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Histology, Bioenergetics and Oxidative Stress in Mouse Liver Exposed to *N*-Diethylnitrosamine

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Abstract. *Background: A mouse model in which N-diethylnitrosamine (DEN) induces Hepatocellular carcinoma (HCC) has histological and genetic resemblance to human tumours. Material and Methods: Male ICR mice were divided into control (n=10) and DEN-treated (n=10) groups. DEN was administered via intraperitoneal injection, once a week, for eight consecutive weeks. Animals were euthanized seven weeks after the last administration of DEN and their livers were collected. Plasma albumin, total bilirubin, alanine transaminase and aspartate aminotransferase activity were all measured and liver mitochondrial bioenergetics and oxidative stress were also evaluated. Results: Histologically, pre-neoplastic lesions were identified in the livers of mice from the DEN group. Total plasma bilirubin increased significantly in the group exposed to DEN and mitochondrial complex I and IV were significantly inhibited (p=0.0403 and p=0.0053, respectively). Conclusion: DEN induced changes in liver bioenergetics and antioxidant capacity towards reactive oxygen species, seven weeks after administration. At this stage, liver tissues in mice exposed to DEN still had the ability to counteract the oxidative effects of DEN by increasing the activity of antioxidant enzymes.*

Hepatocellular carcinoma (HCC) represents 85% to 90% of primary malignant tumours, it is the fifth most common kind of tumours and the third most common cause of death among cancer patients (1). The number of cases of HCC has been steadily increasing, with 600,000 new cases arising worldwide per year. HCC is associated with a poor prognosis and has a high mortality rate, causing up to 500,000 deaths a year. The majority of patients die within a year of being diagnosed with HCC (2).

Over 80% of HCC cases occur in East Asia and Sub-Saharan Africa, whereas the Americas, Northern Europe and Oceania have the lowest rates (<5/100,000). The countries of Southern Europe, including Portugal, have intermediate incidence rates of 5-20/100,000. HCC prevalence is related to the geographical distribution of the associated risk factors (3).

The main risk factors for developing HCC are viral hepatitis, alcoholic cirrhosis, contamination of food by aflatoxins, exposure to certain carcinogenic compounds, certain oral contraceptives, gender and the concomitant existence of other diseases (1, 4).

Several carcinogenic compounds have been identified and associated with the development of different types of cancer, including HCC, in diverse organs using animal models. Synthetic chemicals such as diethylnitrosamine (DEN), dimethylnitrosamine, ethylnitrosurea, 2-acetylaminofluorene, carbon tetrachloride and 1,2-dichloroethane, as well as natural substances such as aflatoxins, are used to induce liver cancer in laboratory animals (5, 6). DEN is an organic substance belonging to the *N*-nitroso group and is widely known for its ability to induce tumours in various organs such as the liver, lung and stomach, and the hematopoietic system. The route and duration of dosage administration may affect the experimental results (7, 9). DEN is also classified as a genotoxic carcinogen and requires metabolic activation. The first biotransformation step is hydroxylation of the α-carbon of an alkyl group forming α-hydroxylnitrosamine. This chemical reaction is mediated by the cytochrome *P450*

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enzyme, with a greater activity in centrilobular hepatocytes (10, 11). As a result of this step, aldehyde and alkyldiazo hydroxide are formed and the resultant compound, in its ionic form, can react not only with nucleic acids but also with the enzymes involved in DNA repair.

The aim of this study was to evaluate the effect of DEN on liver histology, and mitochondrial bioenergetics, and to assess the grade of oxidative stress caused by its action.

Materials and Methods

Animals and experimental conditions. All experimental procedures were developed according to ordinance no. 1005/92, and EU Directive 2010/63/EU on the use of laboratory animals.

Four- to five-week-old male ICR mice were purchased from the Harlan-Interfauna company (Barcelona, Spain). The subsequent quarantine period lasted for one week. The animals were kept under controlled conditions at 23±2˚C, with 12 h light/12 h dark and controlled ventilation. They did not receive any treatment prior to the start of the study. Animals were randomly divided into groups, kept on hardwood bedding and received a standard laboratory maintenance diet (Harlan Global Diet 2014, Barcelona, Spain) and water *ad libitum* throughout the experiment. Maintenance and cleaning were carried out twice a week. Drinking water was replaced weekly or earlier if necessary. Consumption of food and water was measured at the same time as animals' body weight was determined. The animals were observed daily to assess their general health.

Experimental procedure. A total of 20 male ICR mice were divided into two groups consisting of 10 animals each: a control group which did not receive any treatment and a DEN-treated group subjected to treatment with the carcinogen. DEN was acquired from Sigma-Aldrich Company (Portuguese affiliated Office, Sintra, Portugal). The carcinogenic solution was administered *via* intraperitoneal route, once a week, for eight consecutive weeks, at a dose of 3.5 μl/g bodyweight per mouse. DEN solution was prepared at 1% concentration (99 ml of normal saline – NaCl 0.9% to which was added 1 ml of concentrated DEN solution (0.01 μg/μl)).

Animals were checked daily for clinical signs of toxicity and mortality. Their ponderal homogeneity index ${}_iPH=2W_l/(W_l+W_h)$ and ponderal gain $PG=W_2-W_1/W_2\times100$ were calculated, with W_l being the lowest average animal weight, W_h the highest average animal weight, W_1 initial body weight and W_2 final body weight.

Sample collection. Fifteen weeks after the start of the experiment, the animals were euthanased by means of a lethal-dose intraperitoneal injection of pentobarbital sodium. Whole blood samples were collected *via* intracardiac punction and placed in appropriately sized heparinized tubes. At this time, blood samples were also collected into heparinized capillary tubes for microhematocrit procedure. Samples were centrifuged in a Labofuge I (Burladingen, Germany) at 656 ×*g* for 15 min to obtain plasma and kept at −20˚C until used. Complete necropsies were subsequently performed; the heart, lungs, liver, kidneys, urinary bladder and spleen were collected, weighed and examined macroscopically. Relative organ weights (liver, kidney, spleen, lungs, heart and urinary bladder) were calculated as the ratio of the organ weight to the mouse's total bodyweight.

Liver function tests. Automated standardized procedures (RX Daytona, Randox Laboratories, Dublin, Ireland) were used to measure and assess liver function, alanine transaminase and aspartate aminotransferase plasma activity, and plasma levels of total bilirubin and albumin.

Haematology. The capillaries were centrifuged in a Hermle Z320® unit (Gosheim, Germany) at 6000 ×*g* for 5 min, and the hematocrit was measured on a hematocrit ruler.

Histological evaluation. Representative fragments of each organ were fixed in 10% buffered formalin and embedded in paraffin wax. Tissue sections of 2 μm were processed and stained with haematoxylin and eosin (H&E). The livers were sectioned for reticulin and Masson's trichrome staining, according to the techniques described by Jones (12). Liver samples were analyzed *via* optical microscopy to identify lesions. The following parameters were evaluated: architecture, fibrosis, bile ductal hyperplasia, cholestasis, hepatocyte necrosis, the presence of inflammatory cells, hydropic degeneration, hyperplasia, anisokaryosis, binucleated cells, pseudo-nucleoli, apoptosis, focal hepatic necrosis and mitosis. As far as reticulin expression and Masson's trichrome staining were concerned, the following parameters were evaluated: liver parenchyma, portal space, centrolobular space, portal space and the presence of bridges in adjacent centrolobular veins.

Isolation of liver mitochondria. Mitochondrial isolation was performed by conventional methods (13), with minor modifications. The homogenization medium contained 0.25 M sucrose, 5 mM Hepes (pH 7.4), 0.2 mM ethyleneglycol-bis(-aminoethyl ether) *N, N, N, N*-tetra-acetic acid (EGTA), and 0.1% fatty acid-free bovine serum albumin (BSA). Ethylenediaminetetra-acetic acid (EDTA), EGTA, and BSA were omitted from the final washing medium, which was adjusted to pH 7.2. The final concentration of the mitochondrial protein was determined by the Biuret method (14), using BSA as standard protein.

Enzymatic activity of mitochondrial complexes. Complex I or nicotinamide adenine dinucleotide (NADH)-dehydrogenase activity was measured spectrophotometrically at 25˚C by following the oxidation of NADH (reduced nicotinamide dinucleotide) into NAD+ (oxidized nicotinamide dinucleotide), and this decrease in the concentration of NADH was read at 340 nm. The assay was performed in a buffer containing 50 mM potassium phosphate, pH 7.4, 2 mM KCN, 5 mM $MgCl_2$, 2.5 mg/ml BSA, 2 μM antimycin, 100 μM decylubiquinone, and 0.3 mM K_2NADH; 15-25 μg of the mitochondrial sample were used to initiate the reaction. The enzymatic activity was measured for 3 min and values were recorded 30 s after the initiation of the reaction. Specific activities were determined by calculating the slope of the reaction in the linear range in the presence or absence of 1 μM rotenone (complex I inhibitor) and expressed in nmol NADH/min/mg of protein (15). Succinate dehydrogenase activity was measured spectrophotometrically by the reduction of 2,6-dichlorophenolindophenol at 600 nm in the presence of phenazine methasulphate (16). The reaction was performed in 1 ml of the standard reaction medium supplemented with 5 mM succinate, 2 M rotenone, 0.1 g antimycin A, 1 mM KCN, 0.025% Triton X-100 at 25˚C, and 0.5 mg protein of disrupted mitochondria (two cycles of freezing and thawing). Cytochrome *c* oxidase activity was measured polarographically (16) at 25˚C in 1 ml of the standard reaction medium supplemented with 5 mM succinate, 2 M rotenone, 10 M

Table I. *Mouse body weights (g) (mean±SD) and ponderal homogeneity index (iPH) and ponderal gain (PG).*

	Initial body weight	Final body weight	$_{i}$PH	PG
Control group	30.55±1.77	45.40±4.40	2×28.04/(28.04+33.66)=0.909	(454-305.52/454)×100=32.705
DEN-treated group	29.74±1.49	41.88±2.83[a]	2×27.70/(27.70+32.84)=0.915	(418.82-297.42/418.82)×100=28.986

DEN: [a]p=0.048, Statistically different from that of the control group.

cytochrome *c*, and 0.5 mg protein broken mitochondria. The reaction was initiated by the addition of 5 mM ascorbate plus 0.25 mM *N,N,N',N'*-tetramethyl-p-phenylenediamine (TMPD). F_0-F_1 -ATPase activity was determined by monitoring the pH change associated with ATP hydrolysis (16). The reaction was carried out in 2 ml of a medium containing 130 mM sucrose, 50 mM KCl, 5 mM MgCl, and 0.5 mM Hepes (pH 7.2), supplemented with 2 M rotenone and mitochondria (1 mg protein of disrupted mitochondria). The reaction was initiated by the addition of 2 mM Mg-ATP and was completely inhibited by the addition of oligomycin (2 g/mg mitochondrial protein). Proton production was again calculated 2 min after starting the reaction. The final concentration of the mitochondrial protein was determined by the Biuret method (14) using BSA as standard.

Oxidative stress evaluation. Liver homogenization: Livers were placed in freezing medium consisting of 0.2 M mannitol, 0.07 M sucrose, 20% of Dimethyl sulfoxide (DMSO) and subsequently frozen at −70˚C for subsequent homogenization. Livers were weighed and added to 10% (w/v) 50 mM phosphate buffer and were homogenized. The homogenate obtained was centrifuged at 16,000 ×*g* for 20 minutes at 4˚C, the protein supernatant was used to measure antioxidant enzyme activities and protein thiol content.

Determination of protein thiol content: Total protein thiols expressed as reduced glutathione (GSH) were measured as described by Peixoto *et al*. (17). Absorption was measured at 412 nm, using GSH for calibration.

Determination of antioxidant enzymes: Superoxide dismutase (SOD) activity was determined according to the method of Paya *et al*. (18), using the xanthine–xanthine oxidase system. One unit of SOD activity was defined as the amount of SOD inhibiting the reduction rate of nitroblue tetrazolium chloride (NBT) by 50%. Catalase (CAT) was assayed as described by Aebi (19) and the activity was determined without disruption of mitochondria in the presence of 0.5% Triton X-100. Glutathione reductase (GR) activity was performed according to Smith *et al*. (20). The rate was calculated from the linear portion of the curve and expressed as nmol NADPH/min/mg protein. The activity of glutathione S-transferase (GST) was assayed at 25˚C and 340 nm due to the conjugation of GSH to 1-chloro-2,4-dinitrobenzene (CDNB). The reaction mixture contained 650 μl of sodium phosphate buffer (100 mM, pH 7.0), 10 μl CDNB (50 mM), and 25 μl GSH (50 mM). The absorbance was measured at 340 nm, and enzyme activity expressed in μmol CDNB conjugated/min/mg of protein (ε=9.6×10^3/M cm) (21).

Statistical analysis. Continuous data were expressed as mean±standard deviation (SD), with comparisons of continuous data made using a one-way ANOVA, Bonferroni and Tukey's multiple comparison test at the 5% significant level (p<0.05). All tests were performed using the GraphPad Prism, version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Table II. *Weight (g) and relative weight (%) (mean±SD) of liver, kidneys, bladder, lung, spleen and heart of mice.*

	Control group	DEN-treated group
Weight (g)		
Liver	2.417±0.309	2.187±0.182
Right kidney	0.385±0.054	0.315±0.043[a]
Left kidney	0.353±0.027	0.305±0.029[b]
Bladder	0.336±0.227	0.234±0.119
Lungs	0.356±0.032	0.344±0.086
Spleen	0.183±0.060	0.165±0.030
Heart	0.249±0.029	0.209±0.017[b]
Relative weight (%)		
Liver	0.053±5.65E-03	0.052±3.29E-03
Right kidney	0.008±8.47E-04	0.008±7.88E-04[c]
Left kidney	0.008±5.09E-04	0.007±5.57E-04[d]
Bladder	0.007±5.00E-03	0.006±2.62E-03
Lungs	0.008±8.03E-04	0.008±2.09E-03
Spleen	0.004±1.11E-03	0.004±6.83E-04
Heart	0.006±7.69E-04	0.005±4.21E-04

Significantly different from the control group at [a]p=0.005, [b]p=0.001, [c]p=0.016, [d]p=0.045.

Results

In order to evaluate the effects of DEN on mouse liver, we treated mice for eight weeks and euthanased them seven weeks after administration of the final dose. None of the animals showed any clinical signs of disease during the experimental period and at the end of the assay, all the animals were still alive.

Animal growth and water and food consumption. The $_{i}$PH and PG calculated for the two experimental groups are summarized in Table I.

No statistically significant differences in water consumption were recorded during the experimental period, although throughout the experiment, the DEN-treated group manifested lower water consumption compared to the control group (data not shown). Food intake was also lower in the DEN-treated group, however, only at the ninth week this became statistically significant (p=0.045) compared to the control group (data not shown). The mean weight of the animals' livers, kidneys,

Table III. *Biochemical and microhematocrit parameters evaluated (mean±SD).*

	Albumin (g/dl)	Total bilirubin (mg/dl)	Alanine transaminase (U/l)	Aspartate aminotransferase (U/l)	Microhematocrit
Control group	2.79±0.19	0.15±0.04	51.87±9.55	122.88±26.51	40.60±2.43
DEN-treated group	2.77±0.50	0.26±0.12[a]	40.55±14.74	99.77±48.00	40.65±2.58

Significantly different from the control group at [a]p=0.026

Table IV. *Histological lesions identified in animals' livers.*

	Normal liver architecture	Hydropic degeneration	Hyperplasia	Anisokaryosis	Binucleated cells	Mitoses	Pseudo-nucleoli	Apoptosis	Focal hepatic necrosis
Control group (n=10)	10/10	–	–	–	–	–	–	–	–
DEN-treated group (n=10)	10/10	10/10	10/10	10/10	10/10	9/10	7/10	8/10	7/10

urinary bladders, lungs, spleens and hearts, as well as the relative organ weights, are shown in Table II.

Liver function tests and microhematocrit. The results of plasma biochemical analysis and microhematocrit are shown in Table III. Statistically significant differences were found in terms of total bilirubin (p=0.026) between the DEN-exposed group and the control group.

Macroscopic and microscopic evaluation. Macroscopic changes were not identified on the organs collected from the control group; liver from one mouse of the DEN-treated group exhibited a whitish-coloured lesion with a diameter less than 1 mm.

The histopathological liver lesions discovered were confined to the DEN-exposed group and are indicated in Table IV. No histological changes were found in the livers of mice belonging to the control group. Both groups preserved normal liver architecture (Figure 1A).

Livers from animals exposed to DEN presented cytoplasmic hydropic degeneration (Figure 1B); mitoses (Figure 1C); pseudo-nucleoli (Figure 1D); apoptosis (Figure 1E); and focal hepatic necrosis (Figure 1F). Histological changes were not identified in the remaining organs collected. The normal expression of fibrinogen and reticulin (Figure 1G), observed in the livers, means that DEN did not cause the development of hepatic fibrosis. Masson's trichrome staining (Figure 1H) revealed a normal distribution pattern in both groups.

To evaluate the effect of DEN on the group exposed to it, specific activities of complex I, II, IV and V (F_0-F_1-ATPase) were measured on isolated liver mitochondria. Changes in the activity of the enzymatic mitochondrial complexes were identified in the DEN group (Figure 2) compared to the corresponding control group. In DEN-treated animals, there was a 37% inhibition of the activity of complex I (NADH: ubiquinone reductase) compared to the control group (p=0.0403). In addition in the DEN group, there was a noticeable increase in the activity of complex II (succinate-co-enzyme Q reductase) enzymes, however, no statistically significant differences were found compared to the control group. Complex IV (cytochrome *c* oxidase) activity was significantly inhibited (28% to that of the control) (p=0.0053) and F_0-F_1-ATPase activity decreased by 13.6% in mice exposed to DEN, but no statistically significant differences were found compared to the control group.

Quantification of proteins in the liver homogenate indicated a decrease of 12.4% in mice of the DEN-treated group compared to the control group, but this difference was not statistically significant (data not shown).

The results for oxidative stress are summarized in Figure 3. SOD and GR activity increased in the DEN group (6% and 40% compared to of that of control, respectively) but was not statistically significant (p=0.6956 and p=0.1728, respectively). However, concerning Catalase and GST activity, findings suggest an increase of 250% for Catalase (p=0.0001) and an increase of 45% for GST (p=0.0358) in the DEN-treated group. Concerning GSH (total thiols expressed as reduced glutathione), the data show an increase in the content but this was not statistically significant in the DEN-treated group (10% that of the control; p=0.1494).

Mitochondrial bioenergetics and antioxidant enzyme activities suggest effects on liver associated with the biotransformation of DEN.

Discussion

This study aimed to evaluate the effect of DEN on physiological parameters in mice, such as liver histopathology, liver function, mitochondrial bioenergetics and oxidative stress.

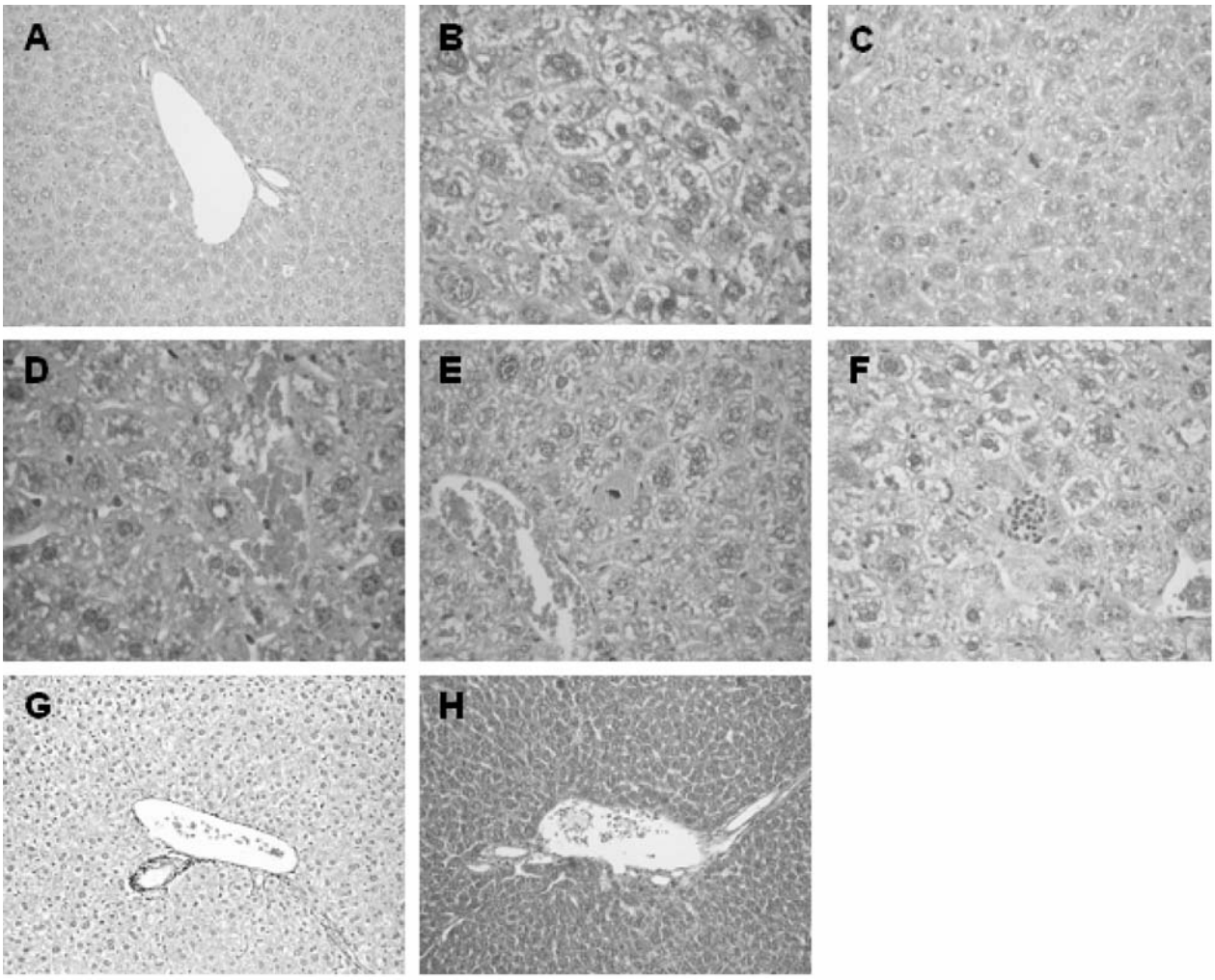

Figure 1. *A: Normal liver architecture (H&E, 200×); B: hydropic degeneration and anisokaryosis (H&E, 400×); C: mitoses (H&E, 400×); D: pseudo-nucleolus (H&E, 600×); E: apoptosis (H&E, 400×); F: focal hepatic necrosis (H&E, 400×); G: reticulin staining expression in liver taken from an animal exposed to diethylnitrosamine (DEN) (200×); H: Normal Masson's trichrome staining expression (200×) in an animal exposed to DEN.*

Regarding liver function, this study showed a statistically significant increase in total bilirubin in the DEN-treated group, which suggests changes in liver function. Related results concerning total bilirubin were described by Al-Rejaie (22).

In this study, histological analysis of the livers of those mice exposed to DEN showed the existence of several histological changes, including areas of necrosis, apoptotic cells and the presence of mitoses, which are extremely rare in normal livers. The existence of mitoses in the livers of animals exposed to DEN was also demonstrated by Sadik *et al*. (23) using the rat as an experimental model. The results described above, combined with the presence of cell hyperplasia and nuclear changes, suggest the formation of pre-cancerous lesions caused by DEN, which is also reported by other researchers (24, 25).

The histological changes induced in liver by DEN exposure may be related to cell energy deficiency, therefore the results of hepatocyte bioenergetics may contribute to our understanding on how DEN affects cellular bioenergetics. Concerning the functional state of mitochondrial bioenergetics, we analyzed the activity of complexes I, II, IV and V. Our findings show that liver bioenergetics are negatively affected by DEN. Complex I activity was significantly inhibited in mice exposed to DEN, which may be associated with the fact that this nitrosamine, or its metabolites, may affect the structure of this peptide complex, by changing its function and reducing its activity. Similar results were obtained by Boitier and colleagues (26).

Regarding complex II, a slight but not statistically significant increase was registered in the DEN-treated group

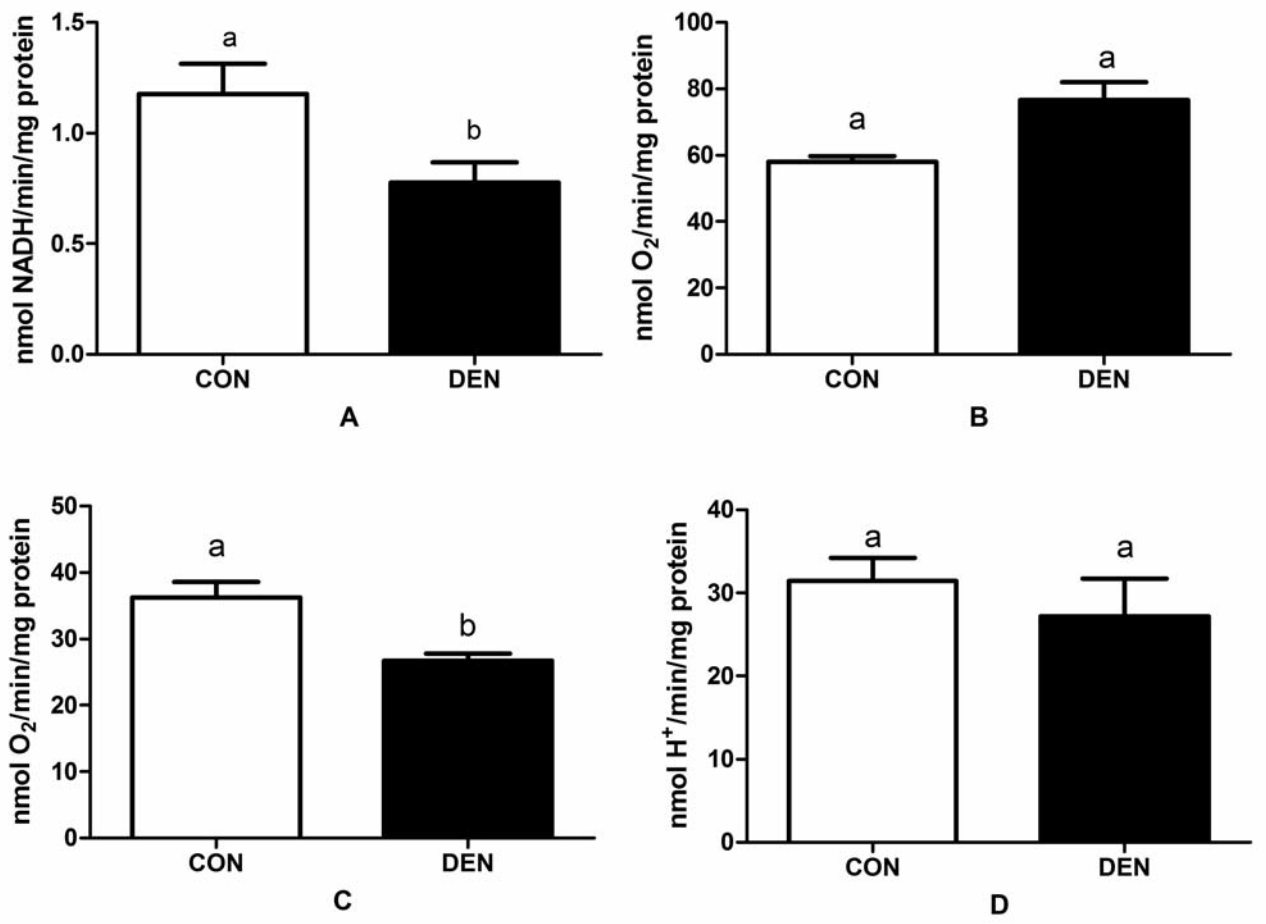

Figure 2. *Effect of treatment with diethylnitrosamine (DEN) on the activity of mitochondrial respiratory chain complexes. A: NADH dehydrogenase (complex I); B: succinate dehydrogenase (complex II); C: cytochrome c oxidase (complex IV); D: F_0-F_1-ATPase activity (complex V) as described in the Materials and Methods. CON: Control group; DEN: DEN-treated group. Values are presented as the mean and standard deviations for all mice treated with DEN (1%) for eight weeks and all control animals. Different letters represent statistically significant differences ($p<0.05$).*

compared to the control group. This increase is likely to be the result of non-specific membrane mechanisms. It is known that certain compounds can interact with the inner mitochondrial membrane, changing its structure, function and dynamics (27). Since mitochondrial respiratory complexes are integrated into the mitochondrial inner membrane, their activity is likely to be affected if the membrane is affected. On the other hand, it is known that mitochondria are able to compensate for such negative effect on one complex by enhancing the activity of the remaining complexes, so that oxidative phosphorylation overall is less affected. Complex IV activity was significantly inhibited for the mice in the DEN-treated group. This result is probably related to structural changes in this complex caused by DEN or its resulting metabolites, consequently leading to a reduction in their activity. The F_0-F_1-ATPase (complex V) activity decreased in mice exposed to DEN. As mentioned before, these results are clear indicators that DEN has a negative effect on mitochondrial bioenergetics. The inhibition of the activity of complexes I and IV in animals exposed to DEN should reduce ATP production *via* mitochondrial phosphorylation compared to the control group. As shown in breast cancer (28), cancer cell mitochondria often exhibit reduced oxidative phosphorylation, increasing the flux through the glycolytic pathway and thereby increasing the Warburg effect. It has already been demonstrated that cancer cells have a significantly decreased complex I activity (29), and a normal or slightly elevated activity of complexes II-IV has also been observed as a compensatory mechanism (30). Nevertheless, our data obtained from mice treated with DEN could reflect a global effect of DEN and its metabolites, as well as the mitochondrial alterations occurring during carcinogenesis.

It is widely recognized that mitochondria are a considerable source of Reactive Oxygen Species (ROS), and intracellular accumulation of ROS over extended periods

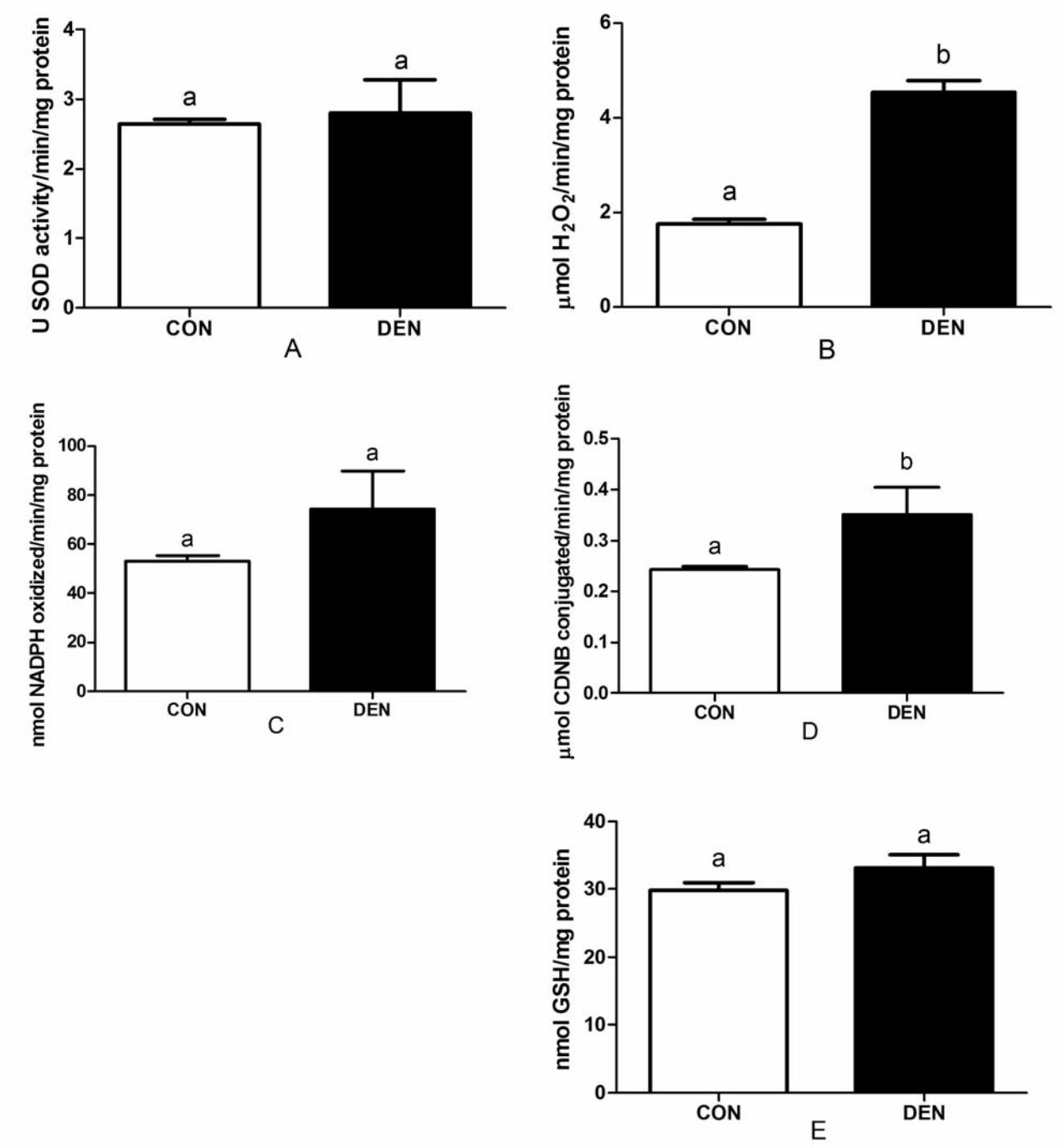

Figure 3. *Antioxidant enzyme activity. A: Superoxide dismutase; B: catalase; C: glutathione reductase; D: glutathione S-transferase; E: total thiols expressed as reduced glutathione as described in the Materials and Methods. CON: Control group; DEN: DEN-treated group. Values are presented as the mean and standard deviations for mice treated with DEN (1%) for eight weeks (N=10) and control animals (N=10). Different letters represent statistically significant differences ($p<0.05$).*

causes the oxidative stress characteristic of many chronic diseases, including cancer and liver dysfunction (31). Enzymes, including SOD, Catalase, GR and GST are successfully used as indicators of oxidative stress and their increased activity is a protective response to ROS (24, 32-34).

The results concerning oxidative stress enzymes may indicate the influence of DEN on the balance between cell oxidants and antioxidants. The activity of SOD was higher in

mice exposed to DEN, however, these results were not statistically significant. SOD catalyzes the dismutation of superoxide anion into hydrogen peroxide. Increased superoxide radical production by mitochondria may lead to increased activity of SOD to offset the abnormal production of the superoxide radical. On the other hand, these results support those obtained for Catalase activity, since Catalase is an enzyme responsible for the conversion of hydrogen peroxide into water and oxygen and an increment in its activity may be a response to increased SOD activity. As expected, Catalase activity was increased due to the addition of a substrate (hydrogen peroxide), a by-product resulting from SOD activity. However, the increase of 250% of this enzyme in animals exposed to DEN cannot be explained exclusively by the action of SOD, since the animals exposed to DEN exhibited only a 6% increase in this enzyme's activity. Therefore, only an unusual production of hydrogen peroxide in the cell would explain the sudden increase in Catalase. The role of cytochrome *P450* should be taken into account since it is responsible for the removal and detoxification of many substances in the liver. As a result of its activity, more reactive metabolites can be formed, as occurs with the metabolism of DEN in this enzymatic system, which leads to the formation of ROS including hydrogen peroxide (35, 36). Inflammatory cells and peroxisomes are also capable of increasing ROS levels. Peroxisomes, specifically, are a source of hydrogen peroxide, which can significantly increase the activity of Catalase (35). GST enzyme activity was also found to be elevated in mice exposed to DEN, which may be related to the function of this enzyme, which is to conjugate xenobiotic substances with GSH. DEN and its metabolites are conjugated by GST in order to be more easily excreted by the organism, justifying a statistically significant increase of 45% in GST activity in the DEN-treated group compared to the controls. GSH content in the DEN-treated group was slightly increased compared to the control group; nevertheless, this difference was not statistically significant.

Concerning GR activity, we found a 40% increase in mice exposed to DEN. Furthermore this increased GR activity may contribute to a rate of GSH production above that required for its conjunction with DEN. These results may have led to a *de novo* synthesis of GSH promoted by DEN treatment, as reported by Marinho *et al*. (37). In identical studies using the rat as an experimental model, similar results for the activity of GST and GR, and GSH content were found (23).

Nevertheless, other studies have shown contradictory results, namely a decrease in the activity of antioxidant enzymes in animals exposed to DEN (33). In fact, an increase in ROS formation and a drop in antioxidant enzyme activity in liver tissue have been reported in several models where HCC was induced by DEN (38, 39). The fact that these studies show a decrease in the activity of the antioxidant system may be related to the experimental design, in that the effects caused by DEN may have been so harmful to cells that they lost the ability to maintain an effective response against ROS. Supporting this idea of cells' incapacity to respond to ROS, Al-Rejaie (22) and Mourão *et al*. (25) showed that the expression of SOD, Catalase and GR was negatively affected in animals exposed to DEN.

Conclusion

This investigation showed that DEN, over the 15-week experimental period, induced changes in animals' biochemical parameters, histological features, liver bioenergetics and antioxidant capability towards ROS. Nevertheless, this work clearly shows that liver tissue cells in mice exposed to DEN, at this stage of the study, still preserve the ability to counteract the oxidative effects of DEN by increasing antioxidant activity. It is also shown that mitochondria play a role in DEN hepatotoxicity.

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Conflicts of Interest

The Authors state that there are no conflicts of interest.

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