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Morphological and Molecular Defects in Zebrafish Embryos (*Danio rerio*) After Chromated Copper Arsenate Exposure

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MORPHOLOGICAL AND MOLECULAR DEFECTS IN ZEBRAFISH EMBRYOS *(DANIO RERIO)* AFTER CHROMATED COPPER ARSENATE EXPOSURE

by

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ABSTRACT

MORPHOLOGICAL AND MOLECULAR DEFECTS IN ZEBRAFISH EMBRYOS *(DANIO RERIO)* AFTER CHROMATED COPPER ARSENATE EXPOSURE

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This investigation identified the morphological defects caused by different concentrations of chromium, copper, and arsenic (CCA) on the developing zebrafish embryo; in addition to defects caused by the three combined components (complete CCA). A change in the DNA of the developing embryos in response to the three components of CCA and complete CCA was also determined. Early *Danio rerio* embryos were cultured for five days with various concentrations of CCA components and complete CCA. Morphological defects were assessed with light microscopy and DNA fragmentation was determined by agarose gel electrophoresis. Embryonic exposure of *D. rerio* to sublethal concentrations of chromium, copper, arsenic, and complete CCA caused a variety of morphological defects including head edema, abnormal finfold development, tail flexures, and cardiac malformations. The two types of morphological defects that were most consistent were abnormal finfold development and tail flexure. The genotoxicity of CCA components and complete CCA was confirmed by induction of DNA fragmentation.

This thesis is dedicated to my mother who
taught me anything is possible with hard work.

ACKNOWLEDGMENTS

Many have contributed to this successful thesis completion. I extend many thanks to my committee chair and committee members for their tolerance and hours of assistance on my research and editing of this document. The tireless effort of my major advisor deserves special recognition as does my fellow graduate students who helped me achieve my goals.

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SECTION I

INTRODUCTION

Chromated copper arsenate (CCA) is a mixture of metallic oxides used as a wood preservative and marketed to protect wood from dry rot, fungi, molds, termites, and other pests that can threaten the integrity of wood products. CCA is injected into wood by a process that uses high temperature and pressure to saturate wood products with the pesticide. CCA-treated wood is commonly used in outdoor settings. Around the home, CCA-treated wood has been used for decks, walkways, fences, gazebos, boat docks, and playground equipment. Other common uses of CCA-treated wood include utility poles, building construction poles, posts and support timber, marine construction, and plywood for highways (Cox, 1991).

On February 12, 2002, the Environmental Protection Agency (EPA) announced a voluntary decision to move away from using CCA to treat wood used in residential settings. The EPA's decision affects virtually all residential uses of wood treated with CCA, including wood used in play-structures, decks, picnic tables, landscaping timbers, residential fencing, patios and walkways/boardwalks. Effective January 1, 2004, no wood trader or manufacturer can treat wood with CCA for most residential uses (National Coalition against the Misuse of Pesticides, 2003). The EPA's decision will facilitate the transition in both the manufacturing and retail sectors to wood preservatives that do not contain arsenic, as well as other alternatives, such as naturally resistant woods and plastic wood. After January 1, 2004 manufacturers were no longer able to use CCA to treat wood products. Although manufacturers abided by this use-restriction in the chemical

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This thesis was modeled after Aquatic Toxicology journal.

treatment of wood, CCA-treated wood was still sold until the remainder of the CCA stock had been diminished. Additionally, despite the fact that the EPA has now banned the use of CCA, CCA treated wood structures will continue to remain in the environment for decades. The EPA has not mandated the removal of the treated structures from the environment even though recent studies have shown that when submerged in water CCA has the ability to leach from the wood.

One such study by Solo-Gabriele et al. (2003) showed that arsenic leaches at a rate of 3.71 mg/L, chromium at 1.82 mg/L, and copper at 10.00 mg/L. The leaching observed occurred over an 18-hour period. Due to the high leaching rate of CCA from wood and the many docking structures and bulkheads found in lake and pond systems, CCA has become an environmental toxicant that is of particular concern to aquatic organisms. Through direct uptake, aquatic organisms collect copper, chromium, and arsenic from the water (Cox, 1991). According to Weis et al. (1991) and Weis and Weis (1996) copper, chromium, and arsenic contamination has been shown to be toxic to an array of marine organisms, including sea urchins, fiddler crabs, and green algae.

The zebrafish, *Danio rerio,* is a small, freshwater fish that is seen as the newest vertebrate model for embryological development studies (Linney et al., 2004). The zebrafish embryos have many advantages as a vertebrate model organism. One such advantage is that the embryos develop transparently during the first few days of development. In particular, all internal organ development can be observed from outside the chorion without disturbing the living embryo (Muda and McKenna, 2004). Another advantage is that they are simple to maintain and care for. These organisms are also easy to breed and spawning large numbers of fish is done effortlessly from week to week. The

zebrafish's reasonably large fertilized eggs make this embryo model an ideal subject to study the affects of toxic substances. Yet another great advantage to using the zebrafish as a laboratory model in toxicology studies is that the embryonic development is quick, in the first 24 hours an observer will be able to see the first stages of development and by day three the embryo will be ready to hatch and swim away (Appelmelk et al., 2004).

The genome of the zebrafish is an ongoing project to sequence the 1.7×10^9 base-pair genome, about half the size of that of the mouse or human genome. The most important value of the mapping and sequencing of the genome is that it will expedite mutation cloning and zebrafish genome comparisons with other species will provide superior starting points for analysis of regulatory elements. The new screens can be applied to the understanding of organ formation and physiology in the developing embryo and evolutionary biology. Behavior, learning, and memory are already the focus of some early phase studies and suppressor and enhancer screens will help define pathway descriptions. Additionally, the relatively ready entrance of many chemicals into the viable embryo makes them targets for chemical screens, relevant to drug discovery and toxicology (Wixon, 2000).

The specific aims of this investigation were to identify and document the morphological defects caused by the components of CCA and combined CCA on the developing embryo and to determine that CCA components and CCA combined affected total DNA in the developing embryos.

SECTION II

MATERIALS AND METHODS

Adult female and male zebrafish were ordered from Aquatic Ecosystems and maintained in two separate 15-gallon tanks. The lab was kept on a 14hr light and 10 hr dark cycle. A light timer would turn on at 8:00 a.m. and turn off at 10:00 p.m. The tank temperature was maintained at 28°C using tank heaters. The night before breeding the mature zebrafish, two males and two females, were transferred to a clean 10 gallon breeding tank lined on the bottom with marbles. The breeding tank was filled with fresh deionized water and the temperature was properly maintained. At the onset of the light cycle, the fish bred in one to two hours with females laying their eggs and males releasing their sperm together for fertilization.

The adult zebrafish were then removed and returned to their separate tanks. A siphon was used to remove the embryos from the bottom of the tank under the layer of marbles. The embryos were siphoned into a mesh nylon cylinder and inverted for transfer into a petri dish containing a solution of egg water (1.5 ml stock salts added to 1 L distilled water = 60 μg/ml final concentration) described by Westerfield (1993). Two washes with the egg water were performed to remove the surrounding debris and then the washed embryos were individually transferred into 24 well plates containing 2 ml of egg water.

Varying amounts of a concentrated (100-mg/L) stock solution of chromium, copper, or arsenic were added to embryo cultures to produce a dilution series that bracketed the effective toxic dose. The 24-well plate was labeled and one row of wells was kept as a control. The plate was placed on supports in a water bath at 28.5°C. The

embryos were observed with a Nikon inverted microscope at 24 hours post-fertilization (hpf), 48 hpf, 72 hpf, and at 96 hpf and the data was recorded in a lab notebook. At 120 hpf pictures were taken of any defects using the Nikon inverted scope with an attached 2.0-megapixal digital Fuji camera and the defects were rated according to type and severity of effect according to Samson and Shenker (2000).

The 120 hpf zebrafish embryo tissues were treated to isolate total DNA using the QIAGEN DNeasy Tissue Kit. Five 120 hpf embryos were placed in a 1.5 ml microcentrifuge tube and 180 µl of buffer ATL was added. Then 20 µl of proteinase K was added and samples were mixed by vortexing. The samples were incubated at 55°C overnight until the tissue was completely lysed. The samples were then mixed by vortexing for 15s, 200 µl of buffer AL was added, and samples were mixed again by vortexing. The samples were then incubated at 70°C for ten minutes. Then 200 µl of 95% ethanol was added to the sample and mixed by vortexing. The mixture was then pipetted into a DNeasy mini spin column placed in a two ml collection tube. The collection tube and mini spin column were then centrifuged at 8000 rpm for one minute. The flow-through and collection tube were discarded. The DNeasy mini spin column was placed into a new 2 ml collection tube and 500 µl of buffer AW1 was added and the sample was centrifuged for one minute at 8000 rpm. The flow-through and collection tube were again discarded. The DNeasy mini spin column was then placed in a new 2 ml collection tube and 500 µl of buffer AW2 was added. The mini spin column and the collection tube were then centrifuged for three minutes at 14,000 rpm. The flow-through and collection tube were again discarded. The DNeasy mini spin column was then placed in a clean 1.5 ml microcentrifuge tube and 100 µl of buffer AE was added. The sample

was then centrifuged for one minute at 8000 rpm to elute. The DNA concentration was determined with an Eppendorf Biophotometer 6131. Five embryos would yield approximately 20 to 40 μg/ml. DNA was concentrated by adding a quantity of 3M Na Acetate equaling 1/10 of the sample volume and then 95% ethanol (2 x sample volume) was added. Samples were centrifuged for 3 min at 8000 rpm with the hinge of the microcentrifuge tube pointing outwards so the supernatant could be removed, making sure to leave the pellet. The pellet was washed with 70% ethanol and centrifuged again for 3 min at 8000 rpm. Supernatant was removed and the pellet was left to air dry at room temperature. Once dry the pellet was resuspended in 20 μl of Buffer AE. A gel was prepared by adding 1.0 g of agarose to 100 ml of TAE Buffer and heated for 30 seconds until boiling started. After removal from heat the gel mixture was dissolved by swirling. Ten μl of Cyber Green from Gelstar was added to the mixture and then poured into the gel rig apparatus and left to solidify for 15 min. The gel was run toward the positive at 140 V for approximately 30 min after loading 20 ng of sample and Tracker Dye into each well. The gels were photographed by using a UV light source and Kodak gel photography camera. Pictures were evaluated and digitally saved.

Statistical difference between various treatment groups was determined with analysis of variance (ANOVA). A p-value of less than 0.05 was considered statistically significant. Graphed results were expressed as mean ± standard error.

SECTION III

RESULTS

Exposing *D. rerio* embryos to sublethal concentrations of chromium, copper, arsenic, and combined CCA caused a variety of morphological defects including head edema, yolk sac edema, abnormal finfold development, tail flexures, and cardiac malformations. The two most consistent morphological defects were abnormal finfold development and tail flexure. The concentration of each component and the CCA mixture affected the frequency and severity of the morphological defects observed. The two defects were exhibited in each component as well as the CCA mixture.

Tissue abnormalities of the finfold

The first defect that was observed was a tissue abnormality of the median finfold region. In normally developing embryos, the median finfold is a clear, thin membrane around the entire trunk region containing unsegmented fin rays (Fig 1A). In treated embryos, the tissue structure of the finfold was disorganized and in the more severe cases, the shape of the finfold and the developing fin rays were altered. With increasing concentrations the chromium, copper, arsenic, and CCA treated embryos displayed increasing defect severity, rated on a qualitative scale (Table 1).

An increasing percentage of abnormal finfold development occurred in chromium treated embryos: 20% in 0.010 mg/L CrO_3, 62.5% in 0.025 mg/L CrO_3, 81.5% in 0.100 mg/L CrO_3, 91.7% in 0.500 mg/L CrO_3, and 100.0% in 1.000 mg/L CrO_3. Exposure to 0.005 mg/L CrO_3 produced no abnormal finfold development. An increasing percentage of abnormal finfold development occurred in copper treated embryos: 9.1% in 0.005

Table 1
Rating in severity of morphological defects in finfold development of 120 hpf *D. rerio* embryos after exposure to components of CCA and CCA

Rating	Description
0	Control, normal tail development (Fig. 1A)
1	Small portion of finfold tissue structure disorganized around outer margin area (Fig. 1B)
2	Majority of tissue near base of tail was abnormal and disorganized, extending to finfold's outer margin with an overall reduction in size (Fig. 1C)
3	Abnormal organization of tissues extended from base of tail to finfold's outer margin and shape of finfold structure was inconsistent with marked size reduction (Fig. 1D)
4	Tissue was abnormal and disorganized throughout entire finfold area with severe size reduction (Fig. 1E)
5	Abnormal tissue was attenuated to the point that no region of finfold development could be identified (Fig. 1F)

mg/L CuO, 20.0% in 0.010 mg/L CuO, 56.5% in 0.025 mg/L CuO, and 100.0% in 0.100 mg/L CuO. Copper treatment at 0.500 and 1.000 mg/L CuO inhibited hatching and thus could not be rated since only hatched embryos were rated in all treatment groups at all concentrations. An increasing percentage of abnormal finfold development occurred in arsenic treated embryos: 5.9% in 0.010 mg/L As_2O_5, 69.0% in 0.025 mg/L As_2O_5, and 84.4% in 0.100 mg/L As_2O_5, 71.9% in 0.500 mg/L As_2O_5, and 100.0% in 1.000 mg/L

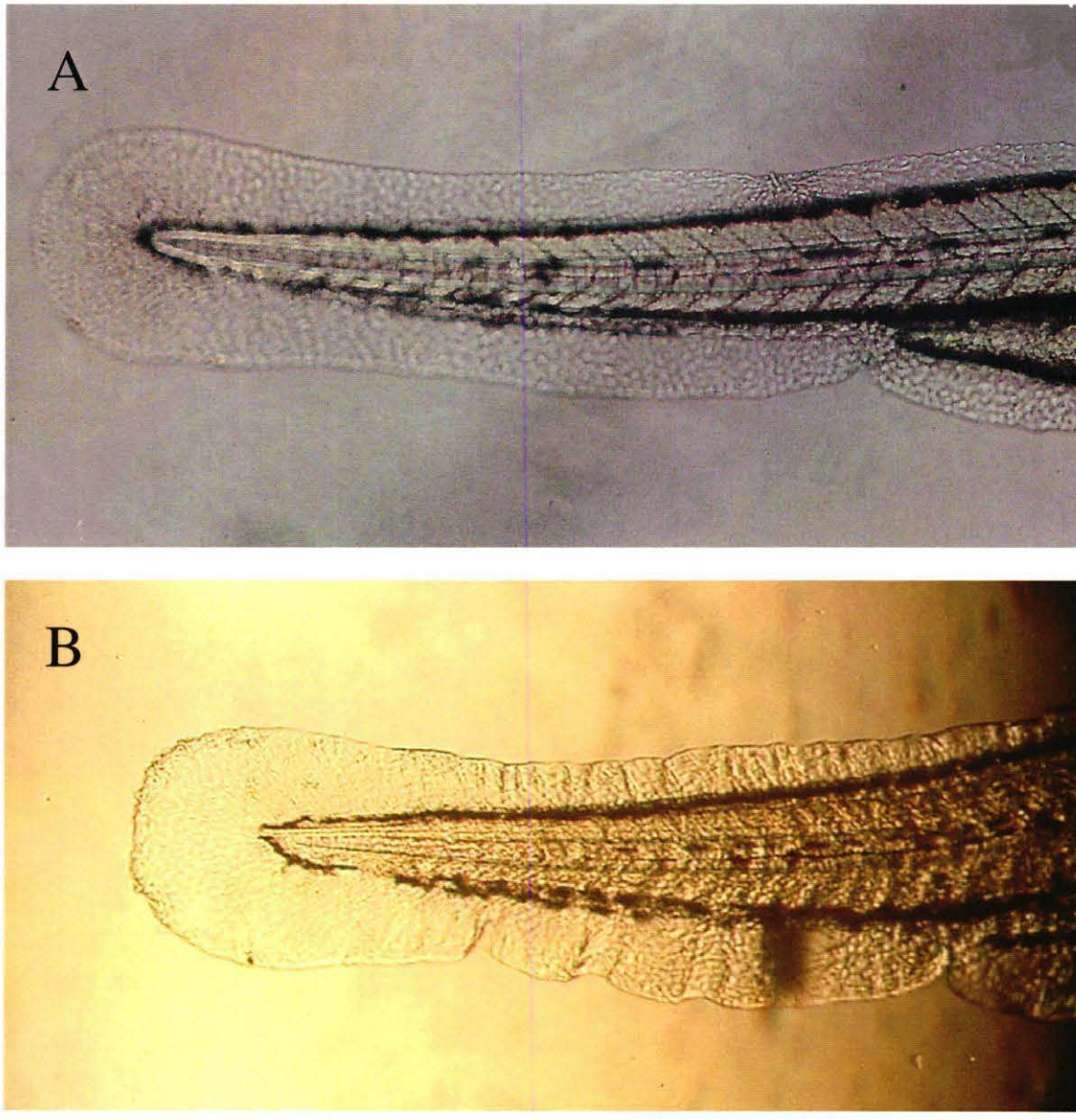

Fig. 1. Range in severity of morphological defects in finfold development of 120 hpf *D. rerio* embryos after exposure to components of CCA and CCA. (A) Control, normal development: morphological score 0. (B) Small portion of the finfold tissue structure was disorganized around the outer margin area: finfold development score 1. (C) Majority of tissue near the base of the tail was abnormal and disorganized extending to the finfold's outer margin with overall reduction in size: finfold development score 2. (D) Abnormal organization of tissues extended from the base of the tail to the finfold's outer margin and the shape of the finfold structure was inconsistent with marked size reduction: finfold development score 3. (E) Tissue was abnormal and disorganized throughout the entire finfold area with severe size reduction: finfold development score 4. (F) Abnormal tissue was attenuated to the point that no region of finfold development could be identified: finfold development score 5.

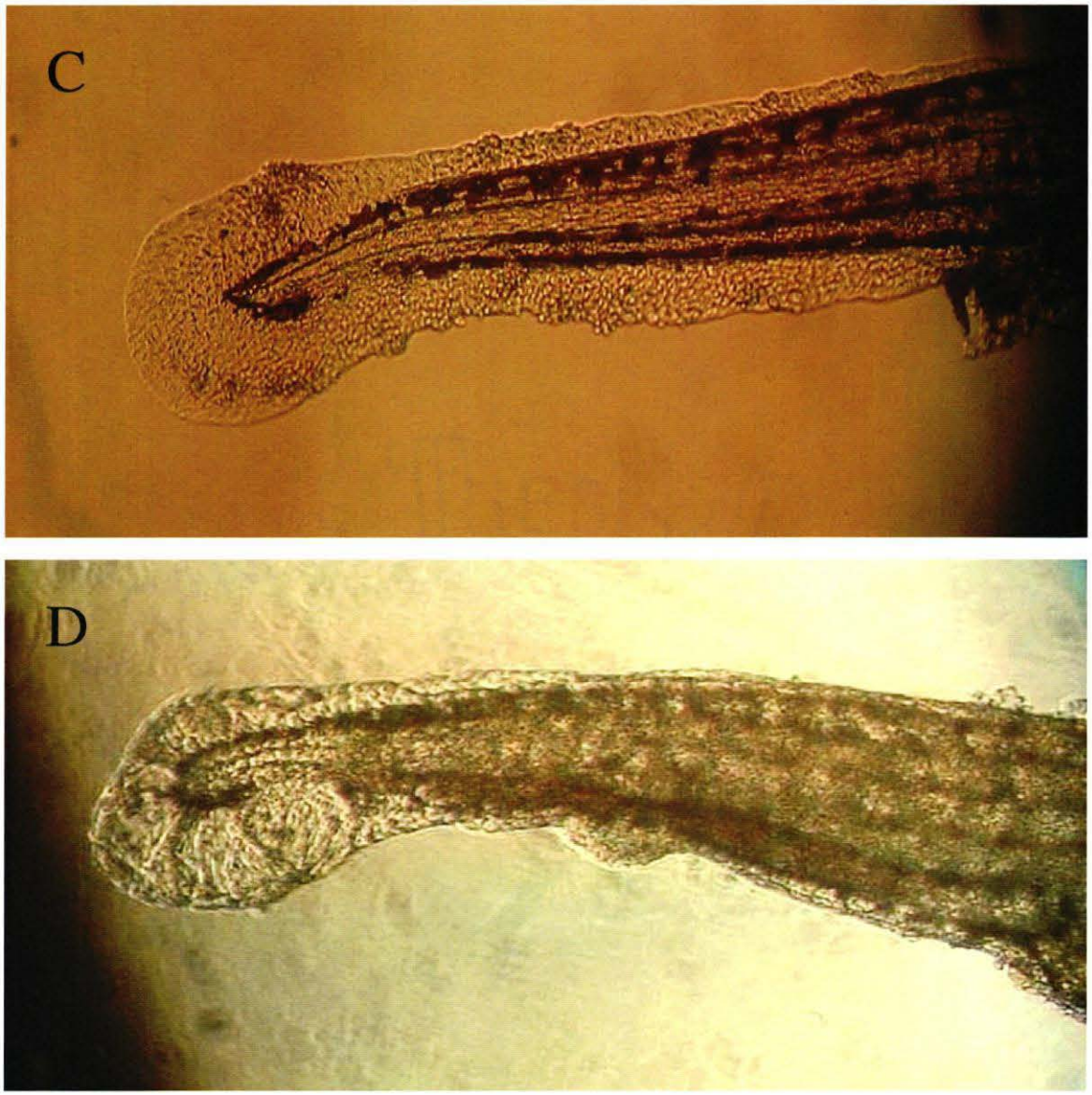

Fig. 1. (*Continued*)

As_2O_5. Embryos exposed to 0.005 mg/L As_2O_5 showed no level of abnormal finfold development. An increasing percentage of abnormal finfold development occurred in combined CCA treated embryos: 28.6% in 0.005 mg/L CCA, 33.3 % in 0.010 mg/L CCA, 100.0% in 0.025 mg/L CCA, and 100.0% in 0.100 mg/L CCA. Abnormal finfold development was never observed in control embryos. Embryos exposed to 0.025 mg/L

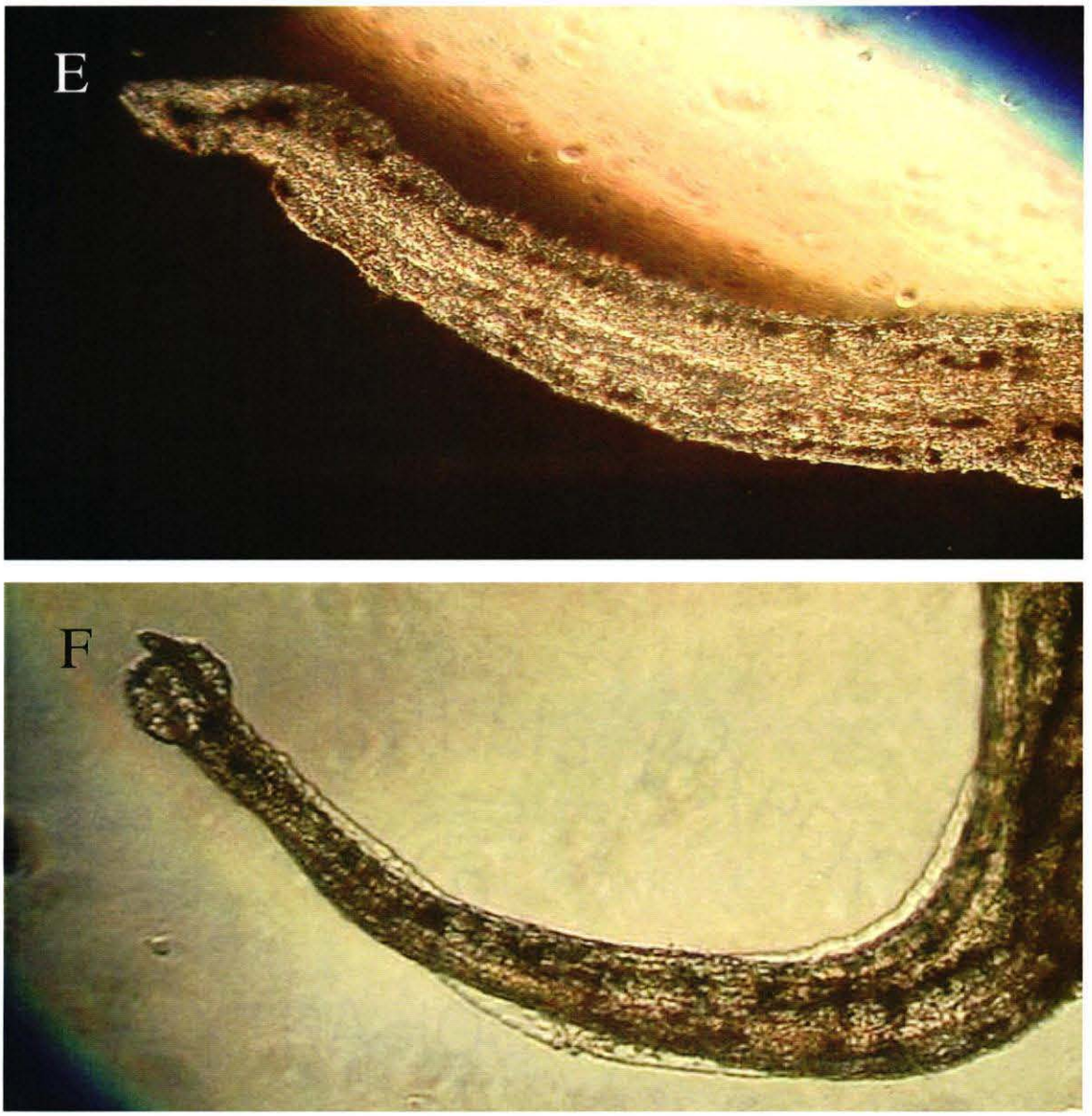

Fig. 1. (*Continued*)

CrO_3 produced a finfold abnormality of 2 at a frequency of 45.8% (Fig. 2). Embryos exposed to 0.100 mg/L CrO_3 also produced a finfold abnormality of 2 at a frequency of 59.3% (Fig. 2). Embryos that were exposed to 0.500 mg/L CrO_3 produced finfold abnormalities of 3, 4, and 5 (Fig. 2). Embryos exposed to 1.000 mg/L CrO_3 also showed that there were no embryos rated 0 (Fig. 2). This suggests that as the concentration of

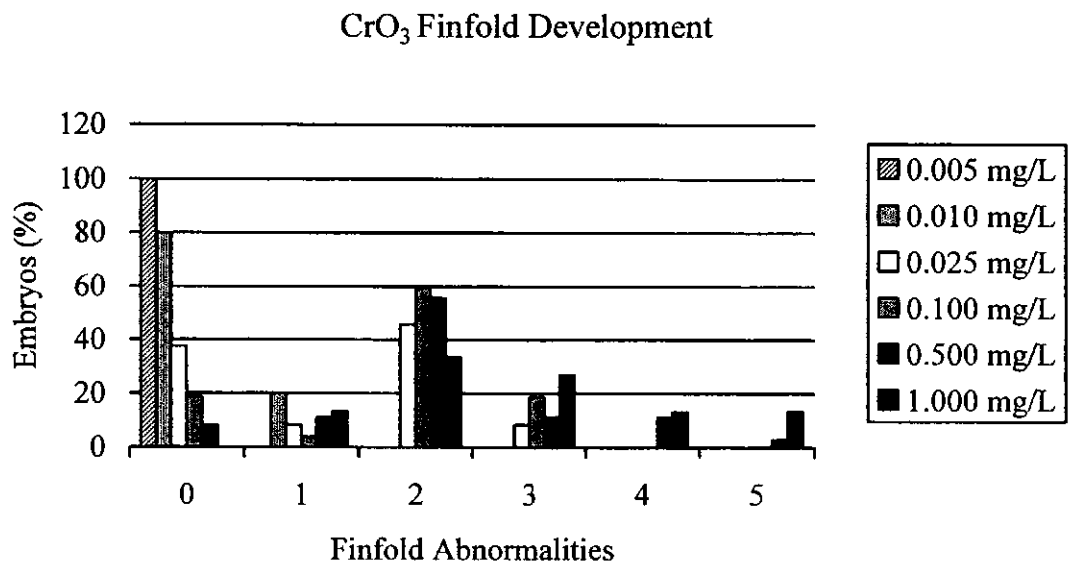

Fig. 2. Histogram showing the frequency of finfold abnormalities for CrO_3 treatment: as the concentration increases there is an increase in the amount of and severity of finfold abnormalities.

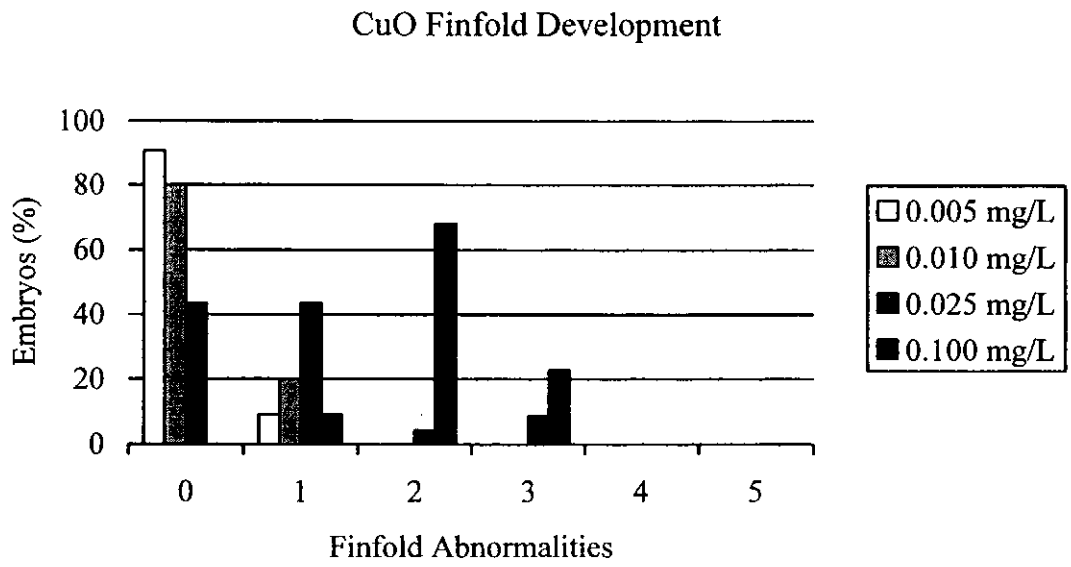

Fig. 3. Histogram showing the frequency of finfold abnormalities for CuO treatment: as the concentration increases there is an increase in the amount of and severity of finfold abnormalities.

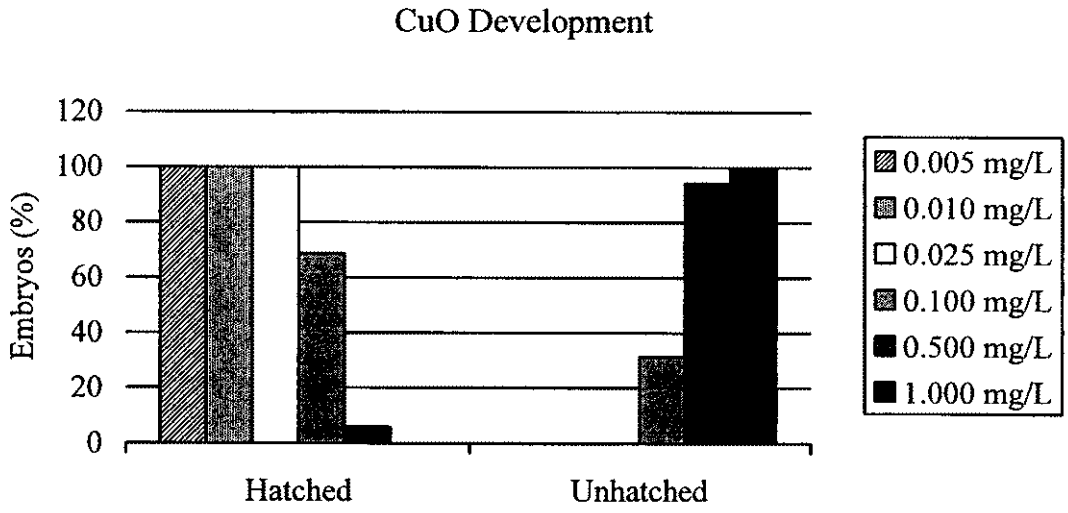

Fig. 4. Histogram showing the frequency of hatching for CuO treatment: as the concentration increases there is an increase in the amount of unhatched embryos.

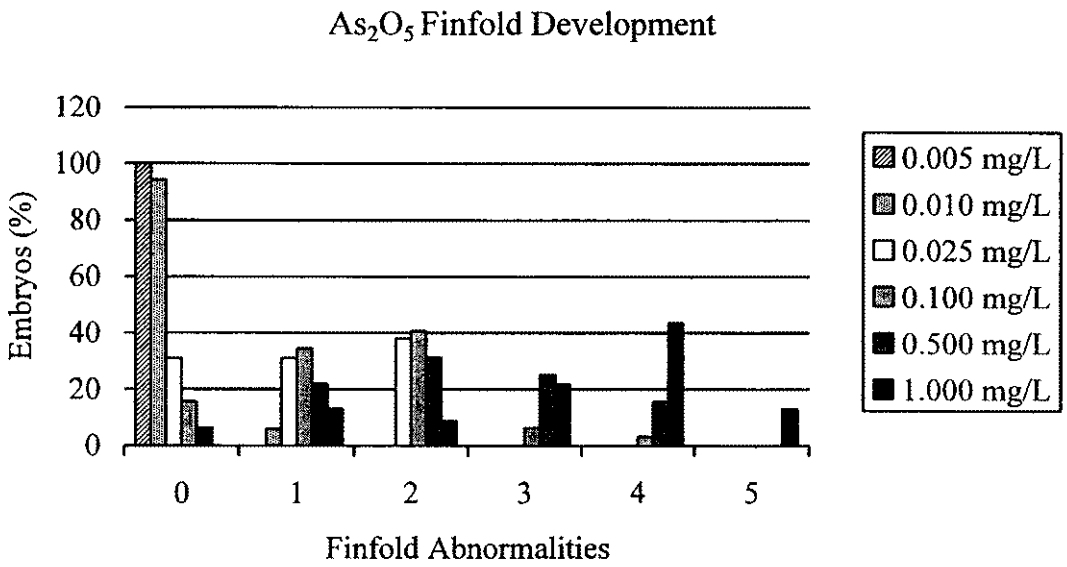

Fig. 5. Histogram showing the frequency of finfold abnormalities for As_2O_5 treatment: as the concentration increases there is an increase in the amount of and severity of finfold abnormalities.

CrO_3 was increased that there was a significant increase in the amount of finfold abnormalities. Embryos that were exposed to 0.025 mg/L CuO produced a finfold abnormality of 1 at a frequency of 43.5% (Fig. 3). Embryos exposed to 0.100 mg/L CuO produced a finfold abnormality of 2 at a frequency of 68.2% (Fig. 3). Embryos exposed to 0.500 and 1.000 mg/L CuO did not hatch and thus were not rated. In CuO as the concentration was increased the embryos would not hatch from the chorion and those that did hatch showed a significant increase in the amount of finfold abnormalities (Fig. 4).

Embryos that were exposed to 0.025 mg/L As_2O_5 produced a finfold abnormality of 2 at a frequency of 38.0% (Fig. 5). Embryos that were exposed to 0.500 mg/L As_2O_5 produced a finfold abnormality rate of 3 and 4 (Fig.5). Embryos exposed to 0.100 mg/L As_2O_5 produced a finfold abnormality of 2 at a frequency of 40.6% (Fig. 5). Embryos exposed to 1.000 mg/L As_2O_5 produced a finfold abnormality of 4 at a frequency of 43.6% and no embryos rated 0 (Fig. 5). This suggests that as the concentration of As_2O_5 was increased there was a significant increase in the amount of finfold abnormalities that occurred. Embryos that were exposed to 0.025 mg/L CCA produced a finfold abnormality of 2 at a frequency of 45.5% and no embryos rated 0 (Fig. 6). Embryos exposed to 0.100 mg/L CCA produced a finfold abnormality of 4 at a frequency of 33.3% and no embryos rated 0 (Fig. 6). Embryos that were exposed to 0.500 mg/L and 1.000 mg/L CCA all died and 0.500 mg/L was determined to be the lethal dose. Thus, as the concentration of CCA increases finfold abnormalities significantly increase and mortality rates increase.

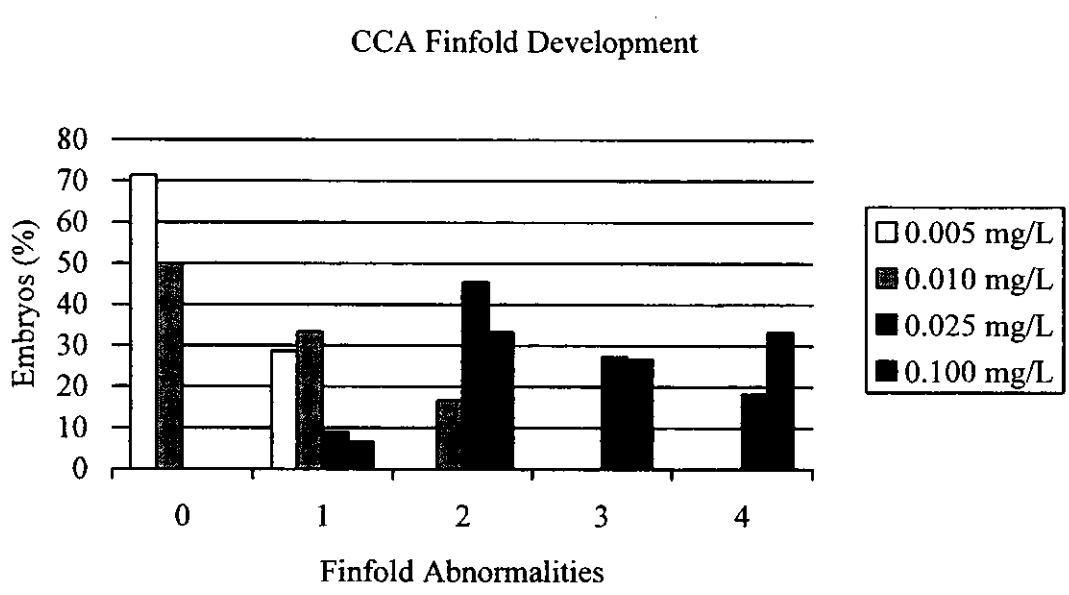

Fig. 6. Histogram showing the frequency of finfold abnormalities for CCA treatment: as the concentration increases there is an increase in the amount of and severity of finfold abnormalities.

An increasing mean finfold abnormality rate occurred in the chromium treated embryos: 0 in 0.005 mg/L CrO_3, 0.20 in 0.010 mg/L CrO_3, 1.25 in 0.025 mg/L CrO_3, 1.78 in 0.100 mg/L CrO_3, 2.14 in 0.500 mg/L CrO_3, and 2.80 in 1.000 mg/L CrO_3. Thus, as the concentration of chromium increases the defect severity increases (Fig. 7). An increasing mean finfold abnormality rate occurred in the copper treated embryos: 0.09 in 0.005 mg/L CuO, 0.20 in 0.010 mg/L CuO, 0.78 in 0.025 mg/L CuO, and 2.14 in 0.100 mg/L CuO. Therefore, as the concentration of copper increases the defect severity increases (Fig. 8). Copper treatment at 0.500 and 1.000 mg/L CuO inhibited hatching and thus could not be rated since only hatched embryos were rated in all treatment groups at all concentrations. An increasing mean finfold abnormality rate occurred in arsenic treated embryos: 0 in 0.005 mg/L As_2O_5, 0.06 in 0.010 mg/L As_2O_5, 1.07 in 0.025 mg/L As_2O_5, 1.47 in 0.100 mg/L As_2O_5, 2.22 in 0.500 mg/L As_2O_5, and 3.35 in 1.000 mg/L

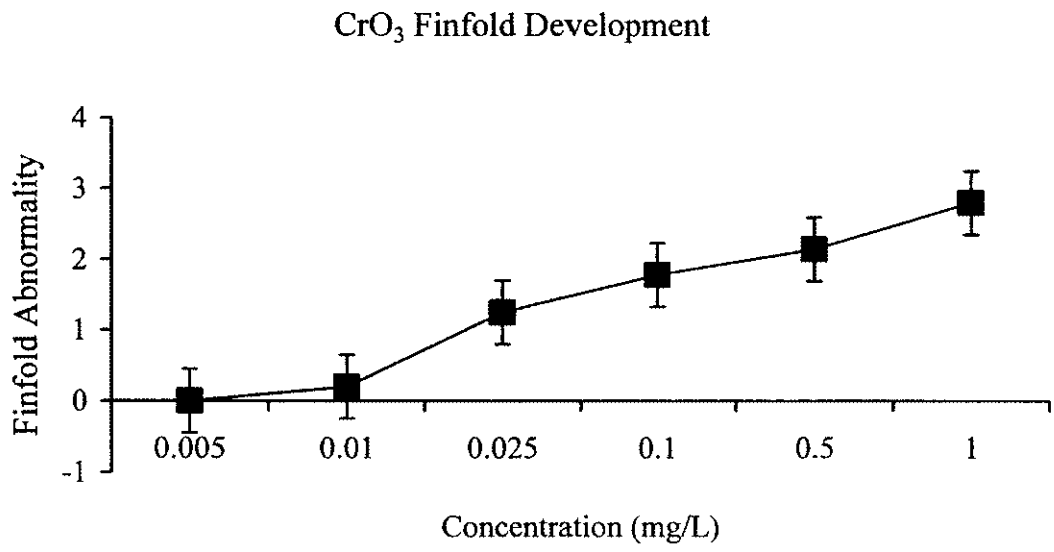

Fig. 7. Graph showing the mean ± standard error finfold abnormality rates for CrO_3 treatment: as the concentration increases there is an increase in the rate of finfold abnormalities.

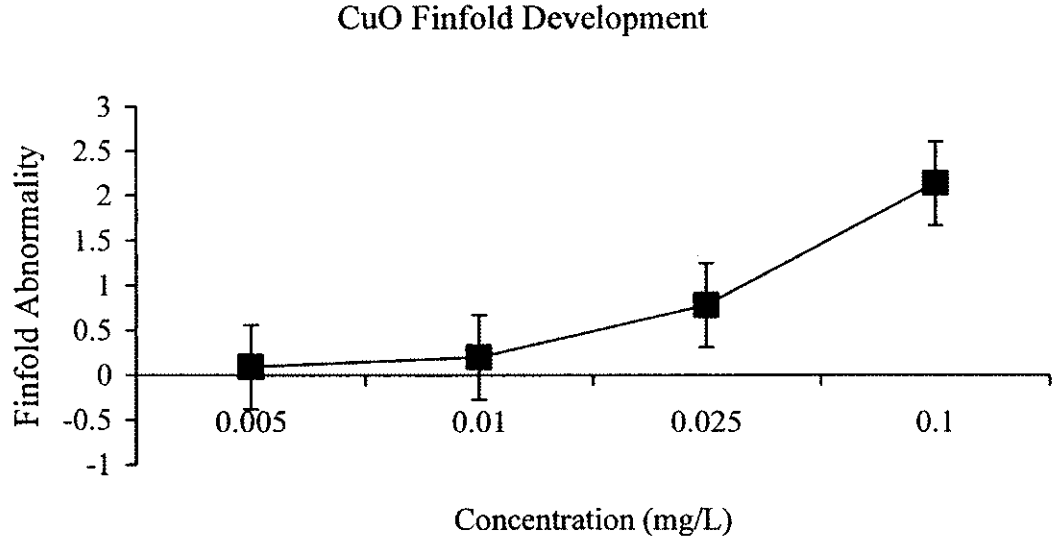

Fig. 8. Graph showing the mean ± standard error finfold abnormality rates for CuO treatment: as the concentration increases there is an increase in the rate of finfold abnormalities.

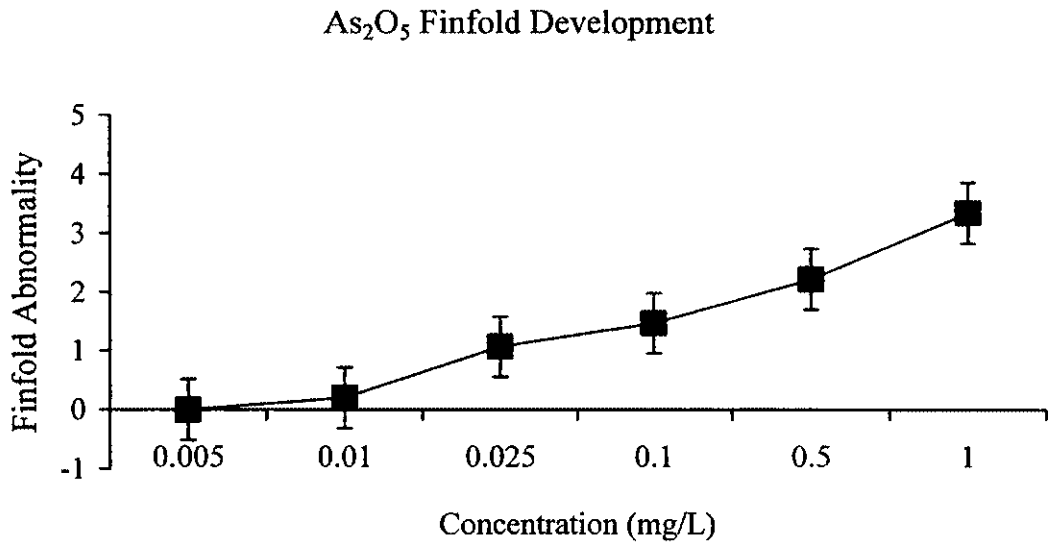

Fig. 9. Graph showing the mean ± standard error finfold abnormality rates for As_2O_5 treatment: as the concentration increases there is an increase in the rate of finfold abnormalities.

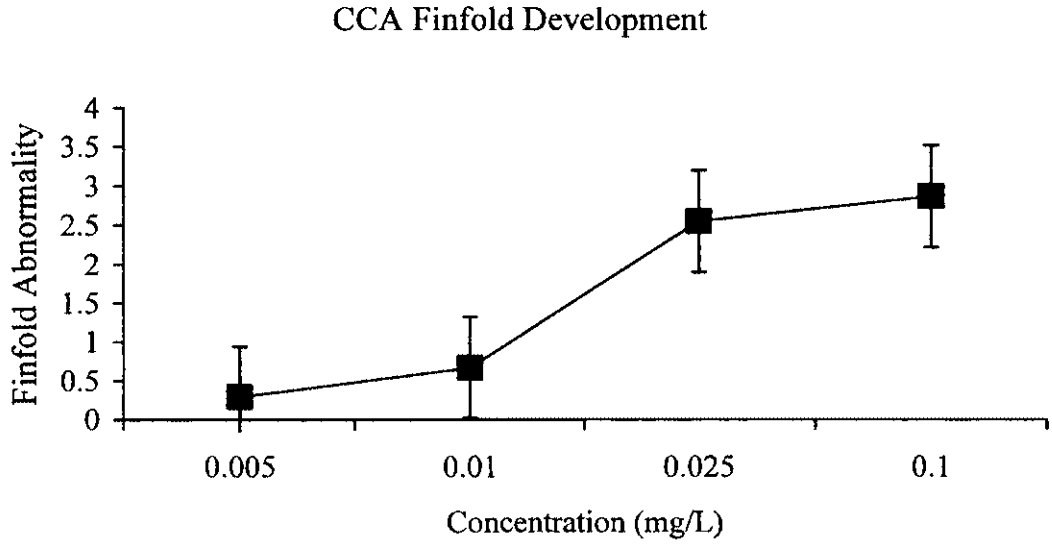

Fig. 10. Graph showing the mean ± standard error finfold abnormality rates for CCA treatment: as the concentration increases there is an increase in the rate of finfold abnormalities.

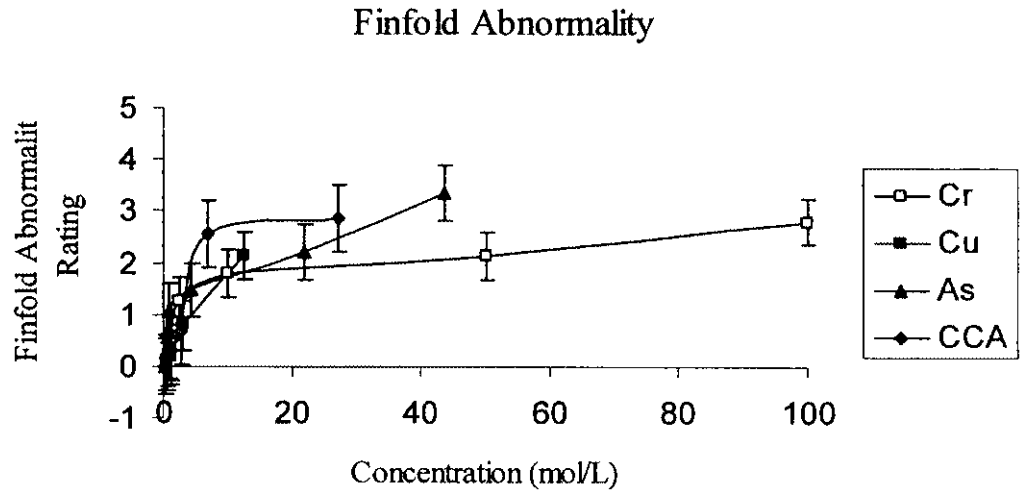

Fig. 11. Graph showing the mean ± standard error finfold abnormality for CrO_3, CuO, As_2O_5, and CCA treatment: as the concentration of each increases there is an increase in the rate of severity of finfold abnormality. The graph also shows that when all three components are together there is an increase in the overall mean finfold abnormality.

As_2O_5. Therefore, as the concentration of arsenic increases the defect severity increases (Fig. 9). An increasing mean finfold abnormality rate occurred in CCA treated embryos: 0.29 in 0.005 mg/L CCA, 0.67 in 0.010 mg/L CCA, 2.55 in 0.025 mg/L CCA, and 2.87 in 0.100 mg/L CCA. Therefore, as the concentration of CCA increases the defect severity increases (Fig. 10), and when all three components are added together the mean rate of finfold abnormality increases at the same concentrations as when the embryos were treated with the individual components of CCA (Fig. 11).

Tail flexures

The second defect that was observed during the exposure of *D. rerio* embryos to chromium, copper, arsenic, and CCA was a flexure of the tail region. When this second defect was observed it also showed tissue abnormalities of the finfold. In normal developing embryos the notochord and spinal cord develop straight to the posterior-most

Table 2
Rating in severity of morphological defects in tail flexure of 120 hpf *D. rerio* embryos after exposure to components of CCA and CCA

Rating	Description
0	Control, normal tail development (Fig. 12A)
1	Very slight flexure in the posterior-most tip of the tail (Fig. 12B)
2	C-shaped flexure of the posterior region of the tail (Fig. 12C)
3	More severe C-shaped flexure of the posterior region of the tail (Fig. 12D)
4	Entire tail region was strongly C-shaped and end was stubby (Fig. 12E)

tip of the tail (Fig. 12A). In the treated embryos however, the tail region was flexed to some extent. In the more severe rated embryos, the flexure was extreme and the overall length of the tail was stubby. With increasing concentrations the chromium, copper, arsenic, and CCA treated embryos displayed increasing defect rated on a qualitative scale (Table 2).

An increasing percentage of tail flexure occurred in chromium treated embryos: 54.2% in 0.025 mg/L CrO_3, 77.8% in 0.100 mg/L CrO_3, 80.6% in 0.500 mg/L CrO_3, and

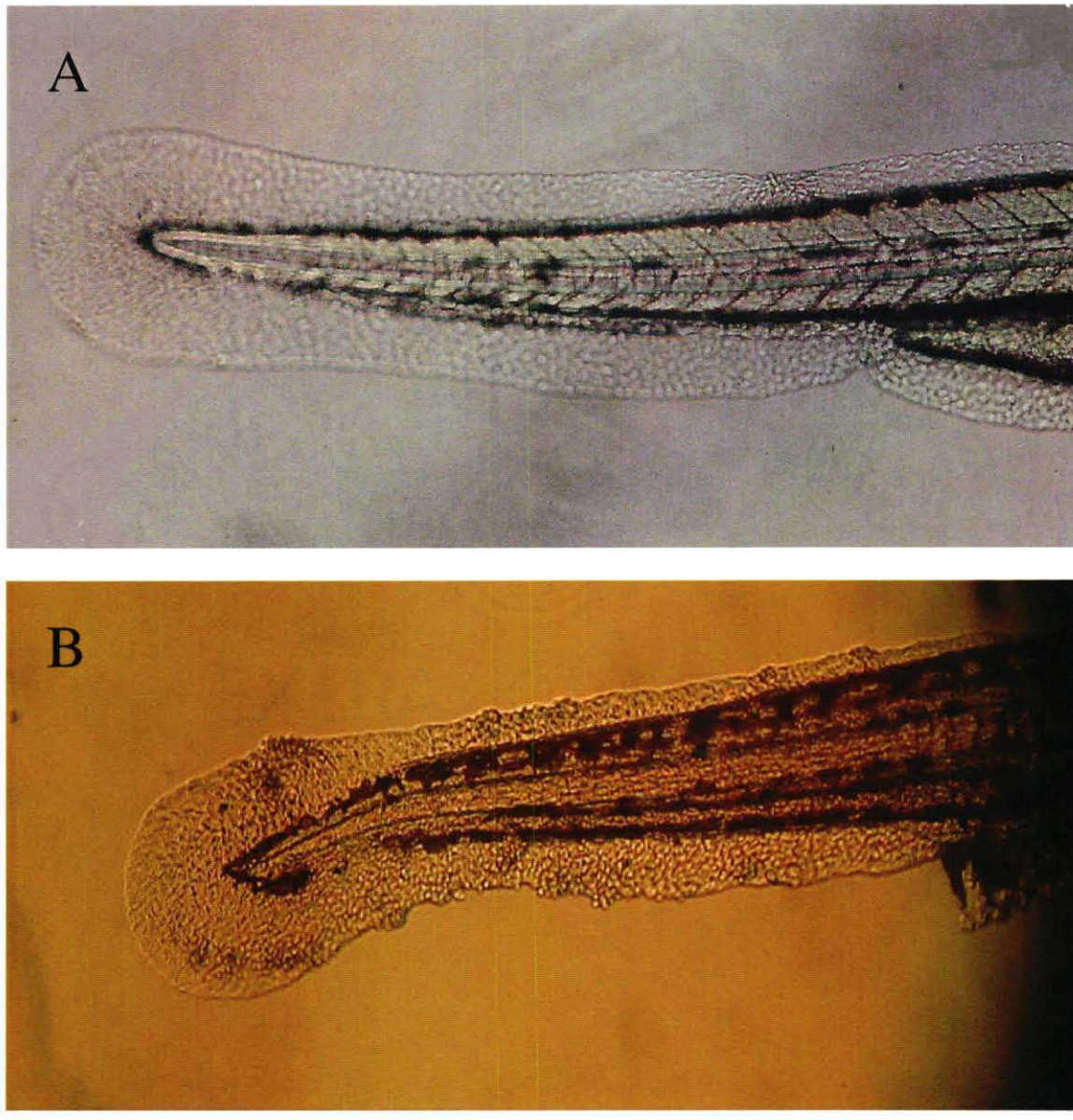

Fig. 12. Range in severity of morphological defects in tail region of 120 hpf *D. rerio* embryos after exposure to components of CCA and CCA. (A) Control, normal development: morphological score 0. (B) Slight flexure in the posterior-most tip of the tail: tail flexure score 1. (C) C-shaped flexure of the posterior tail region: tail flexure score 2. (D) More severe C-shape flexure of the posterior region of the tail: tail flexure score 3. (E) The entire tail region was strongly flexed and the end was stubby: tail flexure score 4.

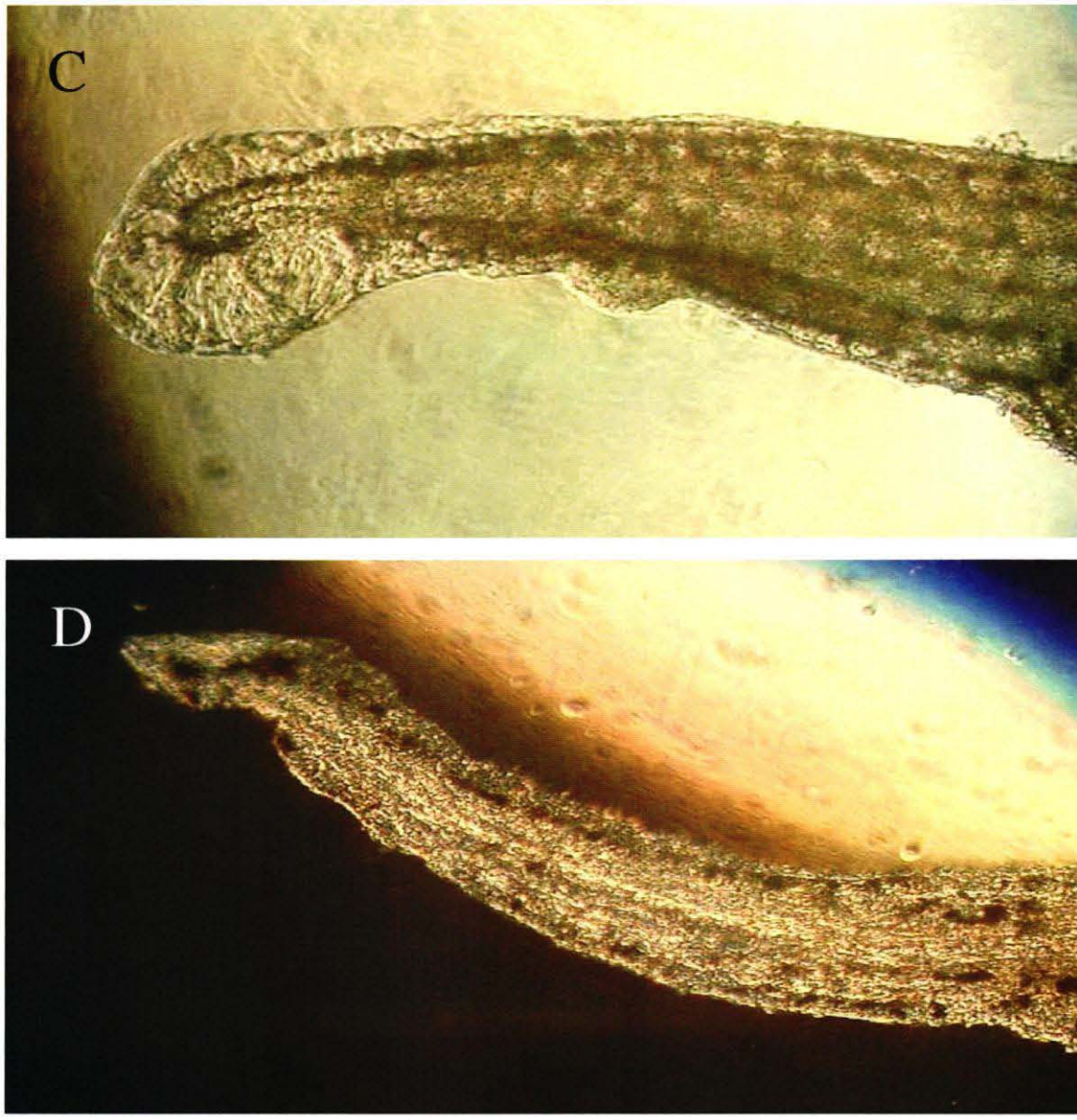

Fig. 12. (*Continued*)

86.6% in 1.000 mg/L CrO_3. Exposure to 0.005 mg/L and 0.010 mg/L CrO_3 produced no level of tail flexure. An increasing percentage of tail flexure occurred in copper treated embryos: 13% in 0.025 mg/L CuO and 90.9% in 0.100 mg/L CuO. Exposure to 0.005 mg/L and 0.010 mg/L CuO produced no level of tail flexure. An increasing percentage

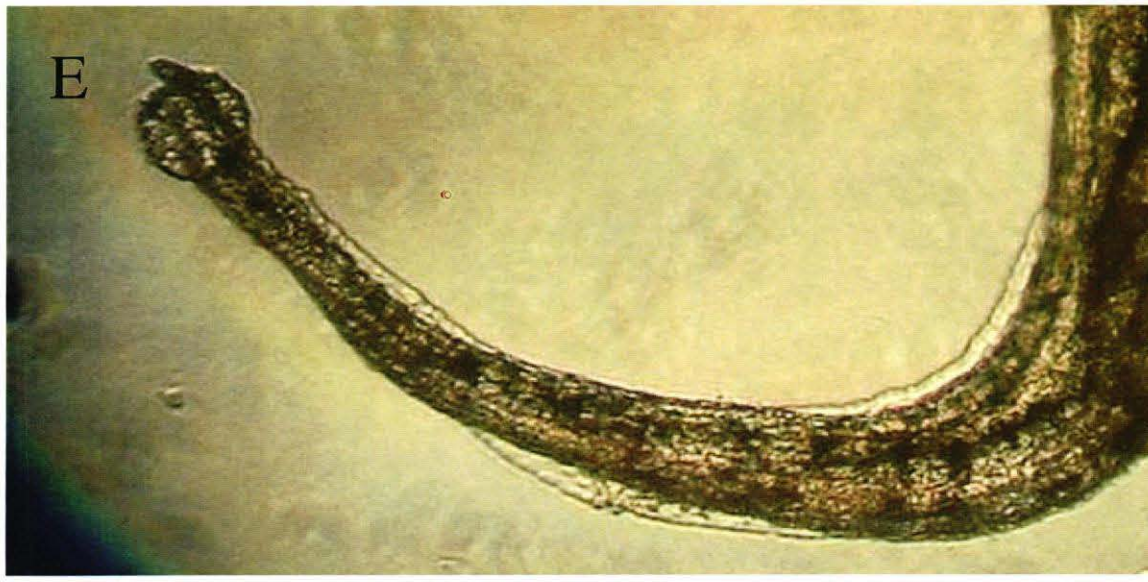

Fig. 12. (*Continued*)

of tail flexure occurred in the arsenic treated embryos: 37.9% in 0.025 mg/L As_2O_5, 50% in 0.100 mg/L As_2O_5, 71.9% in 0.500 mg/L As_2O_5, and 87% in 1.000 mg/L As_2O_5. Exposure to 0.005 mg/L and 0.010 mg/L As_2O_5 produced no level of tail flexure. An increasing percentage of tail flexure occurred in CCA treated embryos: 16.7% in 0.010 mg/L CCA, 91% in 0.025 mg/L CCA, and 93.3% in 0.100 mg/L CCA. Exposure to 0.005 mg/L CCA produced no level of tail flexure. Abnormal tail flexure was never observed in control embryos.

Embryos exposed to 0.025 mg/L CrO_3 produced a tail flexure of 1 at a frequency of 45.8% (Fig. 13). Embryos exposed to 0.100 mg/L CrO_3 produced a tail flexure severity of 1 at a frequency of 59.3% (Fig. 13). Embryos exposed to 0.500 mg/L CrO_3 produced tail flexure severities of 2, 3, and 4 (Fig. 13). Embryos exposed to 1.000 mg/L

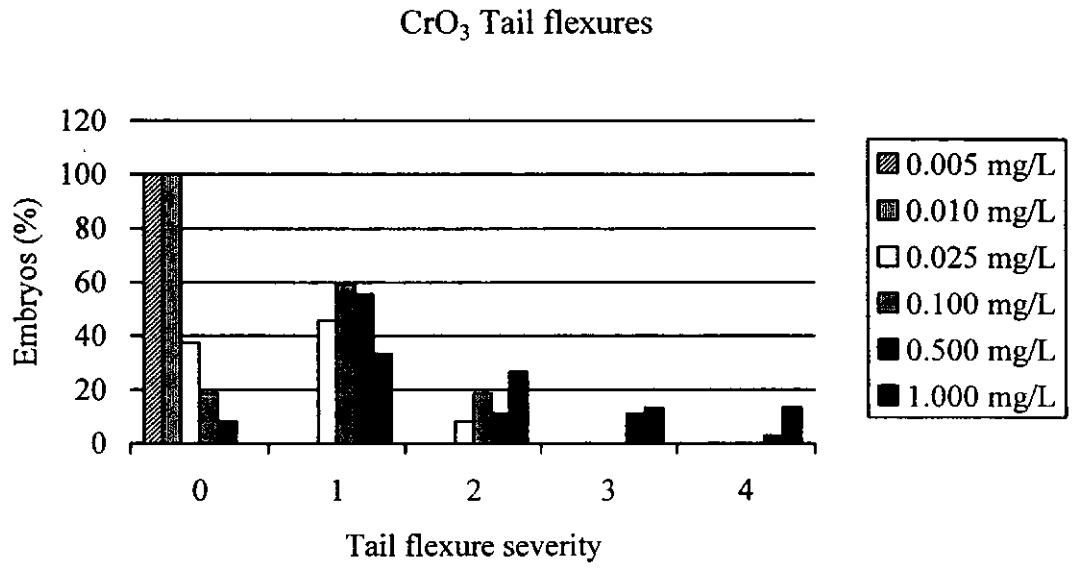

Fig. 13. Histogram showing the frequency of tail flexures for CrO_3 treatment: as the concentration increases there is an increase in the amount of and severity of tail flexures.

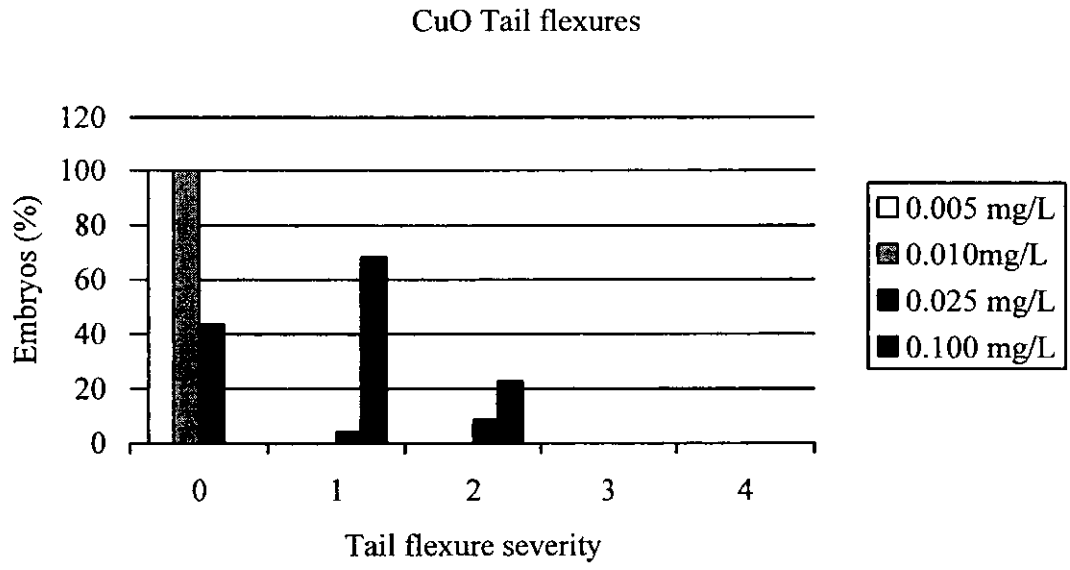

Fig. 14. Histogram showing the frequency of tail flexures for CuO treatment: as the concentration increases there is an increase in the amount of and severity of tail flexures.

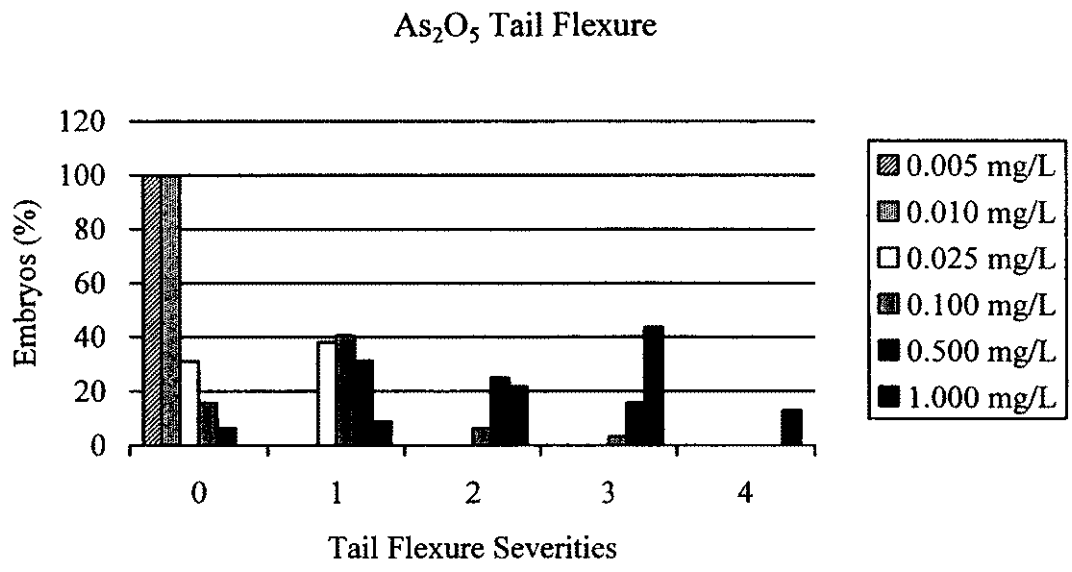

Fig. 15. Histogram showing the frequency of flexures for As_2O_5 treatment: as the concentration increases there is an increase in the amount of and severity of tail flexures.

CrO_3 also showed a significant increase in the amount of tail flexures and showed there were no embryos rated 0 (Fig. 13). This shows that as the concentration of CrO_3 was increased, there was a significant increase in the amount of tail flexures that occurred. Embryos exposed to 0.025 mg/L CuO produced a tail flexure severity of 2 at a frequency of 8.7% (Fig. 14). Embryos exposed to 0.100 mg/L CuO produced a tail flexure severity of 1 at a frequency of 68.2% (Fig. 14). Embryos exposed to 0.500 and 1.000 mg/L CuO inhibited hatching and thus could not be rated since only hatched embryos were rated in all treatment groups at all concentrations. In the CuO treatment groups as the concentration was increased the embryos would not hatch from the chorion and those that did hatch showed a significant increase in the amount of tail flexures. Embryos exposed to 0.025 mg/L As_2O_5 produced a tail flexure severity of 1 at a frequency of 38.0% (Fig. 15). Embryos exposed to 0.100 mg/L As_2O_5 produced a tail flexure severity of 1 at a

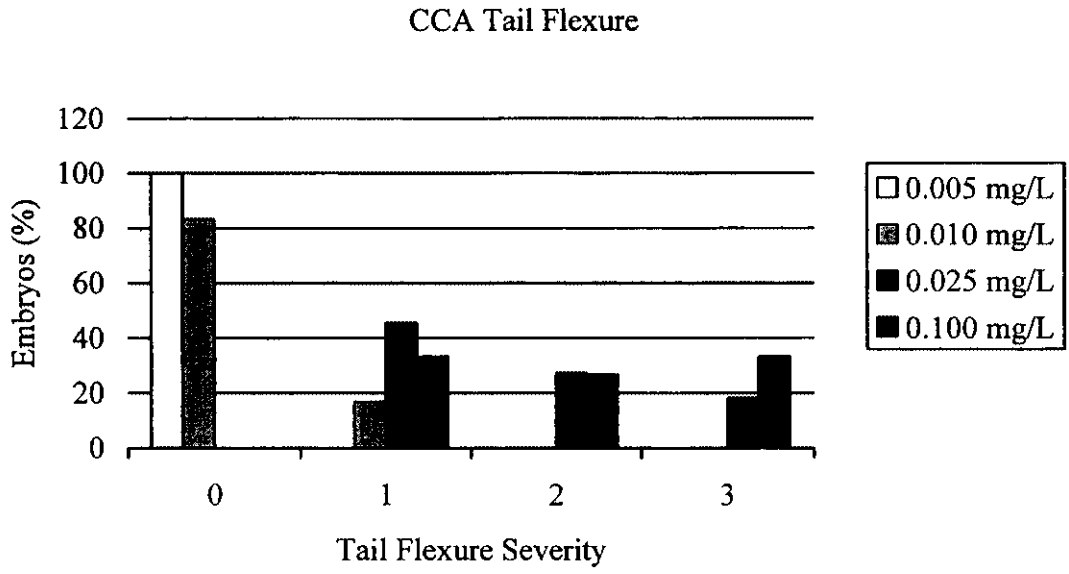

Fig. 16. Histogram showing the frequency of tail flexures for CCA treatment: as the concentration increases there is an increase in the severity of tail flexures.

frequency of 40.6% (Fig. 15). Embryos exposed to 0.500 mg/L As_2O_5 produced a tail flexure severity of 2 and 3 (Fig. 15). Embryos exposed to 1.000 mg/L As_2O_5 produced a tail flexure severity of 3 at a frequency of 43.6% and no embryos were rated 0 (Fig. 15). This suggests that as the concentration of As_2O_5 was increased there was a significant increase in the amount of tail flexures that occurred. Embryos exposed to 0.025 mg/L CCA produced a tail flexure severity of 1 at a frequency of 45.5% and no embryos rated 0 (Fig. 16). Embryos exposed to 0.100 mg/L CCA produced a tail flexure severity of 3 at a frequency of 33.3% and there were no embryos that rated 0 (Fig. 16). Embryos exposed to 0.500 mg/L and 1.000 mg/L CCA were all killed and 0.500 mg/L was determined to be the lethal dose. Thus as the concentration of CCA increases tail flexures significantly increase and mortality rates increase.

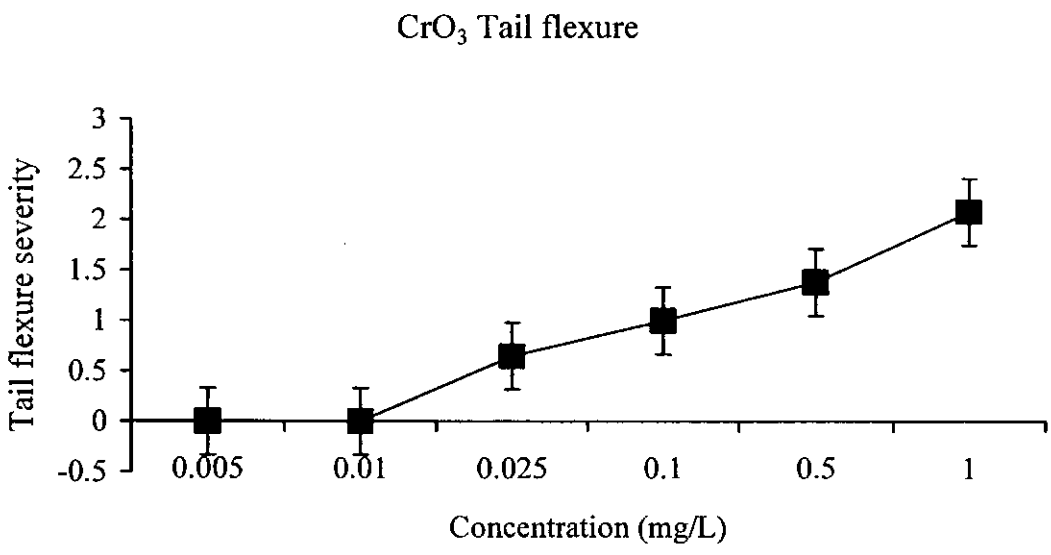

Fig. 17. Graph showing the mean ± standard error tail flexure rates for CrO_3 treatment: as the concentration increases there is an increase in the rate of severity of tail flexures.

An increasing mean tail flexure severity occurred in chromium treated embryos: 0 in 0.005 mg/L CrO_3, 0 in 0.010 mg/L CrO_3, 0.65 in 0.025 mg/L CrO_3, 1 in 0.100 mg/L CrO_3, 1.38 in 0.500 mg/L CrO_3, and 2.08 in 1.000 mg/L CrO_3. Therefore as the concentration of chromium increases the defect severity increases (Fig. 17). An increasing mean tail flexure severity occurred in copper treated embryos: 0 in 0.005 mg/L CuO, 0 in 0.010 mg/L CuO, 0.39 in 0.025 mg/L CuO, and 1.25 in 0.100 mg/L CuO. Therefore as the concentration of copper increases the defect severity increases (Fig. 18). An increasing mean tail flexure severity occurred in arsenic treated embryos: 0 in 0.005 mg/L As_2O_5, 0 in 0.010 mg/L As_2O_5, 0.55 in 0.025 mg/L As_2O_5, 0.95 in 0.100 mg/L As_2O_5, 1.64 in 0.500 mg/L As_2O_5, and 2.70 in 1.000 mg/L As_2O_5. Therefore as the concentration of arsenic increases the defect severity increases (Fig. 19). An increasing

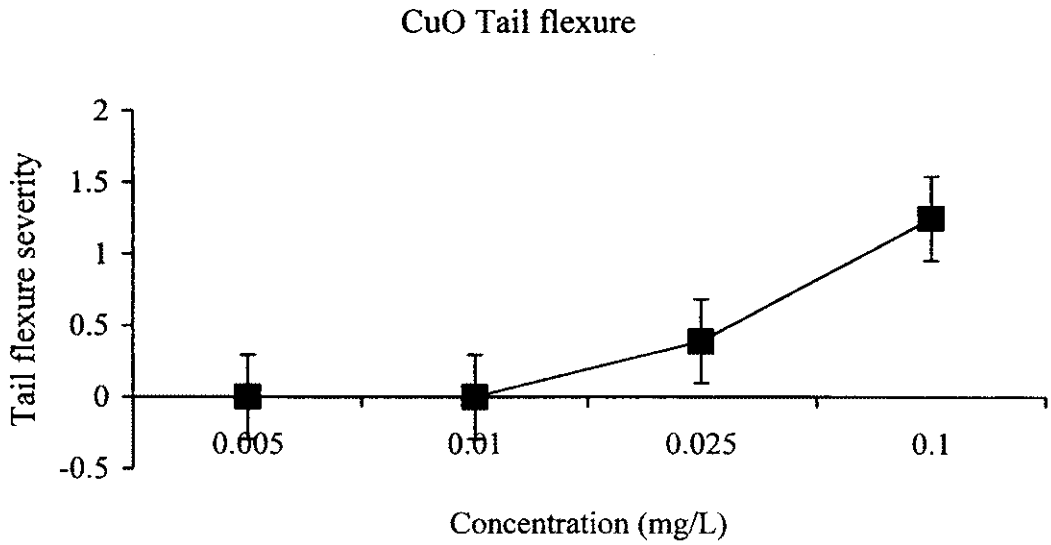

Fig. 18. Graph showing the mean ± standard error tail flexure rates for CuO treatment: as the concentration increases there is an increase in the rate of severity of tail flexures.

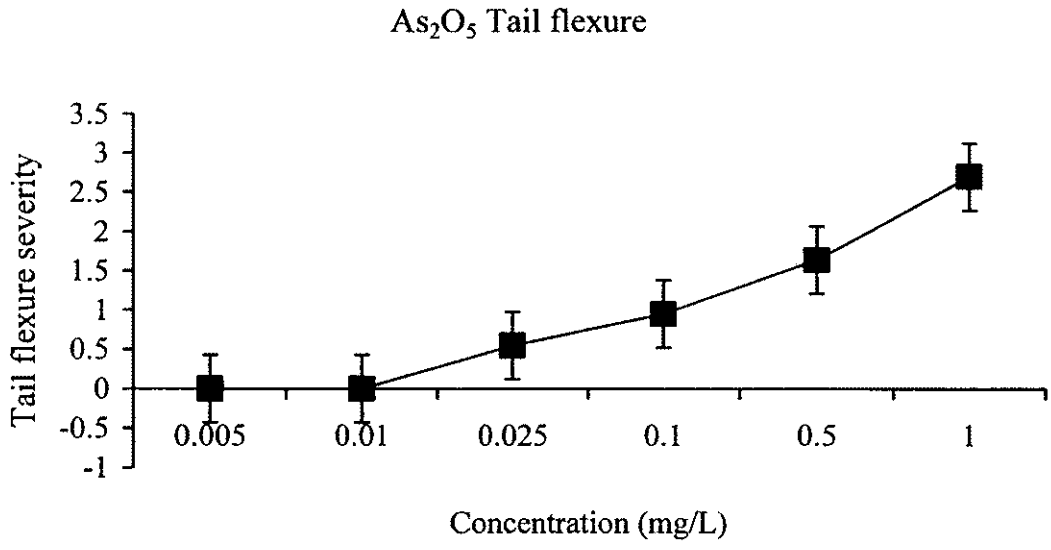

Fig. 19. Graph showing the mean ± standard error tail flexure rates for As_2O_5 treatment: as the concentration increases there is an increase in the rate of severity of tail flexures.

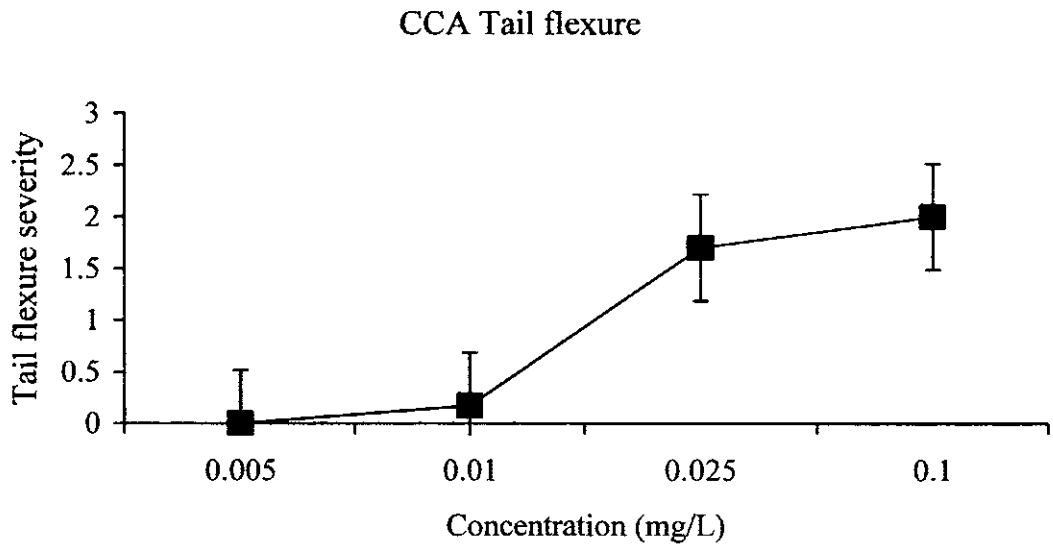

Fig. 20. Graph showing the mean ± standard error tail flexure rates for CCA treatment: as the concentration increases there is an increase in the rate of severity of tail flexures.

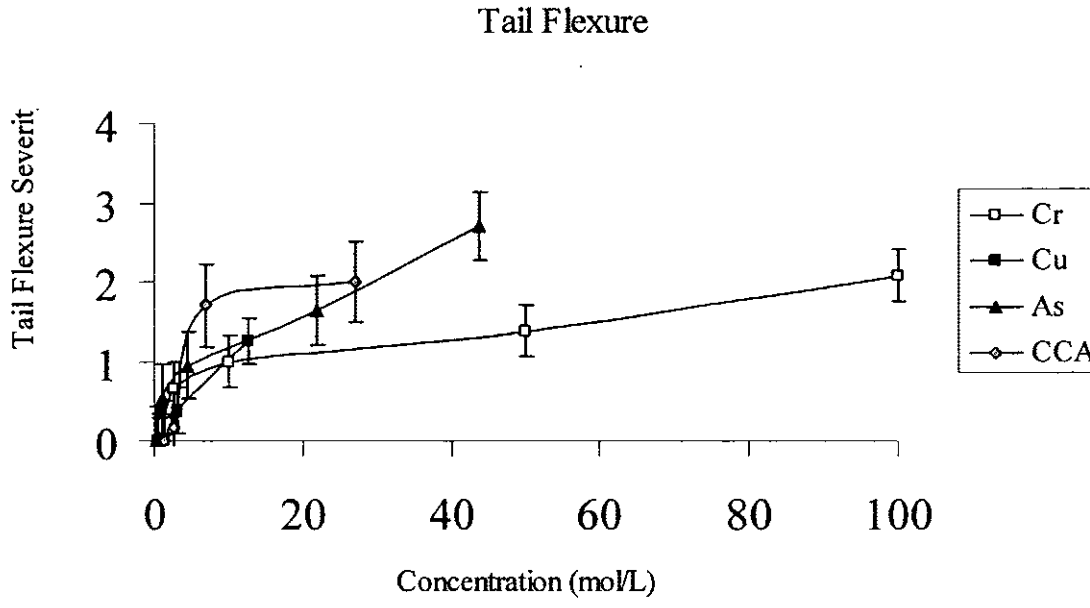

Fig. 21. Graph showing the mean ± standard error tail flexure for CrO_3, CuO, As_2O_5, and CCA treatment: as the concentration of each increase there is an increase in the rate of severity of tail flexure. The graph also shows that when all three components are together there is an increase in the overall mean tail flexure.

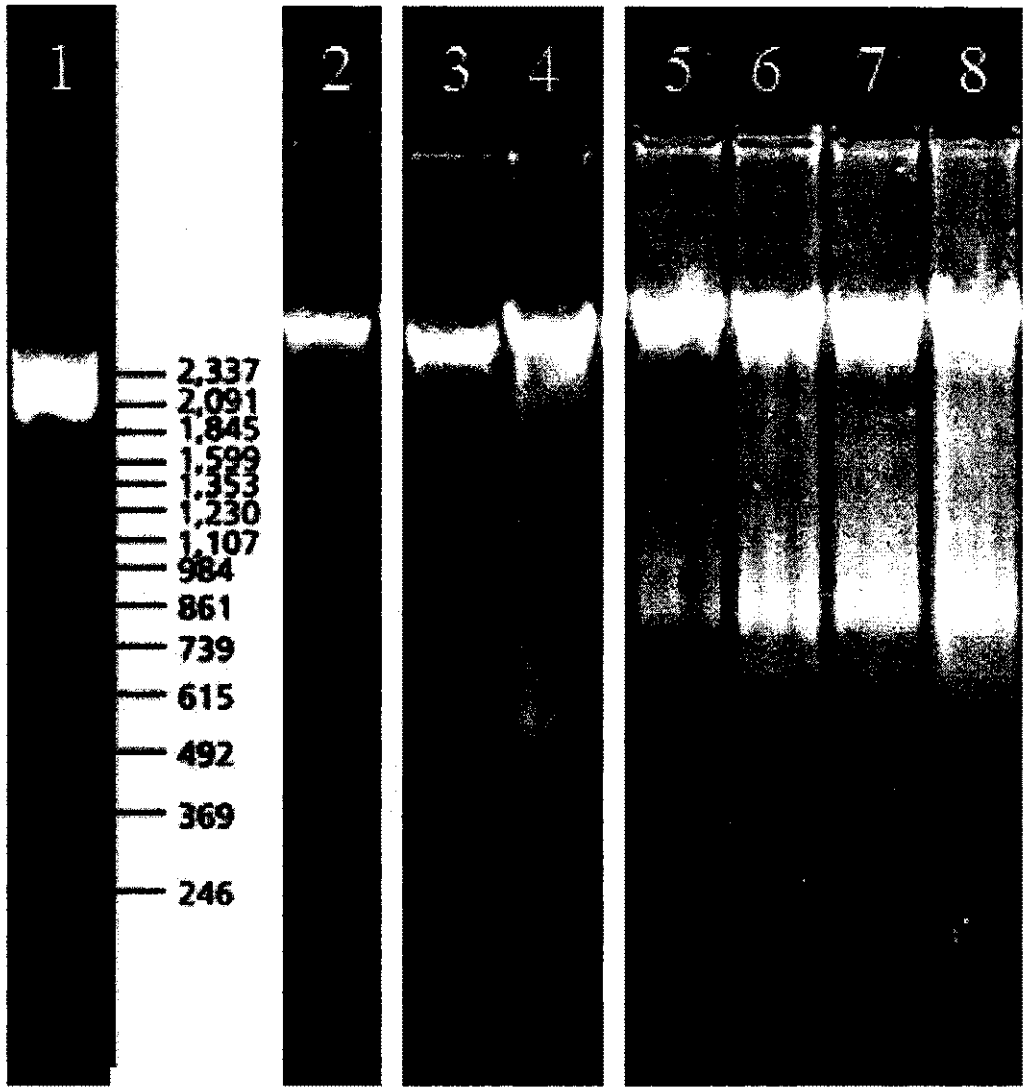

Fig. 22. The gel shows the effects of CrO_3 on DNA fragmentation of zebrafish embryos, 1.0% agarose gel electrophoresis. Lane 1 is 123 base pair DNA marker, lane 2 is control, lane 3 is 0.005 mg/L CrO_3, lane 4 is 0.010 mg/L CrO_3, lane 5 is 0.025 mg/L CrO_3, lane 6 is 0.100 mg/L CrO_3, lane 7 is 0.500 mg/L CrO_3, and lane 8 is 1.000 mg/L CrO_3.

mean tail flexure severity occurred in CCA treated embryos: 0 in 0.005 mg/L CCA, 0.17 in 0.010 mg/L CCA, 1.70 in 0.025 mg/L CCA, and 2 in 0.100 mg/L CCA (Fig. 20). Therefore as the concentration of CCA increases the defect severity increases and when all three components are added together the mean rate of tail flexure increases at the same

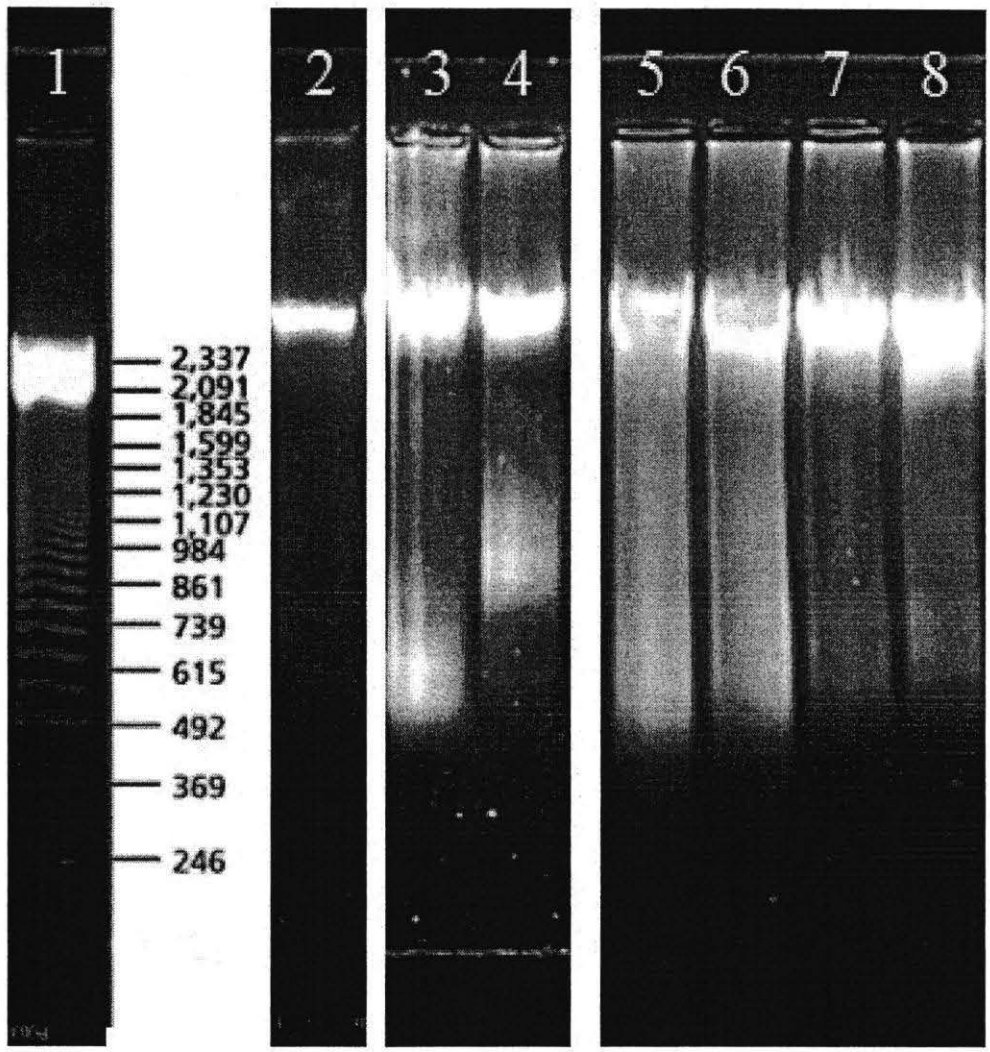

Fig. 23. The gel shows the effects of CuO on DNA fragmentation of zebrafish embryos, 1.0% agarose gel electrophoresis. Lane 1 is 123 base pair DNA marker, lane 2 is control, lane 3 is 0.005 mg/L CuO, lane 4 is 0.010 mg/L CuO, lane 5 is 0.025 mg/L CuO, lane 6 is 0.100 mg/L CuO, lane 7 is 0.500 mg/L CuO, and lane 8 is 1.000 mg/L CuO.

concentrations as when the embryos were treated with the individual components of CCA (Fig. 21).

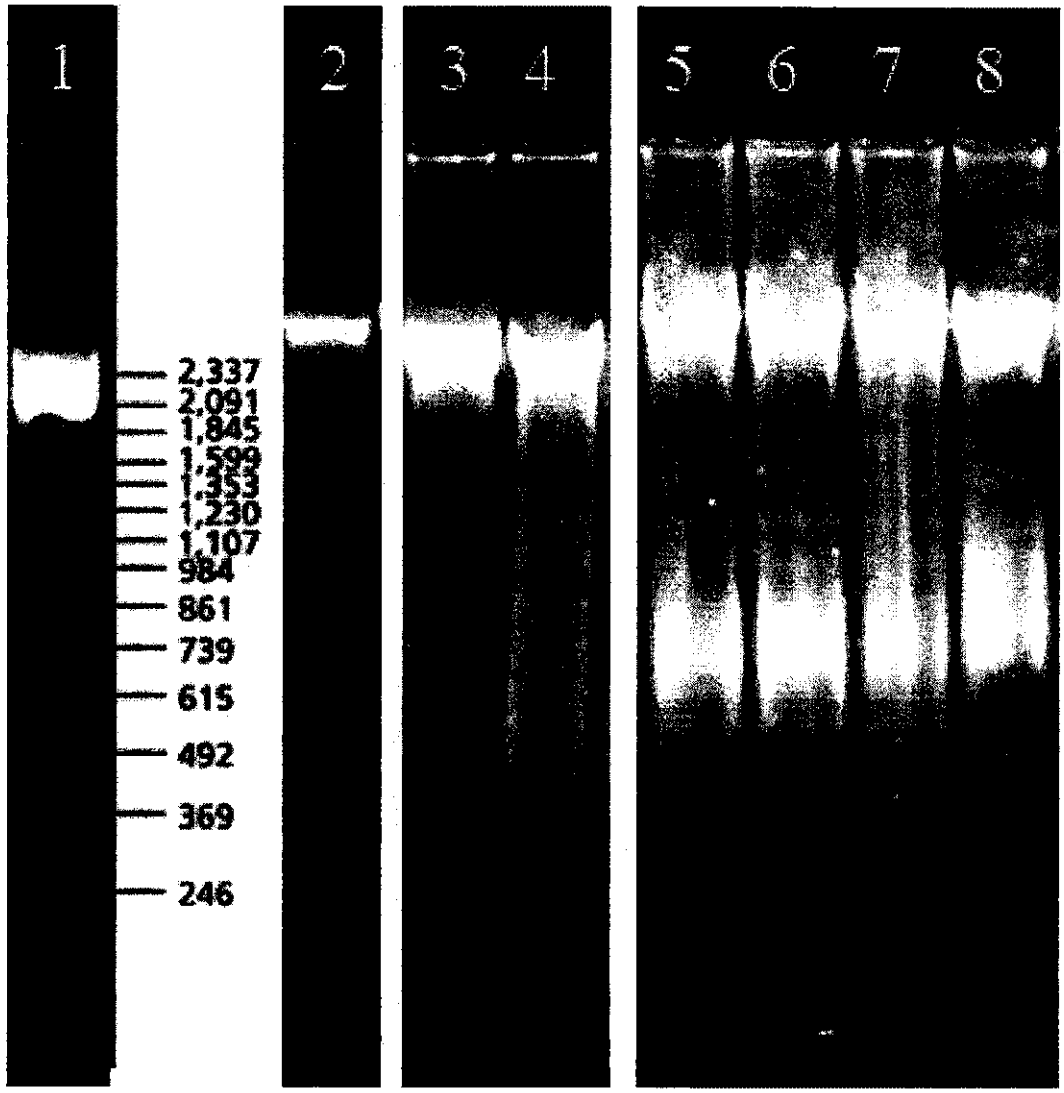

Fig. 24. The gel shows the effects of As_2O_5 on DNA fragmentation of zebrafish embryos, 1.0% agarose gel electrophoresis. Lane 1 is 123 base pair DNA marker, lane 2 is control, lane 3 is 0.005 mg/L As_2O_5, lane 4 is 0.010 mg/L As_2O_5, lane 5 is 0.025 mg/L As_2O_5, lane 6 is 0.100 mg/L As_2O_5, lane 7 is 0.500 mg/L As_2O_5, and lane 8 is 1.000 mg/L As_2O_5.

Detection of fragmented DNA by agarose gel electrophoresis

DNA fragmentation was observed in all concentrations of CrO_3, CuO, As_2O_5, and CCA. The fragmentation was noticeable with all treatment groups and it appeared that

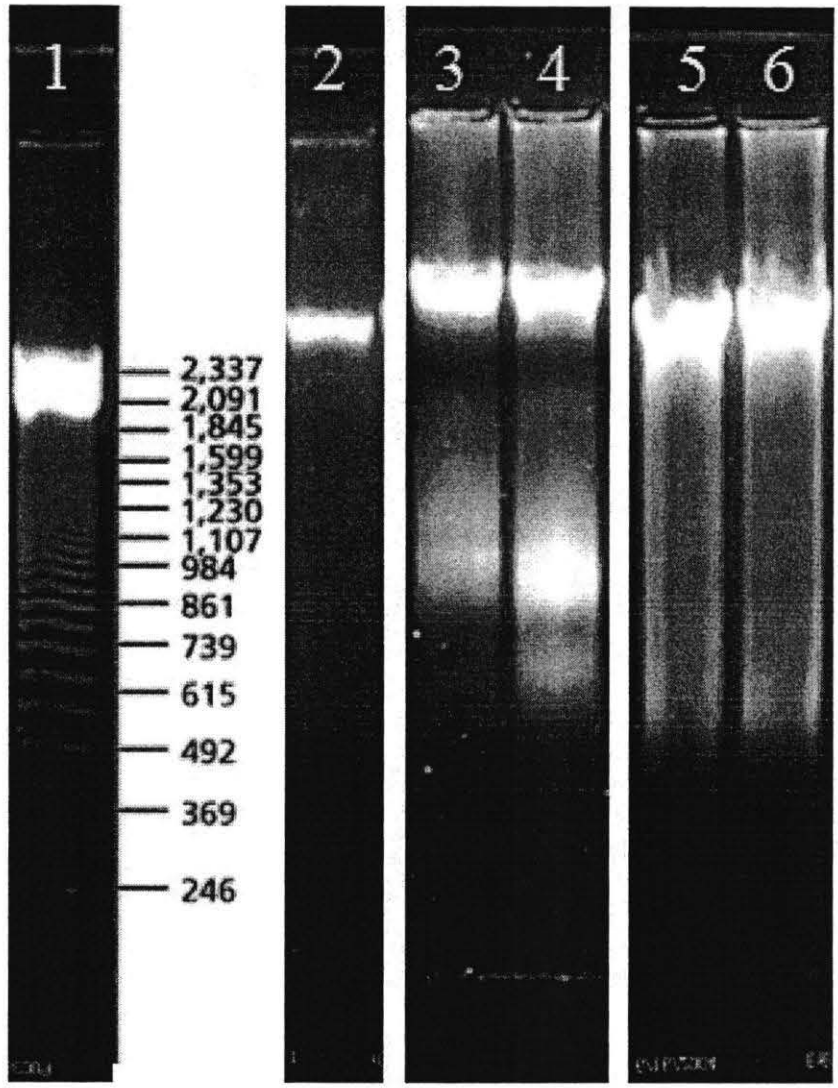

Fig. 25. The gel shows the effects of CCA on DNA fragmentation of zebrafish embryos, 1.0% agarose gel electrophoresis. Lane 1 is 123 base pair DNA marker, lane 2 is control, lane 3 is 0.005 mg/L CCA, lane 4 is 0.010 mg/L CCA, lane 5 is 0.025 mg/L CCA, and lane 6 is 0.100 mg/L CCA.

the fragmentation was not dependent on increase in concentration indicating that the lowest dose used for the morphological study was already above the effective dose for DNA fragmentation. DNA fragmentation showing varying sizes ranging from 400 to 2000 base pairs were visible after gel electrophoresis was performed in all concentrations

of CrO_3, CuO, As_2O_5, and CCA (Fig. 22 - 25). No specific DNA fragments were detected.

SECTION IV

DISCUSSION

Chromated Copper Arsenate (CCA) is of great concern to aquatic organisms, even after the EPA banned the use of CCA. The reason being, CCA-treated docking structures and bulkheads in freshwater pond and lake systems will continue to remain in the environment for decades. The EPA has not mandated the removal of the treated structures from the environment even though recent studies have shown that when submerged in water, CCA has the ability to leach from the wood. Aquatic organisms sequester copper, chromium, and arsenic through direct uptake from the water (Cox, 1991). In this investigation *D. rerio* embryos proved to be a responsive organism for assessing the toxicity of CCA components and CCA. From the results, I concluded that the components of CCA and CCA cause morphological defects in developing zebrafish embryos and are harmful to the normal development of zebrafish. The results also indicated that there was DNA fragmentation, suggesting double stranded DNA (dsDNA) damage. This damage is not easily reversible and is likely related to the morphological changes in the embryo. The results also showed that even in small concentrations, chromium, copper, and arsenic caused evident damage to the dsDNA even when there was a lower occurrence of morphological defects.

Chromium (VI) compounds have been shown to be genotoxic, mutagenic, and carcinogenic in humans, rodents, and a variety of experimental organisms. The reduction of chromium (VI) by intracellular reductions is considered an important step in chromium (VI) carcinogenicity, although the molecular mechanism of this process is not well known (Cox, 1991). The intracellular reduction of chromium (VI) can produce many

reactive intermediates, including chromium (V), chromium (IV), thiyl radicals, hydroxyl radicals, carbon-based radicals, and complexes of chromium (III), the final stable oxidation state. Any of the aforementioned species could potentially target DNA. Numerous studies suggest the possible involvement of reactive oxygen species (ROS), particularly the hydroxyl radical, in chromium (VI) metabolism. The formation of ROS in cells may lead to oxidative stress and related activation of the oxidative-damage-inducible genes (Dubrovskayal, 1998).

Copper is an important component of many proteins, such as cytochrome C oxidase and superoxide dismutase, which are involved in continual cycle of cellular respiration and detoxification of ROS. A deficiency in copper can cause anemia, neutropenia, bone abnormalities, and malabsorption syndromes. Although copper is a vital element, many studies have demonstrated that copper in excess amounts is toxic (Zhai et al., 2000). There has been evidence of acute copper toxicity, for example when children have accidentally eaten CuSO4, which is a mixture commonly used on some crops to keep certain microorganisms off, or when food is prepared in pans that can leach high doses of copper. Chronic copper toxicity is typically marked by the development of liver cirrhosis, along with occurrences of hemolysis and damage to renal tubules, brain, and other organ systems. In addition, copper has been reported to induce the necrosis of liver, esophagus, and stomach, and to hinder the proliferation of mouse zygotes (Cox, 1991). The cytotoxicity of copper has been reported to cause apoptosis. Apoptosis is also referred to as programmed cell death or cellular suicide because this death results from the activation of set programs within the cell by a variety of stimulus. There are distinctive morphologic and biochemical changes that result from the initiation of

apoptosis including chromatin condensation, cytoplasmatic blebbing, and DNA fragmentation (Zhai et al., 2000).

Arsenic, a known human carcinogen, is a worldwide contaminant that is found in soil, water, and air (Cox, 1991). Arsenic is an uncoupler of mitochondrial oxidative phosphorylation that induces generation of ROS (De La Fuente et al., 2002).

Tissue abnormalities of the finfold

An increasing mean finfold abnormality rate occurred in the chromium treated embryos as observed in the results. Showing that as chromium concentration increases, the defect severity increases. An increasing mean finfold abnormality rate occurred in the copper treated embryos as observed in the results. Therefore, as copper concentration increases, the defect severity increases. Copper treatment at 0.500 and 1.000 mg/L CuO inhibited hatching and thus, could not be rated since only hatched embryos were rated in all treatment groups at all concentrations. An increasing mean finfold abnormality rate occurred in arsenic treated embryos as observed in the results. Therefore, as arsenic concentration increases, the defect severity increases. An increasing mean finfold abnormality rate occurred in CCA treated embryos as shown in the results. Therefore, as the concentration of CCA increases the defect severity increases, and when all three components are added together the mean rate of finfold abnormality increases at the same concentrations as when the embryos were treated with the individual components of CCA.

Studies dealing with large range zebrafish mutagenesis screens have identified hundreds of genes controlling embryonic development. One such gene is the *spadetail* (*spt*) gene. This gene was found by mutation and is thought to control cell fate and

morphogenesis of lateral mesodermal cells, especially the somatic muscle cells (Amacher, 1999). The *spt* gene could possibly be one of the genes that are affected by the components of CCA or combined CCA and causing a morphological change in the development of the finfold region. However, to know indisputably, more research should be performed.

Tail flexures

An increasing mean tail flexure severity occurred in chromium treated embryos as shown in the results. This finding suggests that as the chromium concentration increases, the defect severity increases. An increasing mean tail flexure severity occurred in copper treated embryos as observed in the results. Therefore as the copper concentration increases, the defect severity increases. An increasing mean tail flexure severity occurred in arsenic treated embryos. Therefore as the arsenic concentration increases, the defect severity increases. An increasing mean tail flexure severity occurred in CCA treated embryos. Therefore as the CCA concentration increases, the defect severity increases and when all three components are added together the mean rate of tail flexure increases at the same concentrations as when the embryos were treated with the individual components of CCA.

Experiments investigating zebrafish mutagenesis screens have identified several factors affecting the control of embryonic development. A recent study of a zebrafish promoter region known as, *sonic hedgehog* (*shh*), using zebrafish assay systems found the identification of supposed regulatory factors of this gene. The *shh* promoter in transgenic species revealed that this region directly expressed the floor plate and the notochord (Amacher, 1999). The components of CCA and combined CCA could be

affecting the *shh* promoter region causing the morphological findings of abnormal tail flexure. However, more research is needed to confirm this assumption.

Detection of fragmented DNA by agarose gel electrophoresis

The genotoxicity of the components of CCA and CCA has been confirmed because it induced DNA fragmentation. DNA fragmentation is an indicator of dsDNA damage, which can be irreversible damage of the molecular material due to the difficulty to repair. According to Rand (1995) the cellular metabolism of genotoxic substances leads to the formation of highly reactive oxygen intermediates. These free intermediates can undergo attack via nucleophilic centers in macromolecules such as lipids, proteins, DNA, and RNA, which can often be the reason for cellular toxicity. Interaction of these free intermediates with the DNA is marked by structural alterations in the DNA and can take the form of strand breakage. DNA fragmentation was observed in all components of CCA and CCA. The fragmentation was noticeable with all treatment groups and it appeared that the fragmentation was not dependent upon the concentration. All concentrations appeared to affect the DNA equally.

Cells have a built-in cell death program, apoptosis, which protects organisms from potentially damaging cells (Yamashita, 2003). Apoptosis occurs under a wide range of physiological and pathological conditions, such as normal cell turnover, withdrawal of growth factors, and immune-mediated cytotoxicity. Although apoptosis is very important in the homeostasis of the normal functioning system, this type of cell death can be also associated with disease (Mitchelmore and Chipman, 1998). The activation of apoptosis is another possibility for the DNA fragmentation that was observed because this fragmentation has been found to be one of the determining factors in the programmed cell

death pathway. In addition, studies have found that a growing number of agents induce apoptosis in cells, such as chemotherapeutic drugs, irradiation, ethanol, mercury, and copper (Zhai et al., 2000).

Apoptosis can be characterized by fragmentation of the nucleus with condensed chromatin as well as extensive membrane blebbing. However, the most reliable biochemical marker for apoptosis is double-strand cleavage of nuclear DNA (Zhai et al., 2000). The experiment outline in this work demonstrated internucleosomal DNA fragmentation, which could be related to chromium, copper, arsenic, and combined CCA induced apoptosis. Interestingly, in micro amounts, copper has been shown to bind to DNA and enhance DNA damage in several biologic systems. Recently, it has been reported that copper can induce internucleosomal DNA fragmentation which has resulted from directed attack by ROS (Zhai et al., 2000).

Chromium has also been shown to be toxic to a variety of organisms due to the reduction of chromium (VI) by intracellular reactions, although the molecular mechanism of this process is not well known (Cox, 1991). The intracellular reduction of chromium (VI) can produce many reactive intermediates, as stated earlier, and any of these species could potentially target the DNA. As was true with copper, many findings suggest the possible involvement of ROS, particularly the hydroxyl radical, in chromium (VI) metabolism. The formation of ROS in cells may lead to damage in DNA (Dubrovskayal, 1998).

Arsenic toxicity to living species results at a molecular level, because the toxic arsenic mimics phosphate ions, which are an important part of the respiratory process. Arsenic takes the place of phosphate ions and then proceeds to rapidly break down

instead of performing the usual function that the phosphate ion would have performed (Cox, 1991). Arsenic is also a generator of ROS, which may cause damage to DNA (De La Fuente et al., 2002).

Further investigation of the molecular mechanism of apoptosis induced by chromium, copper, arsenic, and combined CCA is likely to provide novel insight into the mechanism involved in the process. In future gel electrophoresis of the genomic DNA, I would like to run a positive and negative control to ensure that the DNeasy kit chemicals are not reacting with the metallic oxides causing the DNA fragmentation but that the treatment concentration is the determining factor. Also I would like to perform terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay to locate specific sites of DNA fragmentation and areas of apoptotic events. I would also like to run a caspase-3 kit looking for this specific executioner caspase to confirm apoptosis induction. Another future endeavor would be to examine micro array assays to pinpoint various genes affected in the treatment process.

SECTION V

CONCLUSIONS

Embryonic exposure of *D. rerio* to sublethal concentrations of chromium, copper, arsenic, and complete CCA caused a variety of morphological defects including head edema, abnormal finfold development, tail flexures, and cardiac malformations. The two types of morphological defects that were most consistent were abnormal finfold development and tail flexure. The genotoxicity of CCA components and complete CCA was confirmed by induction of DNA fragmentation.

Tissue abnormalities of the finfold

An increasing mean finfold abnormality rate occurred in the chromium, copper, arsenic, and combined CCA treated embryos as observed in the results. Showing that as chromium, copper, arsenic, and combined CCA concentration increases, the defect severity increases. When all three components are added together the mean rate of finfold abnormality increases at the same concentrations as when the embryos were treated with the individual components of CCA.

Tail flexures

An increasing mean tail flexure severity occurred in chromium, copper, arsenic, and combined CCA treated embryos as shown in the results. This finding suggests that as the chromium, copper, arsenic, and combined CCA concentration increases, the defect severity increases. When all three components are added together the mean rate of tail flexure increases at the same concentrations as when the embryos were treated with the individual components of CCA.

Detection of fragmented DNA by agarose gel electrophoresis

The genotoxicity of the components of CCA and CCA has been confirmed because it induced DNA fragmentation. DNA fragmentation was observed in all components of CCA and CCA. The fragmentation was noticeable with all treatment groups and it appeared that the fragmentation was not dependent upon the concentration. All concentrations appeared to affect the DNA equally.

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