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Adult Rat Liver After Subchronic Acrylamide Treatment: Histological, Stereological and Biochemical Study

Hígado de Rata Adulta Después del Tratamiento Subcrónico con Acrilamida: Estudio Histológico, Estereológico y Bioquímico

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SUMMARY: Acrylamide (AA) is a widely used chemical and an important monomer in various industrial and laboratory processes. In addition, AA is formed during processing of starchy food at high temperature. The aim of our study was to examine effects of subchronic AA treatment on adult rat liver using histological, stereological and biochemical methods. Adult male Wistar rats were treated with AA at doses of 25 mg/kg b.w. and 50 mg/kg b.w. for three weeks. Stereological analysis showed decrease of volume density of hepatocyte cytoplasm, and increase of volume density of hepatocyte nuclei and nucleocytoplasmic ratio in AA50mg group. Immunohistochemical analysis of the liver sections showed that treatment with AA50mg increase the percentage of PCNA positive cells, while the percentage of caspase 3 positive cells was not affected by AA. PAS-staining showed that glycogen content in hepatocytes was not affected by AA. Serological examination revealed increase of lipid peroxidation in AA50mg group, while total protein concentration, protein thiol group level, as well as, paraoxonase 1 activity were not changed in AA-exposed animals. Stereological and immunohistochemical analyses of adult liver sections suggest increase of proliferation in AA50mg group, while increase of lipid peroxidation in serum of AA50mg group indicates oxidative stress induction.

KEY WORDS: PCNA immunohistochemisty; Stereology; Acrylamide; Liver; Rat.

INTRODUCTION

AA is a monomer used for the production of polyacrylamide (PAA) polymers and has found its application in industry and laboratories. PAA is applied in: wastewater treatment, cosmetics, paper packaging and as a grouting material for underground building structures (Kumar *et al*., 2018). Most cases of human acrylamide intoxication have occurred during occupational exposure. The general population is mainly exposed to AA through food (Parzefall, 2008). AA is formed in various foods cooked at high temperatures, through three mechanisms: (a) from acrylic acid, which is a breakdown product of lipids, carbohydrates or free amino acids; (b) as a product of dehydration or decarboxylation of organic acids; (c) by direct formation from amino acids (Kumar *et al*., 2018). AA is detected in foods commonly consumed for breakfast such as fried potatoes, bread, cookies and cereals but is also found in roasted coffee and tobacco smoke (Loaec *et al*., 2014).

Most of the AA taken into the body is detoxified in the liver, except for a small part, less than 10%, which is a mercapturic acid derivative that is excreted in the urine (Delgado-Andrade *et al*., 2020). In the liver, during the detoxification process, AA is either combined with glutathione (GSH) or metabolized to glycidamide by the enzyme cytochrome P450 2E1 (CIP2E1) (Besaratinia & Pfeifer, 2007). Glycidamide is a genotoxic epoxy product

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of AA, which can form DNA adducts *in vivo* and in vitro (Hartmann *et al*., 2011). Neurotoxicity, genotoxicity, carcinogenicity, reproductive and developmental toxicity of AA have been detected in rats (Wang *et al*., 2010), and in humans AA is classified as a suspected carcinogen (IARC, 1994). AA exhibits toxic effects on liver through the induction of oxidative stress (Markovic´ Filipovic´ *et al*., 2022).

Since toxic AA is metabolized in the liver, the aim of our study was to use histological, stereological and biochemical methods to examine the effects of different doses of AA on this organ, and thus to provide additional data which could be implemented in further studies regarding the AA toxicity in rodents and humans.

MATERIAL AND METHOD

Animals and experimental procedure. The study was conducted on adult male Wistar rats 65 days old at the beginning of the experiments. Rats were housed under controlled laboratory conditions (22-24 °C; 12:12 h light-dark regime) and given standard granulated rodent food and tap water ad libitum. Rats were randomly allocated into three groups (n=10). Acrylamide (Sigma Aldrich) was applied in doses of 25 or 50 mg/kg b.w. by oral gavage for 21 days, while control group received vehicle (distilled water) orally. Selection of the doses applied in this study was made according to other studies concerning AA subchronic treatment (El-Bohi *et al*., 2011).

The research protocol with animal experimentation was approved by the Scientific Ethics Committee of University of Novi Sad (No. I-2011-03). All surgery was performed under sodium pentobarbital anesthesia and every effort was made to minimize suffering.

Histology, stereology and image analysis. The liver sample was taken from the middle lobe, fixed in 10% formalin, standardly prepared for embedding in paraffin and cut into 5 µm thick paraffin sections. For the light microscopy analyses, sections were stained by hematoxylin & eosin (H&E) for general histological examination of the liver tissues while periodic acid-Schiff (PAS) staining was used for glycogen deposits visualization. For analysis of PCNA and caspase 3 (cas 3) expression, liver sections were stained immunohistochemically following Ultravision LP Detection System protocol (TL-125-HD, Thermo Scientific) according to manufacturer's instruction, as described previously in Markovic *et al*. (2018) and Markovic Filipovic *et al*. (2022). IHC was carried out using anti-PCNA antibody (1:5000, Abcam) and anti-caspase 3 (1:100, Thermo Scientific). Digital image of stained sections were taken on Motic™ B3 Series microscope with Moticam 2500 camera (Motic).

Stereological examination of the hepatocytes was carried out by the point counting technique using multipurpose Weibel test grid M42 (Weibel, 1979), under the total microscopic magnification of 400x. Analyses of H&E-stained sections were performed on 20 randomly selected fields of vision per section on 5 sections 30 mm apart per animal. The following stereological parameters were determined: the volume density of hepatocytes (Vvh), volume density of hepatocyte nuclei (Vvhn), volume density of hepatocyte cytoplasm (Vvhc) and nucleocytoplasmic (N/C) ratio. Quantitative analyses of digital images were performed using the ImageJ program (Image J, version 1.50f) as described in Markovic *et al*., (2018), according to the protocol of Varghese *et al*. (2014). Briefly, color deconvolution using appropriate vector for stained sections was applied for the separation of the color spectra. Quantification of purple color intensity in PAS-stained sections was performed using H PAS vector. The optical density (OD) of the PAS dye separated by the H PAS vector was described in detail in previous papers (Markovic *et al*., 2018; Stosic *et al*., 2018; Markovic´ Filipovic *et al*., 2022). Stained percentage color area in digital images of PCNA and cas 3 in IHC-stained sections was determined using ImageJ plugin - IHC profiler as reported previously by Varghese *et al*., (2014). In order to quantify staining intensity, 40 unbiasedly taken digital images of liver of each rat were examined.

Serum analysis. Blood samples were collected from the carcass of each rat after decapitation and centrifuged for 10 min at 2000 × g at 4 °C. Total proteins in the serum were determined by the method of Bradford (1976), the amount of protein thiol groups (-SH groups) was measured according to the protocol of Ellman (1959), while lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA) according to the method of Slater (1984), as previously described by Markovic *et al*. (2018) and Markovic Filipovic *et al*. (2022). Paraoxonase 1 (PON1) activity was measured according to the protocol of Boesch-Saadatmandi *et al*. (2010).

Statistical analysis. Program STATISTICA® version 13.0 (StatSoft, Inc) was used for statistical analysis. Obtained results are represented as means ± standard error of mean. For comparing differences between means, the one-way ANOVA followed by the Bonferroni's post hoc test was applied. Values of p less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Histological evaluation of liver sections stained with H&E demonstrated normal liver structure, without marked morphological changes in AA-exposed animals. However, stereological examination revealed changes in microstructure of hepatocytes in rats exposed to AA50mg. In this animal group the volume density of hepatocyte cytoplasm ($p<0.05$) was reduced, while the volume density of hepatocyte nuclei and nucleocytoplasmic ratio were increased compared to the control ($p<0.05$) (Table I). Volume densities of nuclei and nucleocytoplasmic ratio are parameters associated with cell proliferation (Okamura *et al*., 2000). Therefore, the obtained stereological results suggest that treatment with AA50mg can induce hepatocyte proliferation. The results regarding the volume density of hepatocytes showed that this parameter was not affected by AA treatment ($p>0.05$) (Table I).

In order to confirm the proliferative effect after AA50mg treatment, we performed PCNA-IHC. In control and AA groups, PCNA expression was observed in the nuclei of hepatocytes (Fig. 1a, b). AA treatments led to dose-dependent increase in the number of PCNA positive cells, with most prominent result in the group treated with AA_{50mg} (Fig. 1c). This is the result of great importance since immunohistochemical analysis of PCNA expression

Table I Volume density of hepatocytes (Vvh) (a), volume density of hepatocyte cytoplasm (Vvhc) (b), volume density of hepatocyte nuclei (Vvhn) (c), and nucleocytoplasmic ratio (N/C) (d) in control and acrylamide (AA) treated rats in doses of 25 mg/kg b.w. and 50 mg/kg b.w.

Stereological parameter	Control	AA 25 mg/kg	AA 50 mg/kg
Vvh (mm^3/mm^3)	0.8733 ± 0.0015	0.8838 ± 0.0016	0.8979 ± 0.0007
Vvh_c (mm^3/mm^3)	0.7432 ± 0.0076	0.7363 ± 0.0025	0.6978 ± 0.0043*
Vvh_n (mm^3/mm^3)	0.1501 ± 0.0061	0.1574 ± 0.0013	0.19158 ± 0.0042*
N/C ratio	0.2086 ± 0.1786	0.2084 ± 0.0020	0.2777 ± 0.0065*

Values in charts are means ± SEM, n = 10, *p < 0.05. In statistical analysis, AA-treated animals were compared with the control group.

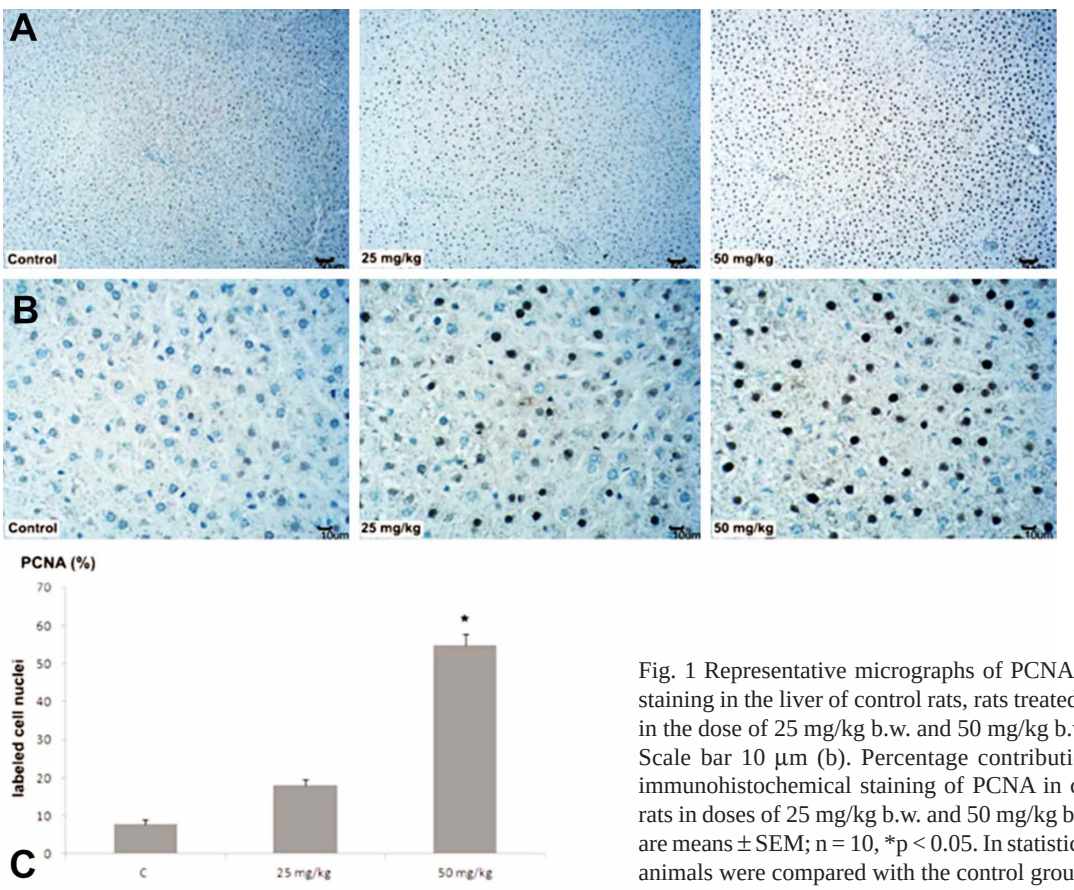

Fig. 1 Representative micrographs of PCNA immunohistochemical staining in the liver of control rats, rats treated with acrylamide (AA) in the dose of 25 mg/kg b.w. and 50 mg/kg b.w. Scale bar 50 μm (a). Scale bar 10 μm (b). Percentage contribution of positive nuclear immunohistochemical staining of PCNA in control and AA-treated rats in doses of 25 mg/kg b.w. and 50 mg/kg b.w. (c). Values in charts are means ± SEM; n = 10, *p < 0.05. In statistical analysis, AA-treated animals were compared with the control group.

confirmed our stereological findings, reporting that AA50mg stimulates cell proliferation in the liver of adult rats. AA-induced hepatic proliferation could be a consequence of AA detoxification that takes place in the liver. Namely, in the liver, AA is either conjugated to GSH or it is metabolized to genotoxic glycidamide. Conjugation of AA with GSH can lead to depletion of cellular GSH stores, and consequently change cellular redox status. Disturbed redox status can affect gene expression directly or via redox-dependent transcription factors which can lead to cell transformation or proliferation (Besaratinia & Pfeifer, 2007). Increased proliferation upon AA treatment has also been reported in the thyroid gland, adrenal gland and testis (Klaunig & Kamndulis, 2005). In line with this, cell proliferation after AA application was also detected in hepatoma cell line - HepG2 (Xu *et al*., 2019). AA induced HepG2 proliferation through upregulation of CYP2E1, EGFR, cyclin D1, NF- KB, p-AKT and miR-21 expression (Xu *et al*., 2019). Apoptotic characteristics were analyzed on cas 3-IHC stained slides. In all animal groups, immunostaining of cas 3 showed weak cytoplasmic immunoreactivity in hepatocytes (Fig. 2a). The percentage of cas 3 immunopositive cells was not affected by AA treatments (Fig. 2b). The results of our immunohistochemical analyses suggest that AA_{50mg} treatment increases proliferation but not apoptosis in the rat liver.

Glycogen deposits were analyzed on PAS-stained liver sections. In all animal groups, intensive cytoplasmic PAS staining in centrilobular hepatocytes was observed (Fig. 3a). Quantification of purple color intensity in PAS-stained liver sections showed that hepatic glycogen content was not affected by the AA treatment (Fig. 3b). Unchanged hepatic glycogen content was consistent with the unchanged serum glucose levels upon AA application (Stosic *et al*., 2018).

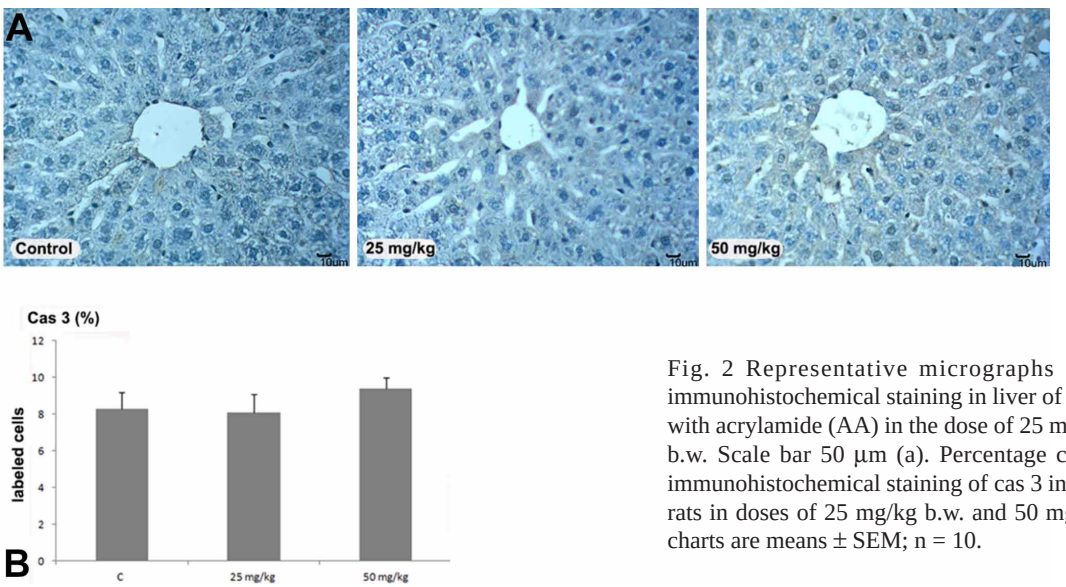

Fig. 2 Representative micrographs of caspase 3 (cas 3) immunohistochemical staining in liver of control rats, rats treated with acrylamide (AA) in the dose of 25 mg/kg b.w. and 50 mg/kg b.w. Scale bar 50 μm (a). Percentage contribution of positive immunohistochemical staining of cas 3 in control and AA-treated rats in doses of 25 mg/kg b.w. and 50 mg/kg b.w. (b). Values in charts are means ± SEM; n = 10.

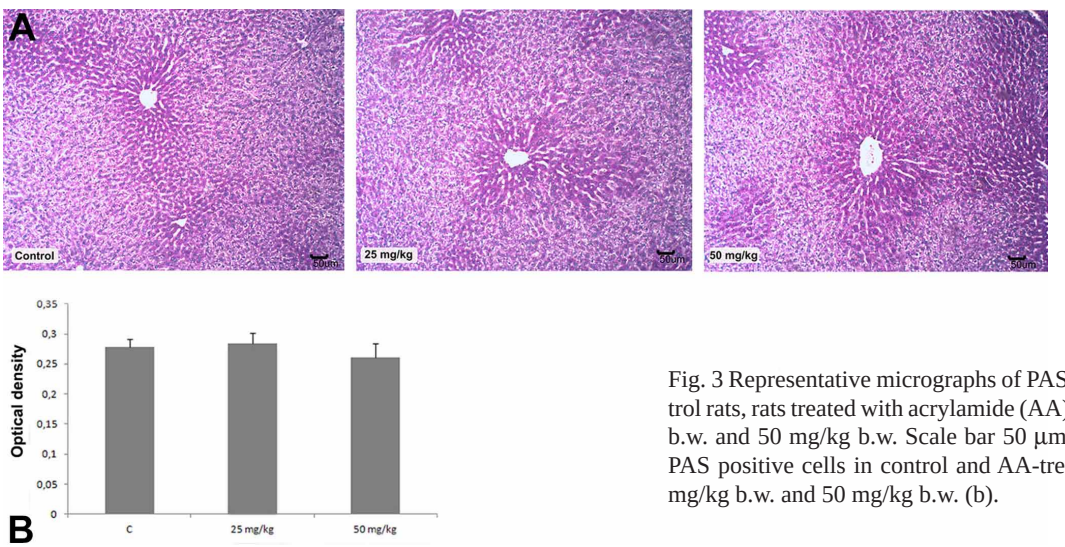

Fig. 3 Representative micrographs of PAS staining in liver of control rats, rats treated with acrylamide (AA) in the dose of 25 mg/kg b.w. and 50 mg/kg b.w. Scale bar 50 μm. (a). Optical density of PAS positive cells in control and AA-treated rats in doses of 25 mg/kg b.w. and 50 mg/kg b.w. (b).

The liver is the major metabolic and detoxifying organ, which by filtering complete body circulation leaves a mark on physiological parameters and markers of damage that can be detected in serum of the animals. After performing serological analysis, we found that AA treatments did not affect the concentration of total serum proteins (Table II). This can be considered as a positive result keeping in mind that unchanged level of serum proteins indicates preserved liver function upon AA application. In addition, serum protein thiol group content (-SH groups), lipid peroxidation and paraoxonase 1 activity were determined as indicators of liver damage since it was shown that liver damage was associated with disturbed oxidative stress parameters in serum (Atamer *et al*., 2008). Exposure to AA led to dose-dependent increase of lipid peroxidation (Table II), however, a statistically significant result was only observed in AA50mg group. Our results are in agreement with El-Beltagi & Ahmed (2016) who also detected an increase MDA level in serum of rats upon AA application. This is a systemic problem, as besides serum, AA-induced lipid peroxidation was also observed in the liver, testes, brain, kidney and lung (El-Beltagi & Ahmed, 2016). MDA concentration proved to be a sensitive parameter of oxidative stress, which is in agreement with Menon *et al*. (2020) who also reported that MDA level is a sensitive oxidative stress biomarker due to its ability to react with lipid membranes. We also showed that AA treatments did not affect protein thiol group content in serum (Table II). Protein thiol groups are significant because they are the main serum antioxidants. The majority of serum protein thiol groups are found in albumin, representing the major reducing agents in body fluids (Kükürt *et al*., 2021). In addition, PON1 is another important biomarker of oxidative stress (Ceron & Tecles 2014), the activity of which was not changed upon AA application (Table II). PON1 is calcium-dependent esterase in serum, that is synthesized in the liver and it protects against lipoproteins oxidation in the serum (Atli, 2013) Taking altogether, our serological analysis reports that increased lipid peroxidation after AA_{50mg} treatment indicates oxidative stress induction in the serum of adult male rats. However, unchanged concentration of protein thiol groups and PON1 activity may suggest mild or low level of AA potential to activate antioxidative defense system in the serum. These results suggest that AA in the dose of 50 mg/kg b.w. can induce disturbance in parameters responsible for redox regulation in the serum.

Table II Total proteins concentration (a), protein thiol (-SH) groups concentration (b), paraoxonase 1 (PON1) activity (c), and malondialdehyde (MDA) concentration (d) in the serum of control and acrylamide (AA) treated rats in doses of 25 mg/kg b.w. and 50 mg/kg b.w.

Parameter	Control	AA 25 mg/kg	AA 50 mg/kg
Total protein (mg/ml)	67.17 ± 3.49	70.31 ± 4.05	67.02 ± 2.88
Lipid peroxidation (nmol MDA/mg proteins)	0.72 ± 0.037	0.83 ± 0.041	0.93 ± 0.038*
SH groups (nmol/mg proteins)	20.26 ± 1.32	19.98 ± 1.03	19.56 ± 1.08
Paraoxonase 1 (U/mg proteins)	1.54 ± 0.07	1.55 ± 0.16	1.69 ± 0.12

Values in charts are means ± SEM; n = 10, *p < 0.05. In statistical analysis, AA-treated animals were compared with the control group.

CONCLUSION

In summary, we conclude that AA treatment in the dose of 50 mg/kg b.w. led to a change in stereological and immunohistochemical parameters in proliferation manner. In addition, AA_{50mg} increased lipid peroxidation in the serum, indicating induction of oxidative stress. Further studies will be conducted in order to elucidate the molecular mechanisms of AA-induced hepatic proliferation and thus contribute to a better understanding of the toxic effects of AA on human health.

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MARKOVIC, F. J.; MILER, M.; KOJIC, D.; VISNJIC, B. A.; MILOSEVIC, V.; KOKORIS, J. C.; DORDEVIC, M.; MATAVULJ, M. Hígado de rata adulta después del tratamiento subcrónico con acrilamida: estudio histológico, estereológico y bioquímico. *Int. J. Morphol., 40(6)*:1618-1623, 2022.

RESUMEN: La acrilamida (AA) es un químico ampliamente utilizado y un monómero importante en varios procesos industriales y de laboratorio. Además, la AA se forma durante el procesamiento de alimentos ricos en almidón a altas temperaturas. El objetivo de nuestro estudio fue examinar los efectos del tratamiento con AA subcrónica en el hígado de rata adulta utilizando

métodos histológicos, estereológicos y bioquímicos. Se trataron ratas Wistar macho adultas con AA a dosis de 25 mg/kg p.v. y 50 mg/kg de peso corporal por tres semanas. El análisis estereológico mostró una disminución de la densidad del volumen del citoplasma de los hepatocitos y un aumento de la densidad del volumen de los núcleos de los hepatocitos y la relación nucleocitoplasmática en el grupo de 50 mg de AA. El análisis inmunohistoquímico de las secciones de hígado mostró que el tratamiento con 50 mg de AA aumentó el porcentaje de células positivas para PCNA, mientras que el porcentaje de células positivas para caspasa 3 no se vio afectado por AA. La tinción con PAS mostró que el contenido de glucógeno en los hepatocitos no se vio afectado por AA. El examen serológico reveló un aumento de la peroxidación de lípidos en el grupo de 50 mg de AA, mientras que la concentración de proteína total, el nivel del grupo tiol de proteína y la actividad de paraoxonasa 1 no cambiaron en los animales expuestos a AA. Los análisis estereológicos e inmunohistoquímicos de secciones de hígado adulto sugieren un aumento de la proliferación en el grupo AA50 mg, mientras que el aumento de la peroxidación lipídica en suero del grupo AA50 mg indica inducción de estrés oxidativo.

PALABRAS CLAVE: Inmunohistoquímica PCNA; Estereología; Acrilamida; Hígado; Rata.

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