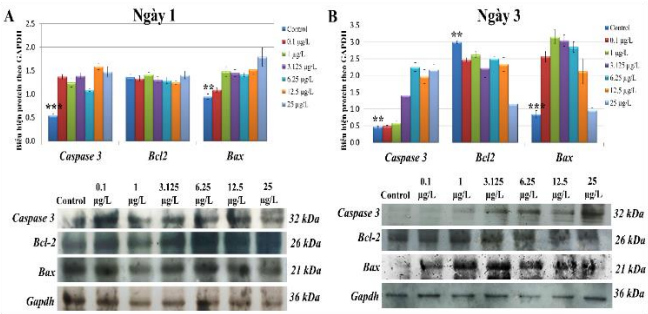


Figure 3.9. The epxression of apoptosis related protein (caspase 3, bcl2, and bax) (n=3). A, B. The western blot analysis was performed in zebrafish embryos from day 1 and day 3.

The Western blot method was applied to analyze the expression of the Bax protein among different experimental groups. The analysis results revealed that zebrafish embryos on the 3rd day (Figure 3.9 B) in the 0.1 µg/L Chromium (VI) concentration group showed increased Bax expression compared to the control group. This elevated expression continued to be observed in the 1 µg/L Chromium (VI) concentration group, the 3.125 µg/L Chromium (VI) concentration group, and the 6.25 µg/L Chromium (VI) concentration group. There was no statistically significant difference in the expression of this protein between the 1 µg/L Chromium (VI) concentration group and the 6.25 µg/L Chromium (VI) concentration group. Furthermore, on the 3rd day, the expression of several proteins related to programmed cell death was also assessed using the Western blot method

3.4.1. Accumulation of Chromium (VI) in the Body of Zebrafish *(Danio rerio)*

First experimental group: At the same LC_{50} concentration, the analysis results showed that the accumulation of chromium (VI) increased over time, with the highest level measured on day 30 (23.9±1.0 mg/kg). This level was four times higher than on day 5 (6.1±0.4 mg/kg), three times higher than on day 10 (9.3±0.6 mg/kg), and 1.5 times higher than on day 20 (17±0.8 mg/kg). In the control group, the chromium (VI) accumulation rate in the fish's bodies was 0%, indicating that chromium (VI) did not accumulate in the bodies of zebrafish.

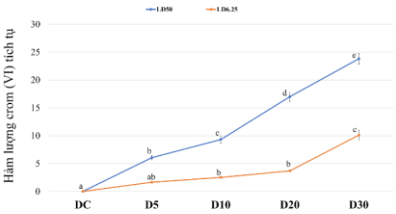
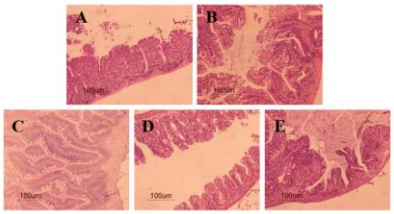


Figure 3.10. Accumulation of Chromium (VI) in the Adult Zebrafish Body

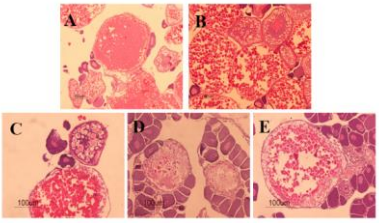
Second experimental group: At the same $LC_{6.25}$ concentration, the chromium (VI) accumulation on days 5, 10, and 20 was 1.7±0.1 mg/kg, 2.5±0.1 mg/kg, and 3.7±0.0 mg/kg, respectively. However, there were no statistically significant differences between these values. The analysis results showed that the accumulation of chromium (VI) increased over time, with the highest level measured on day 30 (10.1±0.9 mg/kg). This level was 5.9 times higher than on day 5 (1.7±0.1 mg/kg), 4.1 times higher than on day 10 (2.5±0.1 mg/kg), and 2.7 times higher than on day 20 (3.7±0.0 mg/kg). In the control group, the accumulation rate was 0%, indicating that chromium (VI) did not accumulate in the bodies of zebrafish.

3.4.2. The Impact of Chromium (VI) on the Tissue Structure of Zebrafish (Danio rerio)

The results of the histological section of the intestine shown in Figure 3.11, observed under a microscope, reveal changes in the tissue structure of zebrafish intestine upon exposure to Chromium (VI) at the LC_{50} concentration. From day 5 to day 20 (Figure 3.11 B, 3.11 C, 3.11 D), there is an increase in villi, compared to the control group, and the villi are most densely packed on day 30 (Figure 3.11 F). Additionally, the adipose tissues within the intestine decrease over time of exposure to Chromium (VI).



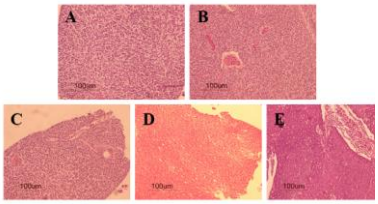
Hình 3.11. Cross-sectional images of the zebrafish intestine layers after exposure to Chromium (VI)



Hình 3.12. Cross-sections of the zebrafish ovarian tissue exposed to different concentrations of Chromium (VI)

The results of the histological section of the ovary (Figure 3.12), observed under a microscope, demonstrate changes in the tissue structure of the zebrafish ovary upon exposure to Chromium (VI) at the LD_{50} concentration. With longer exposure time, the proportion of lipid droplets decreases. From day 5 to day 30, the proportion of lipid droplets consistently decreases compared to the control group.

The results of the histological section of the liver (Figure 3.13), observed under a microscope, demonstrate changes in the tissue structure of the zebrafish liver upon exposure to Chromium (VI) at the LD_{50} concentration. From day 5 to day 30, inflammatory foci appear and become more severe with prolonged exposure to Chromium (VI).



Hình 3.13. Cross-sections of the zebrafish liver tissue exposed to Chromium (VI)

CONCLUSIONS AND RECOMMENDATIONS

Conclusion

In this study, we evaluated the effect of chromium on the development of zebrafish embryos and larvae. According to the findings, there were no significant effects of the chromium treatment at low doses (0.1 g/L and 1 g/L) on embryo survival throughout embryonic development. The chromium exposure at higher concentration (≥3.125 µg/L), however, decreased survival of zebrafish embryo form day 1 to day 7. These findings contribute to our understanding of how chromium affects the development of zebrafish embryo and larvae. The 25 µg/L chromium is the highest concentration at which zebrafish embryos can develop up to day 7. With chromium exposure at the lower concentration, the embryo hatching took place on day 3, while the hatching of embryo under 25 µg/L chromium treatment occur in day 5. Hatching is the result of a combination of many factors including the osmosis, the secreted hatching enzyme, the lashing movement of the embryonic tail (Yamagani, 1988). The changes of embryo size result in the abnormality of the hatching (Jezierska et al., 2009). In the present work, the increase of chromium concentration associated with the decrease of body

length in zebrafish larvae from day 3 to day 7, especially in the group treated with 25 µg/L chromium. This result revealed that the high concentration of chromium induce a delay of zebrafish embryo hatching.

The investigation of apoptosis-related transcript levels revealed the triggering of apoptosis in zebrafish embryos. The previous study reported that the heavy metal induced ROS generation can alter the apoptosis-related gene expression (Di Paola et al., 2021; Capriello et al., 2021). Real time RT-PCR and western blot analyses demonstrated that chromium exposure induced an upregulation of caspase 3 and bax, and the downregulation of bcl2. Caspases are considered to be indicators for oxidative stress-induced apoptosis in zebrafish embryos since they are crucial components of the apoptosis process. One of the convergence points of many different pathways which regulates apoptosis is caspase 3 (Cai et al., 2012). Bcl-2 gene family members play a crucial role in mediating the delicate balance between apoptosis and survival in eucaryotic cells (Kratz et al., 2006). This signaling pathway's dysregulation can cause a variety of diseases relating to development and degenerative disorders (Strasser, 2005). The present investigation showed that chromium treatment induced an increase of caspase 3 expression. In addition, chrommium exposed embryos showed bax upregulation and bcl2 downregulation. These outcomes indicated that an increase of apoptosis occurred in emrbyos under chromium exposure which gave rise to the decrease their development.

Recommendation

Analyze the expression of genes related to the apoptosis and oxidative stress processes in adult zebrafish (Danio rerio).

Evaluate the effects of Chromium (VI) on the structure and function of various tissues, such as the kidney and testes.

NEW CONTRIBUTIONS OF THE THESIS

The novel contributions of this thesis encompass:

This study has demonstrated that Chromium (VI) has the potential to inhibit the development and impede the hatching process of zebrafish embryos at the early stage. It reduces survival rates, induces changes in the length of zebrafish larvae, increases heart rate, and leads to morphological abnormalities in the fish.

Additionally, Chromium (VI) also induces structural damage in vital internal organs such as the intestine, liver, and ovaries of zebrafish. This highlights a direct impact on the endocrine health and digestive system of the fish.

Particularly noteworthy is the finding that Chromium (VI) triggers alterations in the expression of several genes related to the apoptosis process.

LIST OF PUBLICATIONS

Dang, K. D., Ho, C. N. Q., Van, H. D., Dinh, S. T., Nguyen, Q. T. T., Nguyen, T. T. T., ... & Le, L. T. (2023). Hexavalent Chromium Inhibited Zebrafish Embryo Development by Altering Apoptosis-and Antioxidant-Related Genes. *Current Issues in Molecular Biology*, *45*(8), 6916-6926. (SCI, Q2).

Dang Dang Khoa, Nguyen Thi Phuong Thao, Le Thanh Long. Effects of hexavalent chromium on structure of liver, intestine and ovary of Zebra fish. International Journal of Biosciences. 2023, 23 (3): pp 164-169.