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Differential expression of HSP70 and ultrastructure of heart and liver tissues of rats treated with adriamycin: protective role of L-carnitine.

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Key words: HSP70; ultrastructure; adriamycin; heart-liver; L-carnitine.

Abstract. The anticancer drug adriamycin has been associated to tissular oxidative stress. In this regard, the promotion of anti-stress protein synthesis by L-carnitine has been suggested in rat adriamycin-induced cardiomyopathy in the long-term. However, the citoprotective role of L-carnitine in cardiac and hepatic tissues after short-term adriamycin treatment is unknown. HSP70 in the supernatant of the homogenized cardiac and hepatic tissues after short-term adriamycin treatment was determined by Western blot analysis with and without L-carnitine protection and compared to the subcellular characteristics of both tissues by transmission electron microscopic analysis. Female Sprague-Dawley rats (n = 6), body weight 40-60g, were randomized into four groups: control, adriamycin, L-carnitine and L-carnitine-adriamycin. Saline, adriamycin (15 mg/kg body weight) and L-carnitine (20 mg before adriamycin) were given intravenously (0.1 mL). HSP70 accumulation was different between the control and the adriamycin samples of both tissues. HSP70 was higher in the liver than in the heart both with and without L-carnitine protection. The nuclei of heart cells, in the adriamycin group showed alterations including, form and irregular perinuclear cysternae with invaginations of different sizes that were not observed in the L-carnitine–adriamycin samples. Considering the differential expression of HSP70 between liver and heart, our results may be important for understanding the role of these proteins in the adriamycin-induced distinct levels of organ damage and dysfunction. We suggest that L-carnitine exogenous administration might enhance the relationship between the cellular energy state and the activation of heat shock response by an unknown mechanism. L-carnitine may enhance HSP70 in a cellular-type manner.

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Expresión diferencial de HSP70 y ultraestructura de tejido cardíaco y hepático de rata tratado con adriamicina: papel protectivo de la L-carnitina.

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Palabras clave: HSP70, ultraestructura, Adriamicina, corazón-hígado, L-carnitina.

Resumen. La adriamicina, droga anticancerosa, ha sido asociada con el estrés oxidativo tisular. En este sentido, la promoción de la síntesis de proteínas anti-estrés por L-carnitina ha sido sugerida en las cardiomiopatías de largo plazo inducidas por adriamicina en ratas. Sin embargo, el papel citoprotectivo de la L-carnitina en tejido cardiaco y hepático, en la intoxicación por adriamicina en el corto plazo, se desconoce. La HSP70 presente en los sobrenadantes de homogenatos de tejido cardiaco y hepático luego de tratamiento con adriamicina a corto plazo y protección de L-carnitina, fue determinada mediante técnicas de Western blot y comparada con las características subcelulares de ambos tejidos. Ratas hembras Sprague Dawley (n = 6), de peso corporal entre 40-60g, fueron distribuidas aleatoriamente en cuatro grupos: control, adriamicina, L-carnitina y L-carnitina-adriamicina; recibiendo por vía intravenosa 0,1 mL de: solución salina, 15 mg/kg de peso corporal, 20 mg previo a la adriamicina y ambas, respectivamente. Se determinó una acumulación diferencial de la HSP70 entre las muestras del grupo control y adriamicina para ambos tejidos. La mayor acumulación de HSP70 fue en hígado, con y sin protección de L-carnitina. En el grupo adriamicina, las células cardiacas mostraron núcleos de forma no redondeada y cisterna perinuclear con invaginaciones de diferente tamaño, hallazgos que no fueron observados en las muestras del grupo protegido L-carnitina-adriamicina. Tomando en cuenta la expresión diferencial de HSP70 entre hígado y corazón, nuestros resultados pueden ser importantes para entender el papel de las proteínas de choque térmico contra los diferentes niveles de órgano toxicidad inducida por adriamicina. Se sugiere que la administración exógena de L-carnitina podría favorecer la relación entre el estado energético celular y la activación de la respuesta de choque térmico mediante un mecanismo desconocido y de una manera tejido dependiente.

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INTRODUCTION

The widely used anticancer drug adriamycin (ADR) produces toxic effects in numerous organs, the most serious being heart damage. Heart toxicity in human patients is generally expressed as cardiomyopathy following chronic administration whereas the liver is relatively resistant to damage (1-3). ADR has been shown to be a potential source of free radicals. In *in vitro* studies, quinone-containing anticancer agents, including ADR, have been shown to form semiquinone free radical intermediates in the presence of certain flavin enzymes (4). ADR-induced changes in membrane functions have been shown to be accompanied by lipid peroxidation in *in vitro* as well as *in*

vivo studies (5, 6). This increased peroxidation of polyunsaturated fatty acids is recognized as one of the possible biochemical mechanisms for the genesis of membrane injury in the myocardium (7). The role of ADR is associated to the flow of electrons from NADH to molecular oxygen and the production of free radicals. Interaction of ADR with cardiolipin, may contribute to the decline in cardiolipin-dependent enzymes such as coenzyme A and cytochrome oxidase (7, 8). The cardiovascular toxicity of ADR was attributed to a switch in the enzymatic activity of eNOS from a nitric oxide generating enzyme to a superoxide-generating enzyme (i.e. NADHP oxidase activity). In this sense, it have been demonstrated that heart tissue reveals depression in the activities of anti-oxidative enzymes, including catalase and dismutase superoxide (9). Hepatic levels of glutatione (GSH) and GSH peroxidases I and II have been shown to be relatively high and peroxidative damage to hepatocytes was obviated by GSH. In contrast to the liver, it has been demonstrated that the heart has extremely low activities of GSH peroxidase (10). In this regard, it is believed that constitutive cellular protection against acute stress is provided by a variety of intracellular components including the antioxidative enzymatic system, antioxidatives and perhaps heat shock proteins (HSPs) (11). The HSPs constitute a cellular system of endogenous protection which contributes for protein homeostasis and is essential for cellular viability (12). The over-expression of individual HSPs either in cultured cardiac cells *in vitro* or in the intact heart has a protective effect. In particular, HSP70 confers protection against cardiac ischemic damage and against a variety of adverse environmental conditions including certain anticancer drugs (13-17). The expression levels of HSP70 in cells correlated well with their survival following treatments with the tumor necrosis factor, staurosporine and ADR (18, 19). HSP70 rescues cells from apoptosis downstream of some known anti-apoptotic proteins such as caspase 3 (20, 21). However, HSP70 mRNA and protein are induced in different cell types depending on the severity, the nature of the stimulus and the biochemical characteristics of the tissue exposure to stress (22). An example of a cell-type specific stress response may be represented by the findings where rat liver samples showed the greatest accumulation of HSP72 after heat shock in comparison to other tissues including the heart (23). On the other hand, L-carnitine (L-Car), a derivative of natural amino acid and a metabolizing antioxidative agent, promotes fatty acid oxidation by translocating activated long-chain fatty acid into the matrix of mitochondria (24). L-Car has a cardio protective role against many toxic stresses including antimony (25), and promotes the accumulation of HSP25 as a short-term result of L-Car protective strategy, previous to ADR administration which is associated in the long term to alterations of much less severity (26). Nevertheless, it is not known whether there is a relationship between different heart and liver ADR subcellular toxic severity and different accumulation of HSP70 with and without the promotion of HSP70 by L-Car. Seeking to identify molecular clues regarding ADR-induced cardiotoxicity, the aim of this study was to analyse HSP70 response against morphological features in heart and liver of short-term ADR-treated rats. Moreover, given the potential protective role of L-Car against heart pathologies (26-28), we wonder about its relationship with HSP70 response.

METHODS

Animals: Female Sprague-Dawley rats (40-60 g), obtained from the Instituto Venezolano de Investigaciones Científicas

(Caracas, Venezuela), were used. The rats were allowed free access to standard rodent chow and water *ad libitum*. The animals were maintained according to the norms specified in the "Guide to the Care and Use of Laboratory Animals" of the U.S. National Institute of Health (NIH publication No. 85-23, revised 1985).

MATERIALS

Sigma Company (Miami, USA) was the source of all biochemical compounds, monoclonal antibodies and ADR. L-Car was donated by Elmor Laboratories (Caracas, Venezuela).

Experimental design

The animals were randomized in four groups (n = 6): Control, ADR, L-Car and L-Car-ADR. The antitumoral drug was injected i.v. to an accumulated dose of 15 mg/kg body wt, divided into three subdoses of 5 mg/kg body wt (in 0.1 mL of sterile water) applied at 3-day intervals. Control rats received saline solution (0.1 mL). L-Car was administered i.v. at a dose of 20 mg (0.1 mL) before each ADR subdose. 24 hours after the third ADR subdose heart and liver were removed for subsequent biochemical and ultrastructural study.

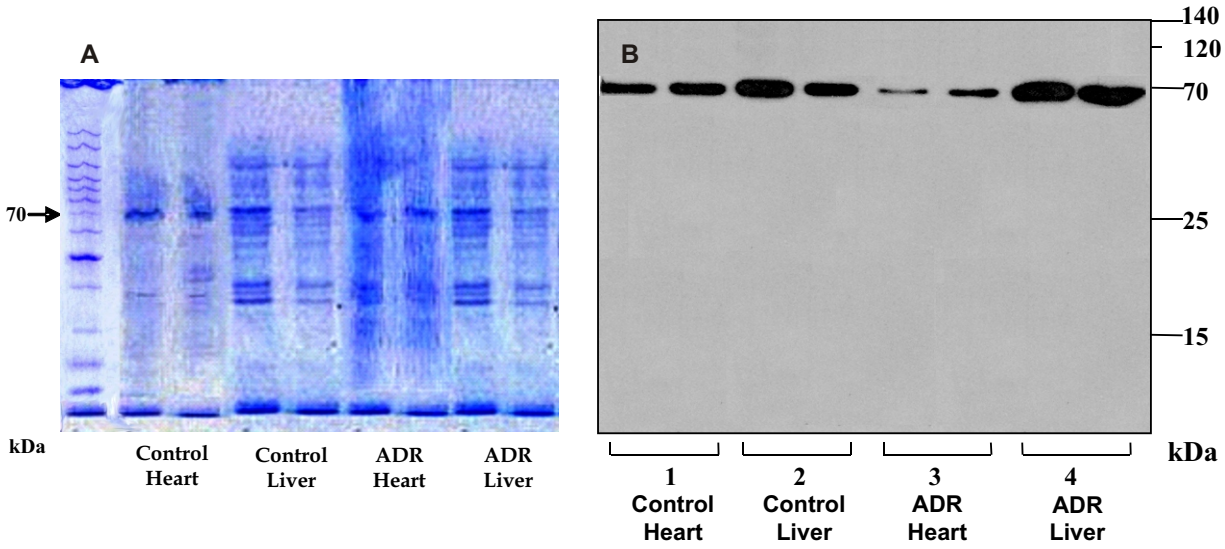
Western blots analysis

Left ventricular free wall and central hepatic lobule samples were homogenized (4°C; 1 mL extraction buffer Tris-HCl 20mM, EDTA 2mM, PMFS 1mM, pH 7.4) using a Potter-Elevehjem tissue grinder. The HSP70 content in the supernatant of homogenized heart and hepatic tissue was determined by Western blot analysis (29). Protein samples were diluted with 4 x Laemmli buffer solution. Equal amounts of protein samples (5 μg per lane) were applied to 1mm thick, 10% polyacrylamide gels and separated by SDS-PAGE (30). The equivalence of loading concentrations and the adequacy of the sample preparation were confirmed by visualization of protein with Coomassie blue stain. Proteins were transferred to nitrocellulose membrane which were washed in PBS with 0.3% low-fat milk to block non-specific binding sites and incubated at 4°C with mouse monoclonal IgG cross-reactive with HSP70 in a 1:5000 dilution at room temperature for 90 min. The immunodetection included: blocking of protein binding sites, binding the primary antibody (1:5000 HSP), washing unbound primary antibody, binding the anti-IgG conjugate, washing to remove unbound conjugate and detection by chemiluminescence. Protein immunoblots were scanned by 690 Bio-Rad Densitometer using the Multi-Analyst program (Bio-Rad).

Tissue preparation and ultrastructural analysis: Samples (2mm) of free left ventricular wall and central hepatic lobule were perfused with saline solution, fixed in Karnovsky (320 mosmol, pH 7.4, 2h, 4°C) and post-fixed in osmium acid (2% osmium tetroxide in Milloning buffer 0.12 M, 320 mosmol, pH 7.4, 2h, 4°C). The samples were dehydrated in graded increasing concentrations of acetone (50%, 70% + uranyl, 80%, 95% 100%; 30 min each) and embedded in polymerizing epoxy resin (60°C, 48h). After embedding, thin sections were cut (ultramicrotomy Reichert OmU3), stained with both saturated uranyl acetate (45min, 60°C) and lead citrate (3min, 25°C), and then examined with transmission electron microscope (Hitachi H-300, 75 Kv). The ultrastructural analysis was only of qualitative type.

Statistical analysis

To test the significance of the data resulting from the optical densitometry of HSP70 recognition among the 4 experimental groups, an analysis of variance by range ordering (Kruskal-Wallis test) was used. Dif-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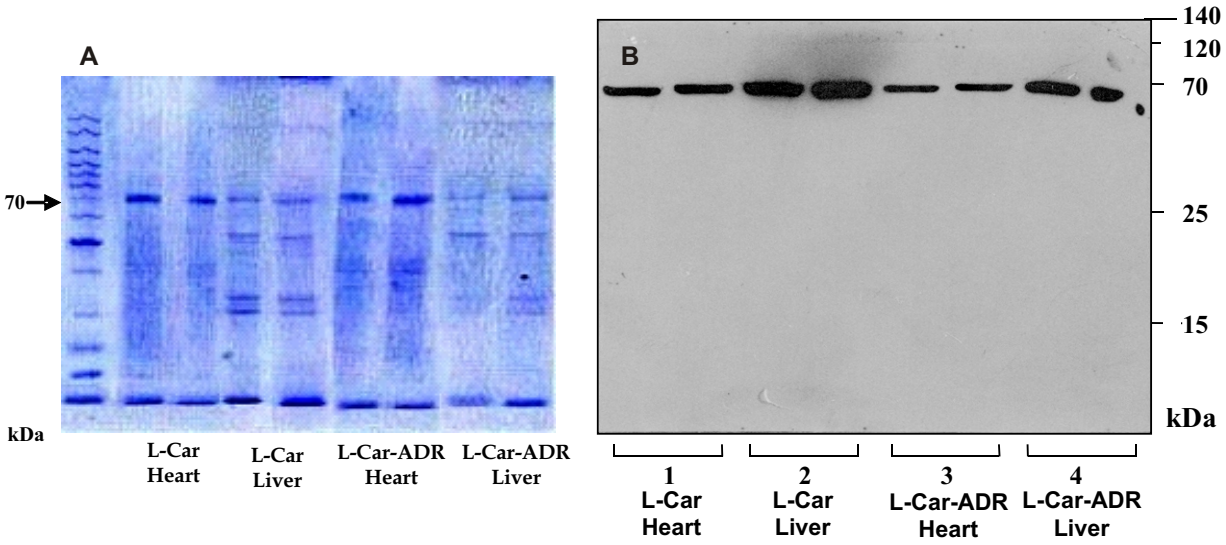

Fig. 2. 2a: Gel stained with Coomassie Brilliant Blue G-250 as a record of loading protein control (2, 3 L-Car and 4,5 L-Car-ADR treated samples from heart and liver, respectively). 2b: A representative Western blot of hsp70 using two contiguous lanes for each group analyzed. The two lanes identified by 1 and 2 correspond to CAR samples from heart and liver respectively, whereas the two lanes identified by 3 and 4 correspond to CAR-ADR samples from heart and liver respectively. All lanes were loaded with 5 μg of the particular protein.

showed myocardial cells with nucleus of normal appearance in Control group (Fig. 3A) whereas ADR group (Fig. 3B) had nucleus alteration including form and irregular perinuclear cysternae with invaginations of different size. The L-Car group (Fig. 3C) resembled the Control, and the L-Car-ADR group (Fig. 3D) showed a continuous perinuclear cysternae like Control and L-Car, and few lipid drops in the cytoplasm.

Hepatic ultrastructural analysis: Hepatic tissue of the four groups (Fig. 4 A,B,C,D respectively) showed hepatocytes with normal appearance.

DISCUSSION

A differential level of HSP70 expression between liver and heart of short-term ADR treated female rats was found in the present study. HSP70 is believed to participate in an array of cellular activities, including cytoprotection (22). Exposure of cardiac myocytes to oxidative stress by H2O2 treatment causes post-translational modification in two protein families involved in cytoprotection: the peroxiredoxins and two small heat shock protein family members: alfa Beta-crystallin and HSP25 (31). In ADR cardio toxicity protected by L-Car, it has been suggested that there is a relation between HSP25 cellular content in the short term and cytoprotection expressed in terms of a heart subcellular pathology of less severity, in the long term (26). A redox mechanism may be involved in the heat-shock signal pathway in oxidative stress, where induction of HSP70 protein has been correlated with a marked depletion of intracellular bound thiols and a decrease in lipid peroxidation (32). In astrocytes treated with acetyl-L-Car the induction of heme oxygenase-1 was related to the up-regulation of HSP60 as well as high expression of the redox-sensitive transcription factor Nrf2 in the nuclear fraction of treated cells (33). Acetyl-L-Car activities in-

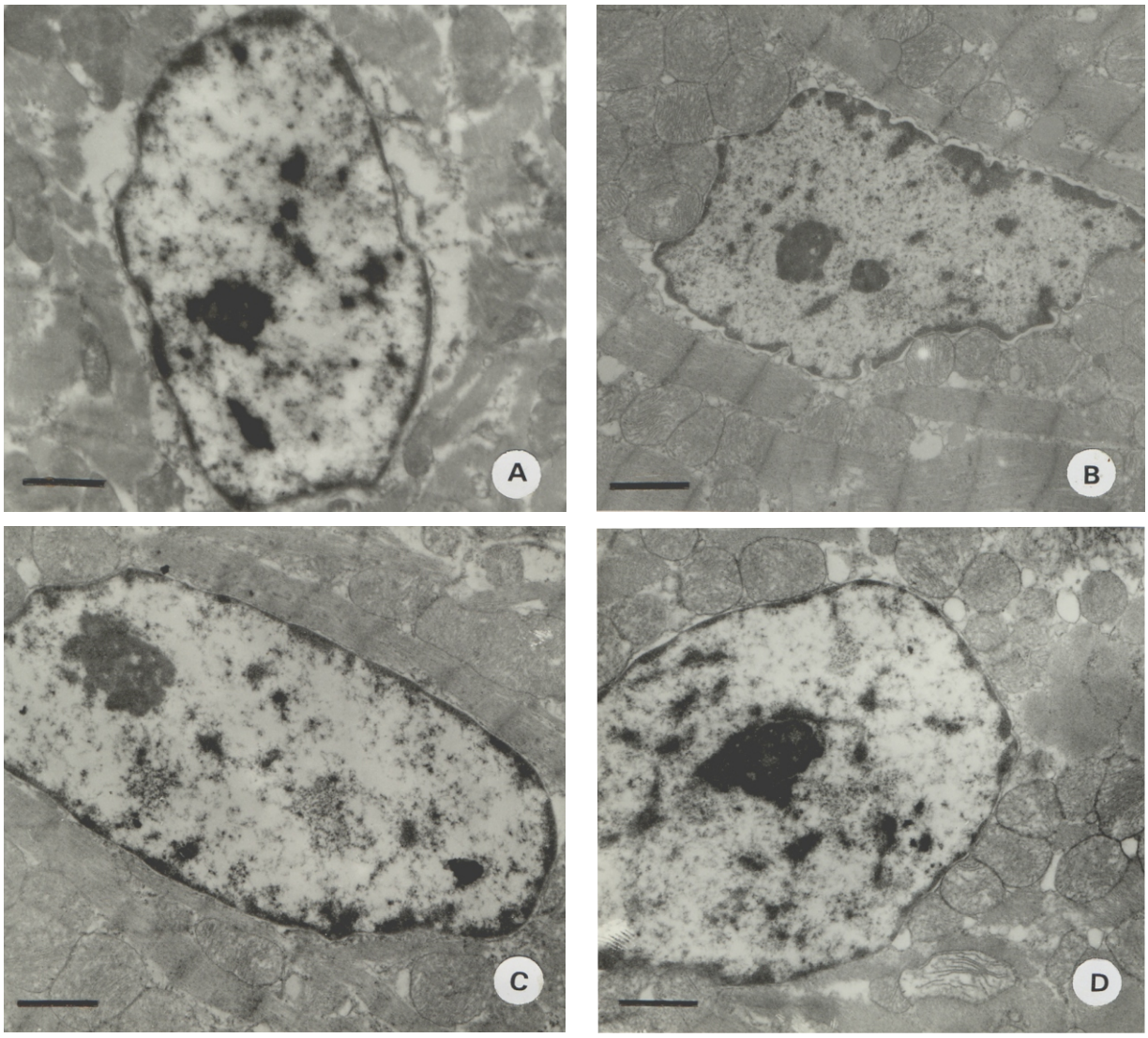

Fig. 3. Micrographs of the free left ventricular wall showed myocardial cells with nucleus of normal appearance in Control group (Fig. 3A). ADR group (Fig. 3B) had nucleus alteration including form and irregular perinuclear cysternae with invaginations of different size. The CAR group (Fig. 3C) resembled the Control, and the CAR-ADR group (Fig. 3D) showed a continuous perinuclear cysternae like Control and CAR, and few lipid drops in the cytoplasm. Bar = 0.7μm

clude acetylation of -NH2 and -OH functional groups in amino acids and N terminal amino acids in proteins; as well as, acting as a molecular chaperone to larger molecules (34). In addiction, a stress-regulated protein, GRP58, a member of thioredoxin superfamily, is a carnitine palmitoyltransferase isoenzyme (35). On the other hand, stress is accompanied by changes in the energy state. As a result, the preferential oxidation of cardiac mitochondrial DNA following acute intoxication with ADR may account for many of the bioenergetic deficits associated with the cardiotoxicity observed *in vivo* (36). Potencial molecular mechanisms of liver L-Car endogenous

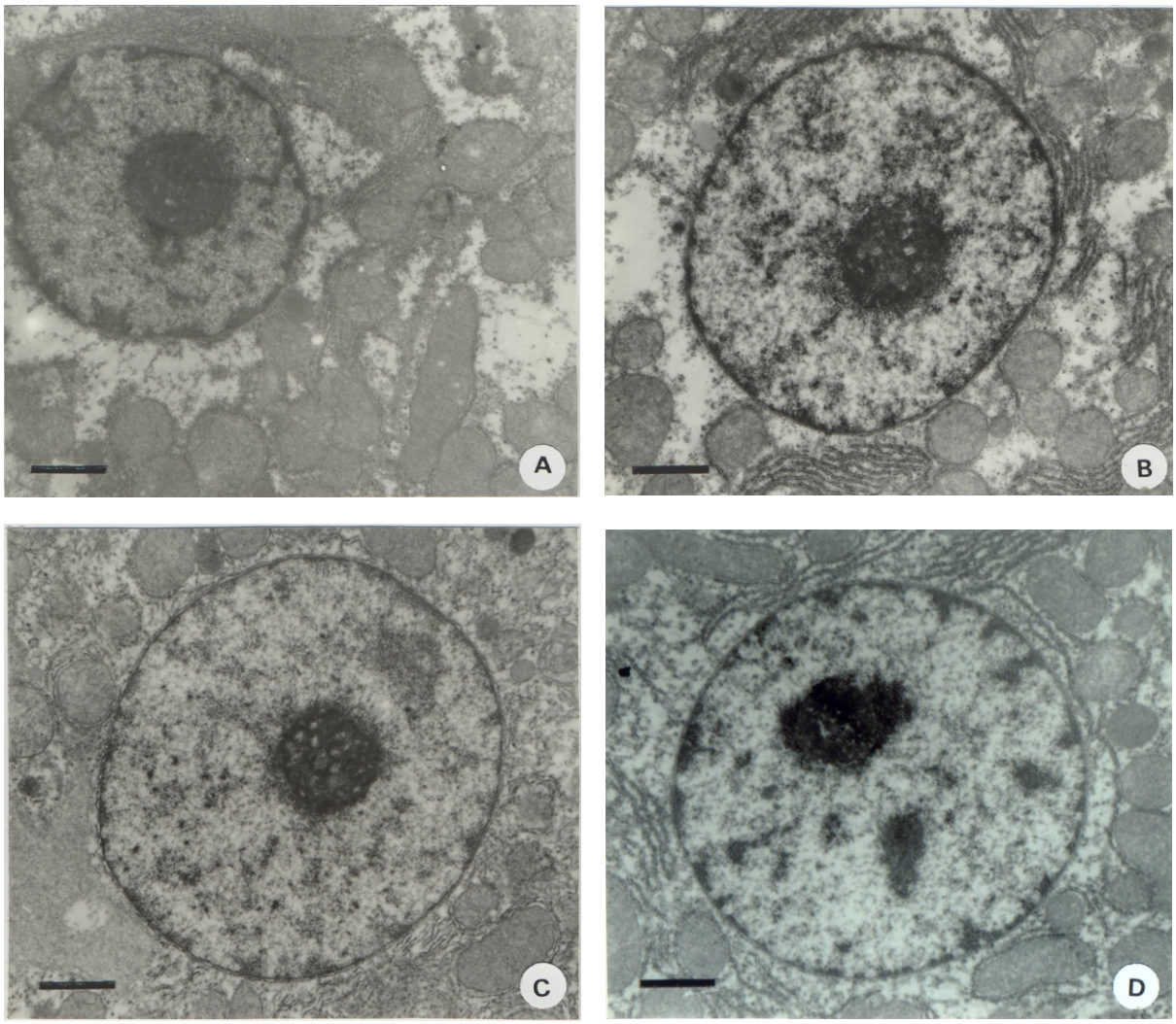

Fig. 4. Hepatic tissue of the four groups (Fig.4 A,B,C,D respectively) showed hepatocytes with normal appearance. Bar = 0.7μm.

biosynthesis might be related to the bioenergetic deficits observed in mitochondria isolated from heart, but no liver (37). A moderate decrease in intracellular ATP correlates with an attenuation in HSF1 in the heart and the restoration of ATP leads to greater activation of HSF1 (38). In this regard, acetil-L-Car acts by stimulating energy metabolism. Futhermore, the redox regulation of mammalian HSF1 is essential for HSP gene activation and protection from stress (39). It is possible that HSP70 plays a chaperone role in the process of ribosome biogenesis. The stabilization of ribosome assembly may have an impact in the preservation of protein synthesis during stress and may be an important component of the general mechanism of cytoprotection mediated by HSPs (40). However, following a stressful condition, HSP70 mRNA and protein are induced in different cell types depending on the severity and the nature of the stimulus (22). In addition, sex, causes differences in drug toxicity in rat, where female Sprague-Dawley hearts have twice as much HSP70 as male hearts, due to

upregulation by estrogen (41). Moreover, correspondence between the expression and/or accumulation of different HSPs has been linked to the establishment of a synergic network related to the maintenance of protein stability (42). The proven stimulation capacity of L-Car in relation to increased HSP25 detection in response to ADR (26), could involve other members of the HSP family including HSP70, in both heart and liver tissue. In addition in ADR cardiomyopathy a depressed protein synthesis including HSPs has also been demonstrated (43) in contrast to the hepatic response. The presence of HSPs may prevent the damaging effects of stress by impeding the precipitation of denatured proteins caused by the attack (44). The difference in HSP accumulation may alter the effectiveness of the organs to respond to stress. On the other hand, it has been suggested that stress contributes to the pathogenesis of proteinopathies through the stimulation of protein aggregation (45). This may be linked to our findings concerning the presence of cardioproteinopathies observed in the heart in comparison to their apparent absence in the liver of ADR treated rats (46). HSP70 content may contribute to the biochemical differences which may determine tissue susceptibility. In conclusion, the determination of the greater amount of HSP70 in liver than in heart ADR female treated rats, and its correspondence to the bigger tisular preservation in liver than in heart, may be important for understanding the function of this protein in organ differential damage. However, since the biochemical experiments were performed with a monoclonal antibody that recognized both the constitutive and inducible form of HSP70, further experiments are required using antibodies for each form of HSP70 with respect to the correspondence between the HSP70 induced form and the toxic stress condition. We suggest that L-Car exogenous administration might enhance the relationship between cellular energy state and activation of heat shock response by the promotion of HSP70 in a cellular-type manner.

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