The *N*-Nitrosodiethylamine Mouse Model: Sketching a Timeline of Evolution of Chemically-induced Hepatic Lesions

RUI M. GIL DA COSTA[1,2], NUNO PAULA-SANTOS[3,4], ANA FAUSTINO ROCHA[3], AURA COLAÇO[3,5], CARLOS LOPES[2,6] and PAULA A. OLIVEIRA[3,4,7*]

[1]*Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE), Chemical Engineering Department, Faculty of Engineering, University of Porto (FEUP), Porto, Portugal;*
[2]*Experimental Pathology and Therapeutics Group, CI-IPOP, Portuguese Institute of Oncology, Porto, Portugal;*
[3]*Veterinary Sciences Dept., University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal;*
[4]*Center for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro, Vila Real, Portugal;*
[5]*Veterinary and Animal Science Research Center (CECAV), Veterinary Science Department, University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal;*
[6]*Department of Pathology and Molecular Immunology, Abel Salazar Institute for Biomedical Sciences - University of Porto (ICBAS-UP), Porto, Portugal;*
[7]*Center for the Study of Animal Sciences (CECA), Food and Agrarian Sciences and Technologies Institute (ICETA), University of Porto, Vairão, Portugal*

Abstract. *Background/Aim: Hepatocellular carcinoma (HCC) is a frequent and aggressive malignancy associated with multiple environmental risk factors. The chemically-induced mouse model of diethylnitrosamine (DEN) provides useful insight into liver carcinogenesis, namely HCC. This work aimed to study the multistep process of hepatocarcinogenesis, providing a systematic framework for animal studies on this subject. Materials and Methods: Male ICR mice were divided into six control and six DEN-exposed groups. Saline solution and DEN were injected intraperitoneally, respectively, for eight consecutive weeks. Two groups (DEN vs. control) were euthanized at 8, 15, 22, 29, 36 and 40 weeks after the first administration. Results: Hydropic degeneration, necrosis and apoptosis were acutely induced at eight weeks and onwards. Hyperplastic foci occurred at 29 to 40 weeks along with diffuse dysplastic areas and hepatocellular adenoma. Peliosis hepatis were also identified at 36 and 40 weeks. HCC were only noted at 40 weeks, showing characteristic histological features of malignancy. Conclusion: Results allowed sketching of a timeline of evolution of DEN-induced hepatic lesions in mice, from initial lesions to malignant neoplasms.*

Correspondence to: Paula Alexandra Martins de Oliveira, Department of Veterinary Science, University of Trás-os-Montes and Alto Douro (UTAD), 5001-911 Vila Real, Portugal. Tel: +351 259350651, Mob: +351 966473062, Fax: +351 259350480, e-mail: pamo@utad.pt

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Liver cancer is largely a problem of developing countries, where 83% of the estimated 782,000 new cancer cases worldwide occurred in 2012 (1). Concerning primary liver cancers, hepatocellular carcinoma (HCC) is one of the most prevalent tumors worldwide, associated with a poor prognosis, showing a great propensity for angioinvasion (2, 3). Its incidence is increasing due to infection with hepatitis B (HBV) and C (HCV) viruses and assumption of cancer-associated lifestyle (*e.g.* smoking, physical inactivity, unhealthy diet, exposure to aflotoxins).

Hepatocarcinogenesis is a multistep process characterized by progressive accretion and interaction of genetic and epigenetic changes leading to unrestrained clonal production, local invasion and distant metastasis (4). Three types of liver cells are regarded as the source of malignant transformation being hepatocytes, cholangiocytes and progenitor cells (5). HCC is preceded in both rodents and humans by the development of numerous microscopic abnormalities, often designated as premalignant lesions (6). These include cytological changes in hepatocytes (*e.g.* large cell change; small cell change), expandable foci of those cytological alterations (dysplatic foci) and macroscopic dysplastic nodules (low and high grade) (7, 8). Other cytological modifications, are collectively termed 'foci of altered hepatocytes', and have been described in several chemically-

induced animal models in early hepatocarcinogenesis. Nevertheless, the role of foci of altered hepatocytes in human hepatocarcinogenesis remains a matter of discussion (4). Well-differentiated early-stage HCCs (early HCCs) arise from these pre-malignant lesions and are thought to emerge either from mature hepatocytes, as mentioned before, or from hepatic stem/progenitor cells, as suggested by the expression of stem cell markers and cholangiocyte markers found in these HCCs (5, 9, 10). Shibuya *et al*. have recently proposed that the expression of stem cell markers in malignant hepatocytes may result from de-differentiation and transdifferentiation into an immature stem cell-like phenotype rather than malignant transformation of stem/progenitor cells (9). This hypothesis was further supported by animal studies using an *N*-Nitrosodiethylamine (DEN)-induced mouse model of HCC (11).

Over the years, animal models have been used to increase knowledge on the mechanisms of pathogenesis underlying HCC (12). The laboratory mouse (*Mus musculus*) is considered one of the best models due to the availability of gene tracking methods, the possibility of xenograft implantation, breeding capacity, size and physiological/molecular correspondence to human patients (6, 13). Genetically-modified mouse strains used as models of liver cancer include transgenic and knock-out mice. Some models take advantage of interspecific or inter-strain differences to study the contribution of different genetic backgrounds to hepatic carcinogenesis; other models, developed using xenografted cell lines, are particularly useful for screening of anticancer drugs. Most recently, human hepatocyte chimeric mice have allowed studies on viral replication and cellular changes induced by HBV and HCV to be carried out (14).

DEN is the chemical most widely used to induce liver cancer in mice (12, 13, 15-17). DEN belongs to the family of *N*-nitroso compounds (NOC), considered highly carcinogenic, and has been found as a contaminant in food, beverages, cosmetic and personal care products, and tobacco among others (18). In fact, dietary exposure to NOC has been associated with increased risk of several types of cancer in human populations (19, 20). Approximately 300 NOCs have been tested for carcinogenicity; 90% of those stimulated carcinogenesis in 40 animal species, including higher primates, and at a variety of sites and organs (20, 21).

DEN is a DNA-alkylating carcinogen that requires metabolic bioactivation in hepatocytes, mediated by cytochrome *P450* (22) and acts as a complete carcinogen. DEN-induced mouse tumors often harbor Harvey rat sarcoma viral oncogene (*H-RAS*)-activating mutations (23). While activation of the Ras pathway is a common event in human hepatocarcinogenesis (24), mutations of the *HRAS* proto-oncogene itself are less frequent and associated with a more aggressive biological behavior (25). Consequently DEN-induced tumors are purported to closely model the more aggressive human HCCs.

Although animal models are particularly useful for studying the pathogenesis of cancer, it is vital that detailed histological descriptions of the induced lesions are made available and updated, using a standard nomenclature, in order to allow inter-study comparisons and adequate interpretation of results in different experimental settings. The present work aimed to evaluate short-term multiple administration induced exclusively by DEN (without any promoter agent), in macro- and microscopic hepatic changes in ICR mouse strain and to report the histological features of pre-neoplastic, neoplastic and non-neoplastic lesions.

Materials and Methods

Animals and experimental conditions. One hundred and twenty male ICR mice (five weeks of age) were acquired from Harlan, Barcelona, and were housed at Trás-os-Montes and Alto Douro University animal facilities, according to National (Portaria 1005/92 dated October 23rd) and European (EU Directive 2010/63/EU) legislation. The subsequent quarantine period lasted for one week. Animals were maintained at a temperature of 23±2˚C, 50±10% humidity, with 12 h light/12 h dark and hardwood bedding (26). Water and a standard diet (Global Diet; Harlan) were provided *ad libitum*. In order to reduce eventual aggressive behaviors conditioned by the long-term experimental procedure, environmental enrichment was provided using ping-pong balls, paper rolls and PVC tubes (27).

Experimental mouse model procedures. Previously to the present procedures, mice did not receive any treatment. At six weeks of age, all mice were identified with ear cuts and randomly divided into 12 groups, as depicted in Figure 1. Groups 1, 3, 5, 7, 9 and 11 (controls) were intraperitoneally (*i.p.*) administered with saline solution for eight consecutive weeks, while groups 2, 4, 6, 8, 10 and 12 were weekly *i.p.* injected with 35 mg of DEN/kg bodyweight per mouse (DEN acquired from Sigma-Aldrich Company, Sintra, Portugal). During the experimental protocol, the animals were daily monitored for signs of distress. Food and water intake were documented weekly. Mouse weights were noted weekly; ponderal homogeneity index ${}_{i}PH=2W_{l}/(W_{l}+W_{h})$ and ponderal gain $PG=W_{2}-W_{1}/W_{2}x100$ were calculated (Wl being the lowest average animal weight, Wh the highest average animal weight, W1 initial body weight and W2 final body weight).

Sample collection and histological processing. The first group (DEN n=9; control n=10) was sacrificed by means of a lethal *i.p.* dose of sodium pentobarbital 18 h after the last DEN injection (designated T1, 8 weeks post-exposure). The remaining groups were sacrificed, correspondingly, at the following weeks: 15 (T2: DEN n=10; control n=10); 22 (T3: DEN n=10; control n=10); 29 (T4: DEN n=10; control n=10); 36 (T5: DEN n=9; control n=10) and 40 (T6: DEN n=8; control n=10) weeks after the first DEN injection by the same method mentioned above.

All animals were submitted to necropsies and internal organs were screened for visible nodular masses. The liver, heart, lungs, spleen, stomach, intestine, pancreas and kidneys were collected and fixed in 10% neutral buffered formalin for 48 h and then liver samples were routinely processed and paraffin-embedded. Relative

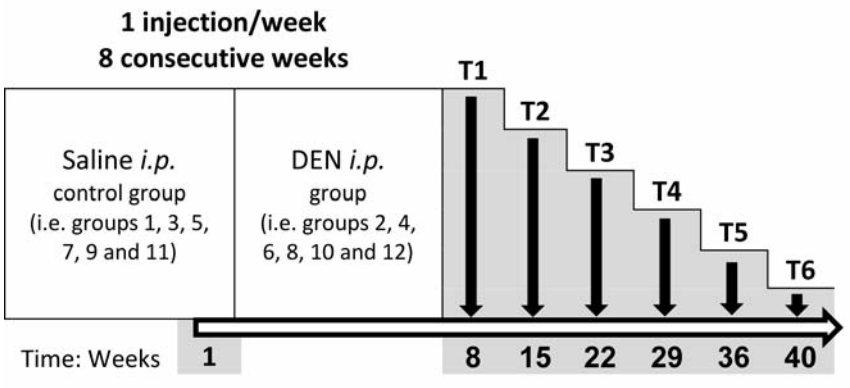

Figure 1. *Experimental design. Time is given in weeks post-exposure. Black arrows indicate time of euthanasia. Control animals received saline solution injections intraperitoneally (i.p.) and were euthanized at the same time as the diethylnitrosamine (DEN) i.p. -treated group.*

organ weights were estimated as the ratio of the organ weight to total mouse bodyweight according to Arantes-Rodrigues *et al*. (28). Macroscopically visible hepatic nodules were counted and measured using a caliper to determine their largest diameters.

Histological evaluation. Representative histological sections (2-μm-thick) were obtained and stained with hematoxylin and eosin (H&E) for examination under light microscopy by two different researchers in a blinded fashion and results were compared. The number of animals in each group presenting hepatic hemorrhage and other vascular disorders, inflammatory cell infiltration, biliary cysts, necrosis, apoptosis, pseudo-nucleoli and mitotic figures were noted. The mitotic index [number of mitotic figures per high power field (HPF) at ×400] in each group was expressed as a range (lowest count - highest count). Proliferative hepatic lesions were classified as hepatocellular hyperplastic foci, hepatocellular adenoma or hepatocellular carcinoma, according to the International Classification of Rodent Tumors (29) and the update on precursors and early lesion on HCC (8). Additionally, multifocal to regionally extensive, poorly delimited dysplastic areas, showing loss or distortion of lobular architecture, irregular hepatocyte plates, moderate cell atypia and mitotic activity were classified as diffuse dysplasia.

Statistical analysis. Data are expressed as the mean±standard deviation (SD) and compared by one-way analysis of variance (ANOVA) followed by 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).

Results

General findings. The mortality rate during the experimental protocol was 6.7% (4 out of 60 mice) and occurred exclusively in the DEN-treated groups. Due to male competitive behavior, despite the environmental enrichment, some sporadic injuries related to the establishment of hierarchy and territory defense were noted, resulting in focal loss of hair (barbering behavior).

Concerning animal growth, the iPH and PG for control and DEN-treated groups were estimated (Table I). DEN-treated groups presented statistically lower mean final body weights when compared to controls at T2 and T5 respectively.

Water consumption variation (DEN-treated groups *versus* control) showed no statistically significant differences; nevertheless, DEN-treated groups presented a lower water consumption compared to controls. Food intake was also lower in the DEN-treated groups. Statistically significant differences ($p<0.05$) were recorded during the assay, concerning DEN-treated *vs*. control group at T4 and T5 (29 and 36 weeks) after the first DEN *i.p*. administration.

Macroscopic and microscopic effects of DEN on mouse liver. The occurrence of hepatic nodular lesions over time and their respective dimensions are summarized in Table II and Figure 2. Exposure to DEN resulted in a sequence of lesions (Table III) that evolved over time, from acute toxic lesions observed from T1 (8 weeks) onwards, to chronic, pre-neoplastic and neoplastic lesions that culminated at T6 (40 weeks) with the occurrence of HCC. Control mice did not show any significant lesions (Figure 3a). As previously reported, acute DEN exposure resulted in regionally extensive to diffuse hepatocellular hydropic degeneration and multifocal necrosis, as well as in increased anisokaryosis, binucleated and mitotic hepatocytes, pseudo-nucleoli and apoptosis (16). Hydropic degeneration (Figure 3b) was most frequently observed at T1 (8 weeks after DEN administration) and diminished onwards. Necrosis (Figures 3c and d) was present in all DEN-treated groups, either in regionally extensive areas or in small foci that formed micro-abscesses, but was most frequently observed in the DEN-treated groups from T1 onwards to T4 (8 to 29 weeks post-exposure). Apoptotic and mitotic figures, as well as pseudo-nucleoli were consistently observed in all DEN-treated groups. Animals euthanized at T1 and T2 (8 and 15 weeks post-exposure, respectively) frequently exhibited abnormal mitotic figures, but the highest mitotic indices were observed at T6 (40 weeks post-exposure).

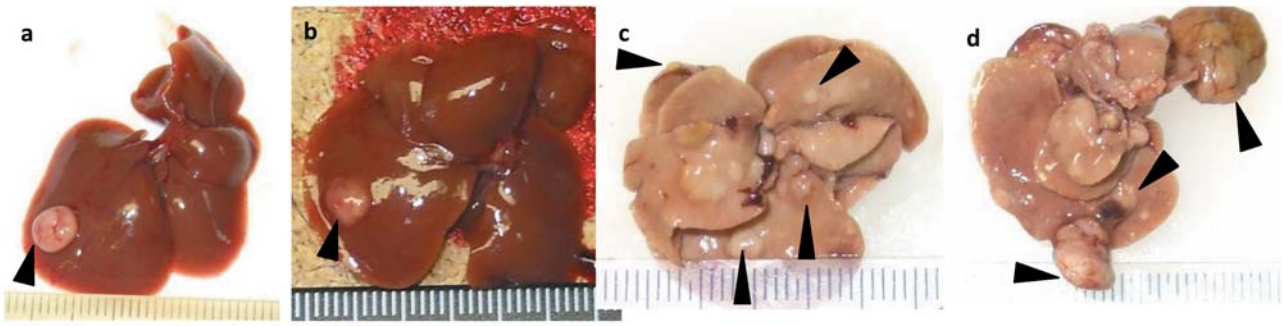

Figure 2. *Evolution of features of livers of diethylnitrosamine (DEN)-treated mice (scale ruler in mm). a: Mouse at 22 weeks (T3), arrowhead points to a single nodule (6×5×2 mm). b: Mouse at 29 weeks (T4), arrowhead points to a small visible nodule (4×5×2 mm). c: Gross morphology of liver tumor from a DEN-treated mouse at 40 weeks (T6), note irregular hepatic surface and several small nodules (arrowheads). d: Gross morphology of liver tumor from DEN-treated mouse at 40 weeks (T6), arrowheads point to differently sized tumors. None of the control mice had any macroscopic changes.*

The late exposed groups, T4 toward T6 (*i.e.* 29 to 40 weeks post-exposure), displayed chronic lesions that distorted normal hepatic architecture (Figure 2c and 2d). Three DEN-exposed animals at T5 and T6 exhibited blood-filled cystic cavities of various sizes (up to 1.0 mm), lined by endothelium, that multifocally replaced part of the hepatic parenchyma. These lesions (Figure 3e), recognized as *peliosis hepatis* (Table III), occasionally contained variably-sized thrombi. Another three mice at T6 exhibited multiple cystic cavities of variable size (up to 5.0 mm) that multifocally replaced the hepatic parenchyma, and contained a lightly eosinophilic fluid and were lined by a low cuboidal (biliary) epithelium, identified as biliary cysts (Figure 3f).

The first hyperplastic foci (six nodules in 4 out of 10 mice) were observed 29 weeks (T4) after last DEN injection (Table III). Such foci were clearly delimited from the adjacent parenchyma and composed of tight hepatocellular plates between compressed sinusoids. Hyperplastic hepatocytes exhibited distinct tinctorial cytoplasmic affinities compared to the adjacent parenchyma, basophilic or mixed. Some animals that developed hyperplastic foci (Figure 4a) also exhibited dysplastic changes in a distant and poorly-delimited area (classified as diffuse dysplasia, Figure 4b), as well as a larger, well-defined nodule classified as a hepatocellular adenoma (Figure 4c). Dysplastic lesions were composed of hepatocytes arranged in irregular and variably-oriented trabeculae which disrupted normal liver architecture without forming distinguishable nodules. Hepatocytes were moderately pleomorphic with significant variations in size and occasional cytoplasmic vacuoles. Pseudo-nucleoli and mitotic figures were present (one per HPF). The hepatocellular adenoma was a large (10.0 mm in diameter) nodule which compressed the adjacent parenchyma and was composed of regularly-oriented hepatocellular trabeculae. Hepatocytes were comparatively small, showed basophilic cytoplasm and occasional mitotic figures (up to one mitotic figure per HPF).

All mice euthanized at T5 (36 weeks after DEN administration) displayed extensive areas of diffuse hepatic dysplasia but hyperplastic foci were noted less frequently (Table III). Dysplastic areas also showed a sinusoidal accumulation of erythroblasts distributed in small foci of two to 20 cells. Occasionally, myeloid precursor cells were associated with such foci. Four out of eight mice at T6 (euthanized 40 weeks after DEN administration) exhibited large hepatic, soft, grey to light brown or, occasionally, hemorrhagic nodules, measuring up to 10.0 mm in diameter. Histologically, these nodules were classified as HCC (Table III). These lesions arose within dysplastic areas and often exhibited invasive growth and a multifocal appearance. Carcinomas were composed of highly pleomorphic cells disposed in an irregular trabecular pattern or, multifocally, in solid nests or pseudo-acinar structures, supported by a fibrovascular stroma. (Figure 4d). Neoplastic cells exhibited moderate nuclear pleomorphism, a prominent nucleolus and up to four mitotic figures per HPF. Cells were often vacuolated, and frequently assumed signet-ring morphology. Multifocally, cells showed variably sized intracytoplasmic hyaline bodies (Figure 4e). Groups of erythroblasts were present in all HCCs and were larger than those observed in dysplastic areas. In one instance, fully differentiated bone marrow developed inside a carcinomatous nodule, with myeloid, erythroid, lymphoid and platelet precursors distributed between bone lamellae and adipocytes (Figure 4f). Additionally, all mice at T6 presented diffuse dysplastic areas and four animals exhibited hepatic hyperplastic foci (Table III).

Table I. *Mouse body weights (g) (mean±SD), ponderal homogeneity index (iPH) and ponderal gain (PG). Time is given in weeks post-exposure: T1: 8 weeks; T2: 15 weeks; T3: 22 weeks; T4: 29 weeks; T5: 36 weeks; T6: 40 weeks.*

No. of weeks	Group	Initial body weight	Final body weight	iPH	PG
8	Control	31.16±2.60	39.86±3.35	2×27.06/(27.06+35.50)=0.865	(39.86-31.16/39.86)×100=21.82
	DEN-exposed	31.16±1.57	39.64±2.17	2×28.90/(28.90+33.06)=0.932	(39.64-31.16/39.64)×100=21.39
15	Control	30.55±1.77	45.40±4.40	2×28.04/(28.04+33.66)=0.909	(45.40-30.55/45.40)×100=32.70
	DEN-exposed	29.74±1.49	41.88±2.83[a]	2×27.70/(27.70+32.84)=0.915	(41.88-29.74/41.88)×100=28.98
22	Control	30.41±2.49	44.39±4.24	2×27.58/(27.58+35.40)=0.875	(44.39-30.41/44.39)×100=31.49
	DEN-exposed	30.55±2.32	48.18±5.98	2×27.16/(27.16+34.00)=0.888	(48.18-30.55/48.18)×100=36.59
29	Control	29.63±1.62	46.01±3.55	2×27.20/(27.20+31.94)=0.919	(46.01-29.63/46.01)×100=35.60
	DEN-exposed	29.84±2.48	44.32±5.81	2×25.62/(25.62+33.86)=0.861	(44.32-29.84/44.32)×100=32.67
36	Control	30.70±2.28	51.13±6.19	2×27.84/(27.84+34.80)=0.888	(51.13-30.70/51.13)×100=39.95
	DEN-exposed	29.22±2.33	42.59±7.02[b]	2×26.04/(26.04+34.02)=0.867	(42.59-29.22/42.59)×100=31.39
40	Control	31.47±2.30	50.07±2.41	2×29.84/(29.84+33.10)=0.948	(50.07-31.47/50.07)×100=37.14
	DEN-exposed	30.30±1.78	48.93±7.11	2×28.02/(28.02+33.76)=0.907	(48.93-30.30/48.93)×100=38.07

Different letters represent statistically significant differences ($p<0.05$). DEN: [a]$p=0.048$, [b]$p=0.016$ Statistically different from that of the control group.

Discussion

The present work aimed to evaluate the macro- and microscopic hepatic changes induced by short-term multiple DEN administration (8 consecutive weeks) in ICR mice, without any promoter agent, in order to report the histological features of pre-neoplastic, neoplastic and non-neoplastic lesions. Chemically-induced cancer in mouse models, such as the DEN mouse model, should be able to recapitulate the multifaceted relationship between the tumor and its surrounding microenvironment which is absent in in vitro systems (30).

Considering the acute stage as a result of the consecutive 8-week repeat-dosing toxicity test, the first mouse liver samples collected (18 h after the last DEN injection) were characterized by recurrent and intense hydropic degeneration, necrosis and apoptosis. Current literature stresses the timing of initiation with DEN as critical due to the fact that hepatocytes are still actively proliferating in infant mice (17, 30-33). Despite the age differences of our DEN-exposed mice (six weeks), the results are consistent with current knowledge regarding DEN pharmacokinetics, which undergoes metabolic activation and acts as a complete carcinogen in mice younger than two weeks (17, 30, 34); when administered in older mice, tumor promotion is required (*e.g.* phenobarbital; carbon tetrachloride) (17, 22). Despite the absence of a promoter agent, and mimicking NOC exposure in older organisms, the increased mitotic index observed in DEN-treated groups reflects reactive cell proliferation as a response to replace destroyed hepatocytes. These findings are in line with our previous report that described acute damage involving major mitochondrial enzymatic complexes and increased activity of enzymes

Table II. *Timeline evolution features from livers of diethylnitrosamine (DEN)-treated mice: Macroscopic nodular masses induced by DEN and estimated dimensions. Macroscopically visible hepatic nodules were counted and measured as described in the Materials and Methods. No macroscopic alterations (nodular masses) were noted at T1 and T2. Time in weeks post-exposure: T1: 8 weeks; T2: 15 weeks; T3: 22 weeks; T4: 29 weeks; T5: 36 weeks; T6: 40 weeks.*

Time of euthanasia	No. of nodular masses per DEN-treated group	Largest diameters measured (mm)
T3	6	2×1×1
	1	6×5×2
T4	1	3×3×1
	1	4×5×2
	1	5×5×5
T5	6	2×1×1
T6	3	2×1×1
	49	2×2×1
	11	3×2×1
	5	3×3×1
	1	3×4×2
	1	4×4×1
	1	4×7×2
	1	5×4×1
	2	5×5×3
	1	6×4×2
	1	10×8×4
	1	10×11×4

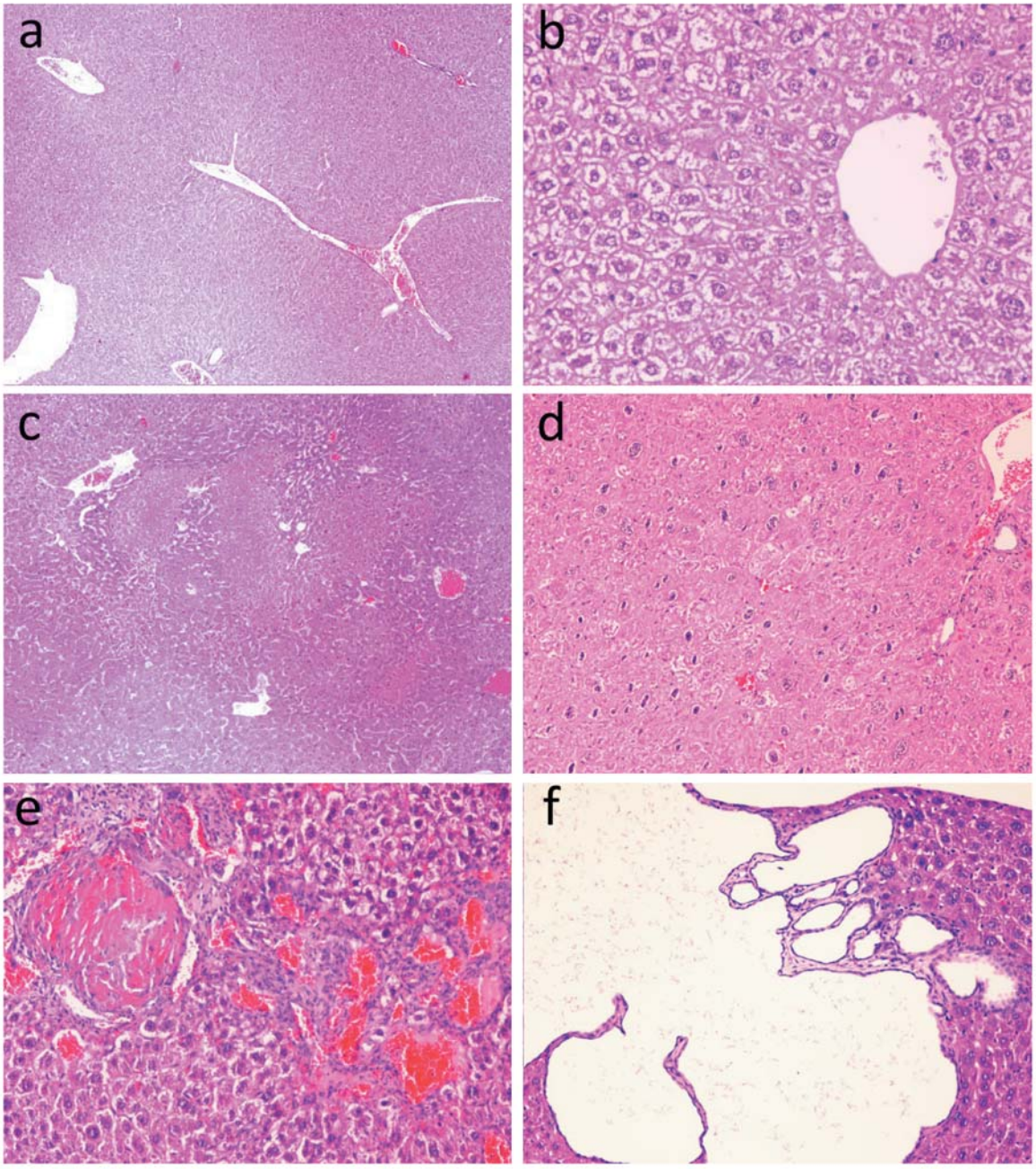

Figure 3. *Histological hepatic changes in diethylnitrosamine (DEN)-treated and control male ICR mice. a: Normal liver histology (control mouse at T6); hematoxylin and eosin (H&E), ×40. b: Hydropic degeneration (DEN-treated mouse at T1); H&E, ×200. c: Regionally-extensive necrotic areas (DEN-treated mouse at T3); H&E, ×40. d: Coagulative necrosis. Note nuclear changes with a condensed chromatin pattern (DEN-exposed mouse at T4); H&E ×100. e: Peliosis hepatis (DEN-treated mouse at T5); H&E, ×100. f: Biliary cysts (DEN-treated mouse at T6); H&E, ×40. Time in weeks (post-exposure): T1: 8 weeks; T2: 15 weeks; T3: 22 weeks; T4: 29 weeks; T5: 36 weeks; T6: 40 weeks.*

involved in controlling oxidative stress (17). Unrepaired DNA damage produced at this stage is likely to trigger the development of later pre-neoplastic and neoplastic lesions. The occurrence of biliary cysts and vascular lesions such as *peliosis hepatis* has also been documented in DEN-treated mice (32).

In the present study, the incidence of hepatocellular hyperplastic foci (up to 6 foci/10 animals) was lower than that obtained by Kushida *et al*. (up to 26 foci per 10 animals) (32). However, the DEN dose used (35 mg/kg) was lower compared to those tested by Kushida *et al*. (25, 50 and 75 mg/kg, in 8

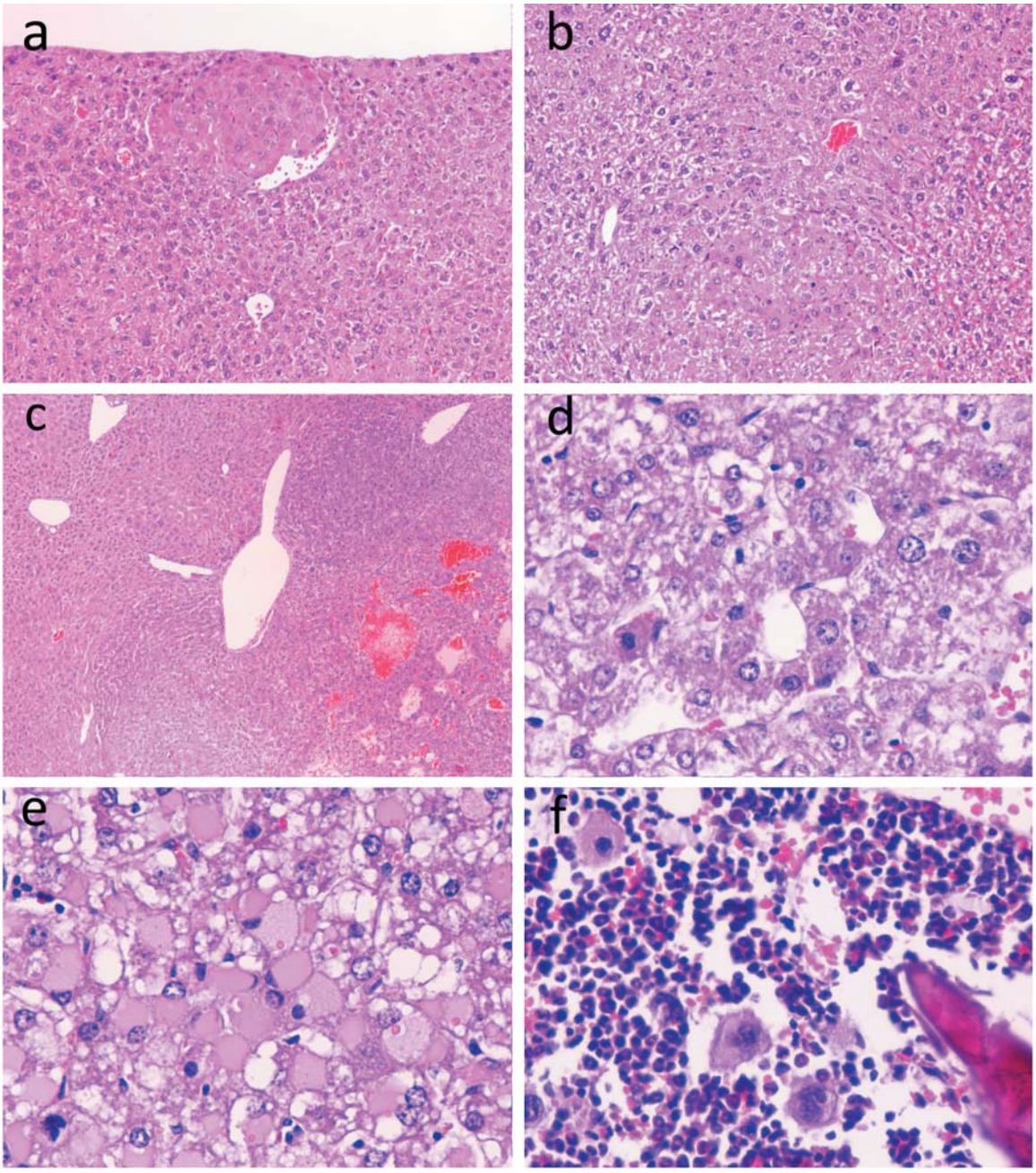

Figure 4. *Proliferative hepatic lesions in diethylnitrosamine (DEN)-treated male ICR mice. a: Hyperplastic focus (DEN-treated mouse at T4); hematoxylin and eosin (H&E), ×100. b: Diffuse dysplasia (DEN-treated mouse at T5); H&E, ×100. c: Hepatocellular adenoma (DEN-treated mouse at T4); H&E, ×40. d: Hepatocellular carcinoma. Note focal pseudo-acinar differentiation and irregular thin trabecular pattern (DEN-treated mouse at T6); H&E, ×400. e: Hepatocellular carcinoma. Note variably-sized intracytoplasmic hyaline bodies and scarce signet-ring morphology (DEN-treated mouse at T6); H&E, ×400. f: Bone marrow focus in hepatocellular carcinoma. Note bone trabeculae and several bone marrow precursor cells, with prominent megakaryocytes (DEN-treated mouse at T6); H&E, ×400. Time in weeks (post-exposure): T1: 8 weeks; T2: 15 weeks; T3: 22 weeks; T4: 29 weeks; T5: 36 weeks; T6: 40 weeks.*

weeks' consecutive treatment), a difference which might explain this discrepancy associated with the age of mice used in our research (three weeks older). On the other hand Kushida *et al*. (32), reported hyperplastic foci and adenomas occurrence at 33 weeks after the end of the treatment. At a similar time point, the present work at 32 weeks after the last DEN injection (T6 *i.e*. 40 weeks after first DEN exposure), our group identified HCCs. The occurrence of HCC in four out of eight exposed animals at

Table III. *Time-related evolution of histological liver lesions induced by diethylnitrosamine (DEN). Time is given in weeks post-exposure: T1: 8 weeks; T2: 15 weeks; T3: 22 weeks; T4: 29 weeks; T5: 36 weeks; T6: 40 weeks.*

	Toxic hepatic changes (no. affected animals/N)						Proliferative lesions (no. of identified lesions)					
Time of euthanasia	N	Necrosis	Apoptosis	Hydropic degeneration	Pseudo-nucleoli	Mitosis per HPF	Bile cysts	*Peliosis hepatis*	Hyperplastic foci	Difuse dysplasia	Hepatocelular adenoma	Hepatocellular carcinoma
T1	9	7/9	8/9	9/9	7/9	0-1, 9/9	0	0	0	0	0	0
T2	10	7/10	8/10	10/10	7/10	1-2, 9/10	0	0	0	0	0	0
T3	10	7/10	3/10	8/10	7/10	0-1, 2/10	0	0	0	0	0	0
T4	10	10/10	5/10	5/10	5/10	0-1, 3/10	0	0	6	1	1	0
T5	9	5/9	5/9	1/9	7/9	0-1, 4/9	0	1	3	9	0	0
T6	8	4/8	7/8	1/8	8/8	0-4, 8/8	3	2	4	8	0	4

HPF: High power field.

T6 also seems to diverge from results presented in a review by Minicis *et al*. suggesting a time of 100 weeks for HCC tumor development induced by DEN without any promoting agent (31). In our opinion these opposing results highlight the influence of the strain's genetic background on carcinogenesis. The tumors exhibited characteristic features, including cell ballooning, intra-cytoplasmic hyaline bodies and pseudo-acinar structures. Intra-cytoplasmic hyaline bodies from human HCCs have been suggested to consist of p62 and show variable positivity for ubiquitin and for cytokeratins (35). The presence of extramedullary hematopoiesis, with formation of intratumoral bone marrow foci is a particularly interesting feature of these tumors, as it points towards their primitive phenotype and may provide leads concerning their histogenesis.

Taking into account the existing similarities between DEN-induced lesions in experimental models and those observed in patients with cancer, the standardization and detailed characterization of experimental lesions becomes a priority, in order to allow adequate interpretation of results and inter-study comparisons.

The consecutive time points chosen for euthanasia and data analysis allowed us to provide a wide-ranging timeline overview of DEN-induced hepatic lesions, comprising acute toxic lesions to malignant neoplasms. The protocol proposed in the present work [ICR strain and age of mice (six weeks) at first *i.p*. exposure to DEN, without any promoter agent] accomplished the goal of inducing HCC. However, in order to refine this mouse model of DEN-induced HCC, it is important to increase knowledge concerning the timeline of histological features as a consequence of NOC exposure.

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