Trabajo Original | Toxicología Experimental

Haemotoxicity activity caused by acrilamide on the peripheral erythrocytes of ZEBRAFISH *(Danio rerio).*

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Abstract

Acrylamide (ACR) is a 2 alkene monomeric reagentused to synthesize polymers for industrial application. Evaluating its haematological toxicity action has been a preponderant objective of worldwide concern since the chemical was detected in human umbilical cord blood. In this work, its toxicity (ACR) in adult zebrafish (*Danio rerio*) peripheral blood was tested maintaining the fishes during 8 days in a 0.50 mM ACR LC_{50} and normal controls. Haemoglobin (Hb) analysis was performed by cellulose acetate electrophoresis.The activity ofδ-aminolevulinic acid dehydratase (ALAD) determination by spectrophotometric method was used as a toxicity biomarker. DNA analysis of nucleated erythrocytes in blood extensions contrasted with Feulgen staining was also performed. All these data were processed through the Fiji-ImageJ program platform and the GraphPad Prims statistic-7. The controls, from the peripheral blood electrophoretic profiles showed two main bands of globin, and one secondary minor band. Otherwise, those treated with ACR showed one main globin band and three minor bands, in the electrophoresis. The ALAD enzyme activity showed a 20% reduction compared with the controls. The erythrocytes presented nuclear DNA fragmentation in 80% of the analysed erythrocyte population, with respect to the control erythrocyte population. The haematocrit evaluation was higher in peripheral blood treated with ACR. The current study showed that ACR altered the structure of peripheral blood Hb, probably through the generation of ACR-Hb and / or ACR-GB type adducts, which may be expressed in the weakest bands found. In addition, it was possible to demonstrate that ACR can inhibit the enzymatic activity of ALAD, serving as a possible biomarker of haemotoxicity. The preceding was also accompanied by an apoptosis process evidenced through Feulgen staining. In conclusion, the ACR has haemotoxic activity in addition to the well-known neurotoxicity already reported, which could jeopardize the activity of oxygen supply in the blood and compromise processes in human foetal development.

Key words: *Apoptosis, Acrylamide, Erythrocytes, Haemoglobin adducts, Toxicity.*

Resumen

Actividad de hemotoxicidad causada por acrilamida en los eritrocitos periféricos de ZEBRAFISH (*Danio rerio*).

La acrilamida (ACR) es un reactivo monomérico de alqueno tipo 2 utilizado para sintetizar polímeros para aplicaciones industriales. La evaluación de su acción de toxicidad hematológica ha sido un objetivo preponderante de preocupación mundial desde que se detectó el químico en la sangre del cordón umbilical humano. En este trabajo, se analizó su efecto (ACR) en sangre periférica de pez cebra adulto (Danio rerio) manteniendo los peces durante 8 días en un LC_{50} ACR 0,50 mM y controles normales. El análisis de hemoglobina (Hb) se realizó mediante electroforesis con acetato de celulosa. La actividad de la determinación de la deshidratasa del ácido δ-aminolevulínico (ALAD) por método espectrofotométrico se usó como un biomarcador de toxicidad. También se realizó un análisis de ADN de eritrocitos nucleados en extensiones de sangre en contraste con la tinción de Feulgen. Todos estos datos se procesaron a través de la plataforma del programa Fiji-ImageJ y la estadística GraphPad Prims-7. Los controles, de los perfiles electroforéticos de sangre periférica mostraron dos bandas principales de globina y una banda secundaria. De lo contrario, los tratados con ACR mostraron una banda de globina principal y tres bandas menores, en la electroforesis. La actividad de la enzima ALAD mostró una reducción del 20% en comparación con los controles. Los eritrocitos presentaron fragmentación de ADN nuclear en el 80% de la población de eritrocitos analizada, con respecto a la población de eritrocitos de control. La evaluación del hematocrito fue mayor en la sangre periférica tratada con ACR. El estudio actual mostró que ACR alteró la estructura de la Hb de sangre periférica, probablemente a través de la generación de aductos de tipo ACR-Hb y / o ACR-GB, que pueden expresarse en las bandas más débiles encontradas. Además, fue posible demostrar que ACR puede inhibir la actividad enzimática de ALAD, sirviendo como un posible biomarcador de hemotoxicidad. Lo anterior también fue acompañado por un proceso de apoptosis evidenciado a través de la tinción de Feulgen. En conclusión, el ACR tiene actividad

hemotóxica además de la conocida neurotoxicidad ya informada, lo que podría poner en peligro la actividad del suministro de oxígeno en la sangre y comprometer los procesos en el desarrollo fetal humano.

Palabras clave: *apoptosis, acrilamida, eritrocitos, aductos de hemoglobina, toxicidad.*

INTRODUCTION

In recent years, the toxicity of acrylamide (ACR)(CH2=CHC(O)NH2)studies has gained great interest mainly because its presence in carbohydrate-rich foods cooked at high temperatures and a recent concern about acrylamide existence in the peripheral umbilical cord blood of new-borns[1]. ACR is a type 2 alkene monomer, presented as a white crystalline powder, highly soluble in water and in a variety of organic solvents. Exposure of biological organisms by ACR has been considered, an environmental risk factor due to its high neurotoxic potential, demonstrated in clinical and experimental studies[2]. It should be noted that due to the hydrophilic nature of ACR and its main metabolite Glycidamide (GD) ($C_3H_5NO_2$), generallythe tertiary structure of proteins is highly sensitive to both molecules. The reaction of ACR and Glycidamide with N-terminal valine residues defines interactions that yield products called protein adducts. Recently, haemoglobin and human DNA adducts generated by ACR have been quantified[3,4]. These products, accompanied by other actions, at the biochemical and cellular level, have been suggested as biomarkers of one of the first routes of the ACR toxic action, which could suggest a possible haemotoxic activity, which has been little explored. In recent years, the zebrafish (*Danio rerio*) has been positioned as an animal model par excellence in toxicological studiesZeGlobalTox system[5].This animal model has complete functional systems, including the hematopoietic, even from early stages of development[6]. Particularly, the biosynthesis of haemoglobin (Hb) begins approximately 15 hours post-fertilization (hpf) and the erythrocytes, cells containing Hb, are present in the circulation after 24 hpf.This protein (Hb) has two alpha chains and two beta globin chains, found in erythrocytes, which greatly increases the oxygen capacity in the blood, allowing efficient

transport of this gas from the respiratory surfaces, such as the lungs, gills and skin, towards internal organs. The zebrafish erythrocytes are nucleated and oval cells that use aerobic metabolism, instead of anaerobic metabolism, to generate adenosine triphosphate.All these previous features make zebrafish blood tissue an ideal model in addressing the objective of the present investigation, which determined the toxic effect of ACR in peripheral blood of adult zebrafish, treated with a lethal concentration fifty (LC_{50}) of ACR

MATERIALS AND METHODS

Zebrafish Husbandry

Adultszebrafish both sexes from wild strains were used and maintained in our laboratory as described[7]. Fish were gathered from natural breeding, maintained at 27 °C and performed according to standard protocols[8]. All assays were carried out following animal use international protocols.

Ethical approval

This project was approved by Anatomical Institute(José Izquierdo) Ethical Committee of the Universidad Central de Venezuela (N°.26032018), done in accordance with the standard procedure described in the literature[9,10].

The ACR lethal concentration fifty (LC_{50})

The lethal concentration fifty (LC_{50}) of the ACR used was 0.85 mM, estimated at 30% and 15%,in accordance with the LC_{50} used in previous works[11,12,13]. Zebra fishes were exposed to the proposed concentrationsduring 8 days, in 2-liter fish tanks and in number of four individuals/fish tank, under conditions of adequate oxygenation and 27 ° C. The experimental treated and control fishes received food in flakes *ad libitum*. The experiments were performed in triplicate. The average weight of the animals, control and treated, was obtained after the daily change of the ACR solution.

Blood sample extractions from the adult zebrafish

After 8 days to be exposed to theLC_{50} ACR, the peripheral blood analysis of the population of control and treated fishes was carried out, collecting blood from the zebrafish under anaesthesia scheme, which consisted of the induction of hypothermia at a temperature of approximately 4 °C, for 3 to 6 s depending on the fish, until it did not respond to stimuli.Once anesthetized, a lateral incision in the region of the dorsal aorta, approximately 0.3 cm in length was made with a steel blade. When the blood began to flow out, it was gently aspirated with a micro pipette tip; depending on the fish, it is yielded a volume of approximately 5 to 20 µL.The blood collected by each specimen was placed in a previously heparinized Eppendorf cryotube, from which the sample was distributed, according to the volume required in the different bioassays.

Electrophoresis of haemoglobin from erythrocytes peripheral blood.

Haemoglobin electrophoresis was performed with controls erythrocytes from peripheral blood without prior treatment. The samples were loaded directly onto 60 x 76 mm Titan III-H cellulose acetate plates (Helena Laboratories), and subsequently the electrophoretic run was performed on the Consort EV265 equipment. Individual Hb specimens were defined by electrophoresis in 0.018 M/L Tris/0.1 M/L boric acid / 2 mM /L EDTA solution, pH, 8.4, for 1 h at 300 V. After running, the respective plates were revealed with 1% Ponceau S Red in 5% trichloroacetic acid, for 15 minutes. Then, several washes were performed in 5% acetic acid for plate clearance and photographic recording was performed under indirect light. The images obtained from the respective control, and treated sample gels were digitised and analysed with ImageJ Fiji. The respective visible bands were selected in a rectangular way, enclosing the peaks corresponding tothedescribed gel bands. Next, the density histogram that was expressed in units of pixels / µm2 and graph was estimated using the GraphPad Prims Program-7.

Evaluation of the δ-aminolevulinic acid dehydratase (ALAD) enzyme activity

The ALAD enzyme activity evaluation has been used as a biomarker of the possible haemotoxic effect of ACR. Firstly, the haematocrit was measured, since it is a required data for the enzymatic activity estimation. Once this value was obtained, 100 µL of zebrafish blood was subjected to rapid haemolysis using 1.4 mL of Triton X-100 reagent.

Then, to this mixture was added 1 mL of ALA substrate buffered, in citrate phosphate buffer, pH 6.65 and mixed vigorously. One mL from this mixture 1 mL (v/v) of N-ethylmaleimide in trichloroacetic acid (a deproteinising agent)was added and incubated at 38 °C for 1 h. Finally, both blank and sample were centrifuged for 10 min at 2000 g. Then,1.5 mL of Ehrlich reagent, which is the reaction developer, was added to 1.5 mL of the supernatant (blank and/or sample) and waits nearly 13 min, for colour development. After this period, the absorbance was read in the next 10 min at 555 nm, using distilled water to adjust the equipment to zero.

Peripheral blood smears

Peripheral blood smears of the control and treated fish were carried out by spreading a drop of blood 3-4 mm in diameter on the surface of a slide, in order to assess the morphology of the blood cells. The respective smears were fixed

RESULTS

Haemoglobin (Hb) of zebrafish erythrocytes exposed to ACR

The control electrophoresis profile showed two dense main and a third lower density bands(Fig. 1A). TheACR treated samples electrophoresis profile showed a main band and three minor bands (Fig. 1B).The run profiles showed this (Fig. 1', 1B').An analysis of the average density of the Hb bands of both groups was done.Itwas possible to estimate that there were significant differences in the density of the respective bands, when comparing controls and ACR treated (Fig. 2).

Activity of the δ-aminolevulinic dehydratase (ALAD) enzyme

The ALAD activity in control fish was 0.70 U/l, while the activity of this enzyme in fish ACR treated was 0.56 U/l. A 20% reduction was evidencing in the ALAD enzymatic activity.

Peripheral blood smears

The control smears showed oval red blood cells in perfect core/cytoplasm relationship and strongly contrasted (Fig. 3).The image analysis of the transformation from the

original to colour image (RGB in digital language) (Fig.3a), an 8 bit-white image (Fig.3a ') was showed. The nuclei were centred, with nuclear material compact (Fig.2a ''). On the other hand, although the nucleus/cytoplasm ratio was maintained, it showed an obvious hypochromic staining; the nuclei were highlighted with a manifest fragmentation of nuclear material (Fig. 3b'and 3b'').

DISCUSSION

In recent research, only the neurotoxic activity of ACR has been given importance, and little is known about the other harmful effects that it is capable of producing in different human and animal tissues[14,15]. Here, the haematotoxicityfoundwas characterised by a modification of the tertiary structure of Hb of peripheral blood of zebrafish (*Danio rerio*), generating possible Hb adducts.In this regard, the normal electrophoretic profile of Hb in three bands coincides with the results obtained by other authors[16]who have shown the same pattern of Hb separation in peripheral blood of adult zebrafish. The band pattern corresponds to different globins that permits to suggest that in addition to the α and β globin genes that have been isolated, other globins may be present. For instance, the so-called β A1-globin and β A2-globin, the amino acids at position 105 are arginine and lysine, respectively. This difference probably can explain the different migration patterns in Hb electrophoresis, as the authors have suggested[16]. The other bands may represent tetramers between other α-globin and β-globin chains.Concerning, the electrophoretic profile of the peripheral blood of adult zebrafish treated with ACR, it was evident that the high reactivity of the ACR and/or Glycidamide (GD), towards the N-terminal valine groups present in it, seems to have been conjugated to forming structures of lower density, such as those shown, transforming globin into complexes of ACR-Hb and GD-Hb type adducts respectively.Alternatively, the ACR was able to inhibit the enzymes δ-aminolevulinic dehydratase (ALAD) of erythrocytes, the enzymatic inhibition after exposure to ACR could be considered an effect biomarker of this type of alkene. This is very similar to the biomarker situation, in the well-known case of lead toxicity[17]. The

reduction in the enzymatic activity of ALAD, a regulating enzyme of Hb biosynthesis was evident. A decrease in the activity of the ALAD enzyme demonstrated in the presence of ACR, could be linked to its reactivity with sulfhydryl groups present in the catalytic site of the enzyme. The adduction mechanism probably inhibits its activity in a similar modusas reported in heavy metal envenomations, such as lead. Finally, it should be noted that the haematotoxicity induced by ACR, not only compromised the functionality of this Hb, but also the structure of the main Hb of theerythrocytes. In this sense, the erythrocytes hypochromia was evident as well as the DNA nuclear fragmentation of these nucleated cells, these results so far not previously reported by other authors. These findings could be associated with the high affinity of ACR for cysteine groups of macromolecules such as nuclear DNA, as some epidemiological studies of ACR intake[18]. have been described. A positive association between the intake of ACR in the diet and ovarian cancer risk when studying gen-acrylamide interactions for ovarian cancer risk has been proposed. The alteration of the cell nucleus could affect the production of adenosine triphosphate (ATP), which compromises the energy contribution and the functionality of the erythrocyte.Here it has been demonstrated that the ACR has haemotoxic activity, in addition to the well-known neurotoxicity already reported.

Fig. 1. Peripheral blood electrophoresis of adult zebrafish. A) Controls: two strongly dense main bands and one minor (arrows) B) Treated with ACR-CL50: a strong main band less dense and three weak (arrows). **Graphic profile of the respective.** Control (A) and ACR (B ') runs. ImageJ J Fiji program.

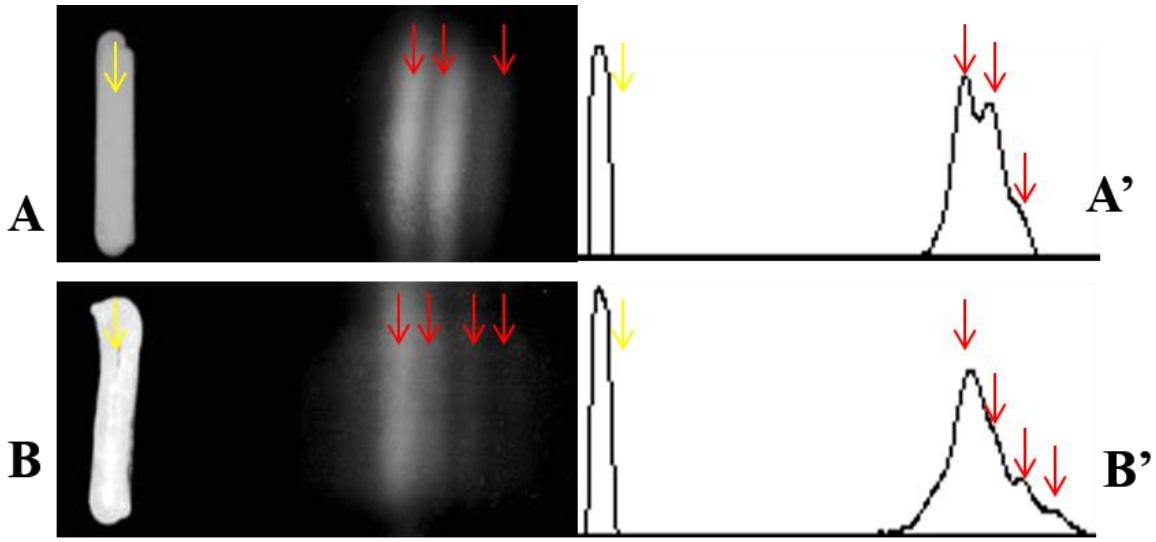


Fig. 2. Density histograms. Significant differences between the columns. ACR-Control. (B1-B1) p <0.0003. (B2-B2) p <0.0001. (B3-B3) there was no significant difference between them. It is X ± DS. GraphPad Prims Program. 7. ANOVA was applied for differences.

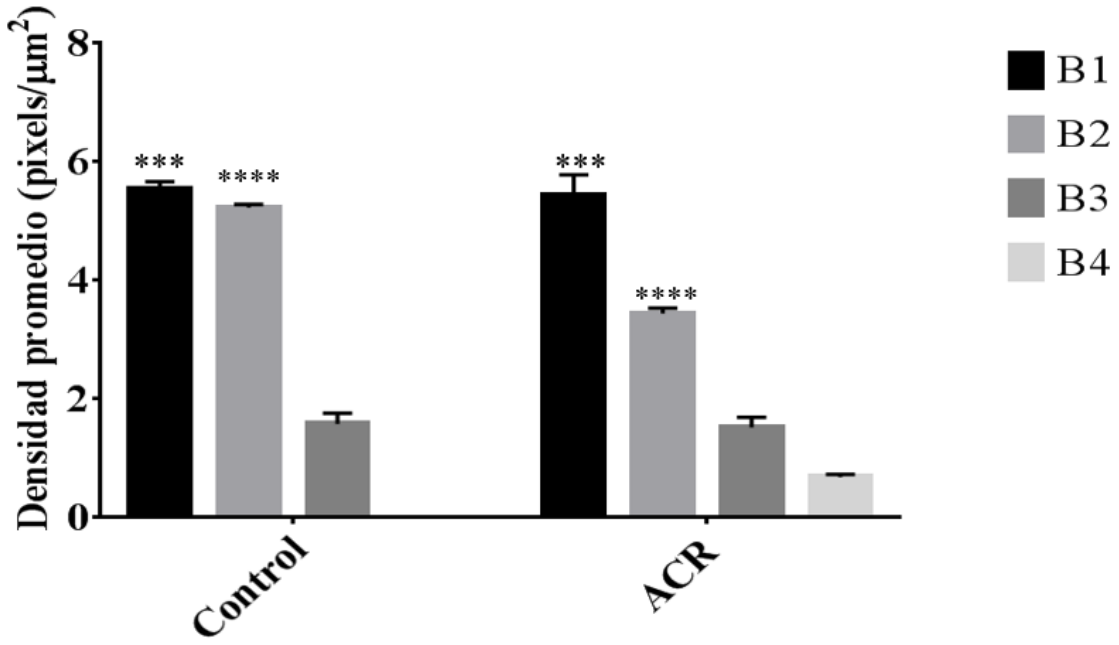
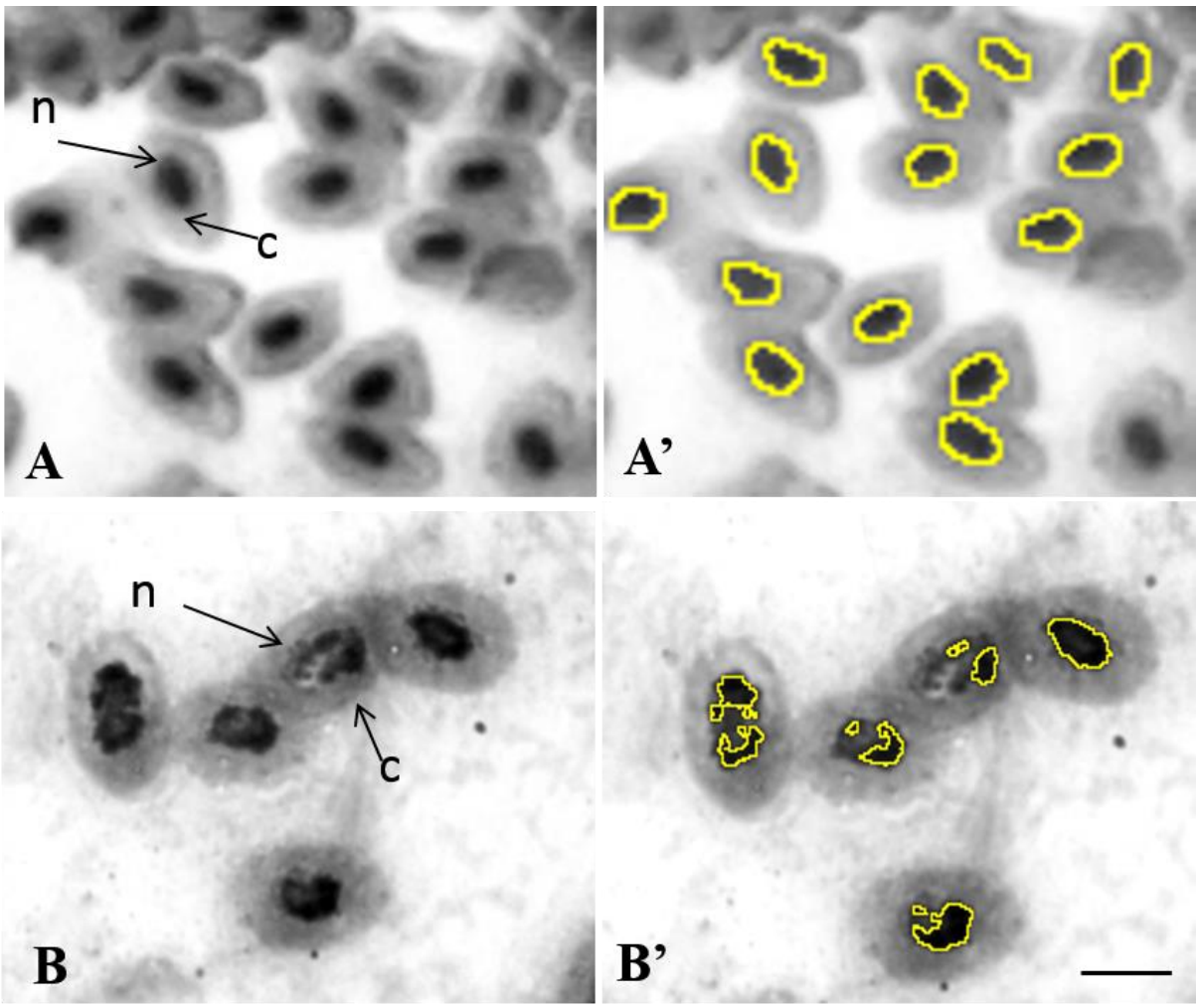


Table I. ALAD enzyme activity. Reading of Optical Density at 555 nm.

Sample	Optical density (555nm)	Control ALAD activity	ACR on ALAD activity
Control blank	0.17	-	-
Control	0.24	0.70 U/mL of GR	-
Sample blank	0.20	-	-
Sample	0.27	-	0.56 U/mL of GR

Fig. 3. Fragmentation of nuclear material. Original images in grayscale control (A) and treated with ACR-CI50 (B). Recognition of nuclear form and dimension (A ', B'). ImageJ J. Barra = 20µm. n = core c = cytoplasm.



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