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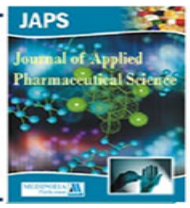

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Expression of Ki 67 in hepatocellular carcinoma induced by diethylnitrosamine in mice and its correlation with histopathological alterations

Magda Ismail Youssef, Hala Maghraby, Eman Ahmed Youssef and Mohammed Mahmoud El –Sayed

Magda Ismail Youssef,
Eman Ahmed Youssef,
Mohammed Mahmoud El –Sayed
Histochemistry & Cell Biology Department, Medical Research Institute, Alexandria University, Alexandria, Egypt.

Hala Maghraby
Pathology Department Medical Research Institute Alexandria University, Alexandria, Egypt.

For Correspondence
Eman Ahmed Youssef,
Email:Emankhalifa_124@yahoo.com

ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and the prognosis still remains dismal, so the present work was planned to assess the prognostic value of Ki67 in mice model of HCC induced by diethylnitrosamine (DEN), in addition to its correlation to the histopathological changes. Forty male Swiss albino mice were randomly divided into two groups; group I: 10 mice were served as controls and group II: 30 mice were injected i.p with DEN at a dose of 75 mg/kg b.w once /week for three weeks then 100 mg/kg b.w for another 3 weeks. Mice were sacrificed after 6, 12 and 18 weeks from the beginning of the experiment. Liver specimens were processed for histopathological examination and immunohistochemical expression of Ki67. **Results** of histopathological study revealed spotty necrosis with enlarged nuclei and cholestasis 6 weeks after DEN injection. Proliferation of bile ducts, perivenous focal apoptosis and increased number of cells acquiring large nuclei were prominent after 12 weeks. Deleterious effect of DEN was obvious after 18 weeks; where HCC features were seen as sheets of malignant hepatocytes, multinodular areas of coagulative necrosis and nodule of ghosts' necrotic hepatocytes. Collagen deposition was time dependent and showed maximum level around and within nodules in HCC after 18 weeks. Immunohistochemical expression of Ki67 showed increased positivity after 6 and 12 weeks and the highest increase in the number of Ki67 positive cells after 18 weeks of DEN injection. Conclusion: Based on the previous data, it could be concluded that ki67 can be used as a biological marker for prognosis of HCC.

Keywords: HCC, DEN, Ki 67, histopathology and immunohistochemistry.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy in liver and the third leading cause of cancer deaths worldwide, with few effective therapeutic options for this severe disease (Ferenci *et al*., 2010 and Llovet & Bruix , 2008) . Most HCC appears in cirrhotic livers after years of chronic liver inflammation caused by hepatitis viral infection, alcoholic and non-alcoholic steatohepatitis (El-Serag & Rudolph, 2007 and Asahina, 2010)**.** HCC detected after the onset of symptoms has a dismal prognosis (0%-10% 5-year survival) (Llovet *et al*., 2003).

The common denominator in HCC of different etiology is the induction of oxidative stress by inflammatory cells, resulting in chronic hepatic injury and cell death, followed by oncogenic transformation of surviving hepatocytes and compensatory proliferation that leads to tumorgenesis (Fausto, 1999 and Maeda *et al*., 2005). Diethylnitrosamine (DEN) is a representative chemical of a family of carcinogenic n-nitroso compounds (Liao *et al*., 2001). DEN was carcinogenic in all animal species, and that there was sufficient evidence of a carcinogenic effect to classify DEN as a probable human carcinogen, despite the lack of epidemiologic data (Poirier and Beland, 1994). Administration of DEN to animals has been shown to cause cancer in liver and, at lower incidences, in other organs as well (Verna *et al.*, 1994). N-nitroso compounds can act as alkylating agents, either directly or after metabolic activation by cytochrome P450 enzymes which are the key enzymes in tumorgenesis (Saffhill, 1985). Increased generation of reactive oxygen species (ROS) and decreased antioxidant enzymes in liver tissue has been reported in many models of diethyl nitrosamine -induced HCC (Ramakrishnan *et al*., 2006 and Sivaramakrishnan *et al*., 2008). ROS play a major role in tumor promotion through interaction with critical macromolecules including lipids, DNA, DNA repair system and other enzymes (Kweon *et al*., 2003 and Yadav & Bhatnagar, 2007).

Cell proliferation is considered to play an important role in the several steps of carcinogenesis process. Ki67 is one of the many antigens protein has been used as proliferation marker for cancer cells (Lin *et al*., 2000). The assessment of the presence of cell cycle-related proteins may yield important information about the biological behavior of a tumor (Mazen *et al*., 2004). Ki67 is a protein associated with active cell proliferation and expressed in all phases of the cell cycle, except G0, with the highest expression seen in G2/M (Guzman *et al*., 2005). The monoclonal antibody of Ki67 has been developed and used in evaluating cellular proliferation rates of malignant tumor (De Riese *et al*, 1993 and Barbareschi *et al*, 1994). Ki67 expression was associated with prognosis in prostate, breast and lung cancer (Bubendorf *et al*, 1998; Scholzen & Gerdes, 2000; Mathieu, 2004 and Martin *et al*., 2004). In contrast, an inverse association was observed with cervical cancer prognosis (Graflund *et al*., 2002) and no association was found with prognosis in patients with colon and pancreatic cancer (De-Jong *et al*., 1998 and *Stanton et al*., 2003). Ki67 expression could represent a valuable tool in the understanding of hepatocellular carcinoma. The evaluation of ki67 in hepatocyte proliferative index has been suggested as a useful tool for analyzing liver regeneration and carcinogenesis processes, identifying cirrhotic patients at risk for developing HCC, discriminating between normal, regenerative, and neoplastic liver in cytological or microhistological samples, and predicting survival hepatectomized patients with HCC (Tarao *et al*., 1992 and Ojanguren *et al*., 1993) . **The aim** of the present study was to assess the prognostic value of Ki67 in mice model of hepatocellular carcinoma (HCC) induced by diethylnitrosamine (DEN) and their correlation to the histopathological findings.

MATERIALS AND METHODS

The present study was carried out on forty male Swiss albino mice weighting 15-18 g. obtained from Animal Unit of Medical Research Institute, Alexandria University. They were housed in stainless steel cages at a constant temperature (24 $\pm 2^0$C) with free access to food and water. Mice were then randomly divided into two groups. Group I (10 mice) was served as normal control. Group II (30 mice) was injected intraperitoneally with 75 mg/Kg b.w of diethylnitrosamine (DEN) once / week for 3 weeks, then 100 mg/Kg b.w for another successive 3 weeks (Oshi *et al*, 1999). Three control and five treated mice were sacrificed after 6, 12 and 18 weeks from the beginning of the experiment and the livers were immediately excised. Liver specimens were fixed in 10% neutral buffered formalin for histopathological and immunohistochemical studies.

Histopathological Studies

Hematoxylin and Eosin Stain (H&E)

Paraffin sections of 4µm thick were cut, processed and stained with hematoxylin and eosin (H&E) for studying histopathological changes (Bancroft and Steven, 2002)

I- Masson's Trichrome Stain

Sections were processed down to distilled water, mordant in Bouin's solution, stained with Weigert's hematoxylin and rinsed in running tap water. Sections were stained in Biebrich scarlet-acid fuchsin solution, washed in distilled water, differentiated in phosphomolybdic- phosphotungstic acid solutions, then lastly stained with fast green FCF and processed to be mounted (Kiernan, 2001)**.**

II- Immunohistochemical Study of Ki67

Deparaffinized sections were hydrated in a graded series of alcohol solutions. Sections were incubated in antigen retrieval (boiling the sections at 98°C for 20 minutes in 10 mmol/L sodium citrate buffer), treated with 3% H_2O_2 to block endogenous peroxidase. Monoclonal antibody (Anti-ki67, DAKO Corp.) were applied on the slides and incubated in humid chamber overnight in refrigerator at 4^0C. Secondary biotinylated antibody was then applied, followed by incubation with streptavidin peroxidase (DAKO Corp.). Sections were washed with phosphate buffer saline (PBS) three times after each step. Sections were stained with diaminobenzidine chromogen solution (DAB), and counterstained with hematoxylin (Horiguchi *et al*., 2007).

A semi quantitative estimation of Ki 67 based on the staining intensity and the percentage of positive cells was performed to evaluate the labeling index, where 5-8 fields per specimen were randomly selected (meanly 500 hepatocytes). Positive cells were counted in sequential high-powered fields (X400) and the results were expressed as the mean number of positive cells per limited surface area .Statistical analysis were determined using independent t-test and considered statistically significant at the $P< 0.05$

RESULTS

Histopathological results

Hematoxylin and Eosin Stain (H&E)

Control liver sections stained with H&E showed classical hepatic lobules. Each lobule showed anastomating plates of hepatocytes radiated from the central vein toward the periphery of the lobule. The liver sinusoids were seen in between the adjacent plates, Kupffer cells were also seen associated with the sinusoidal lining cells (Fig. 1).

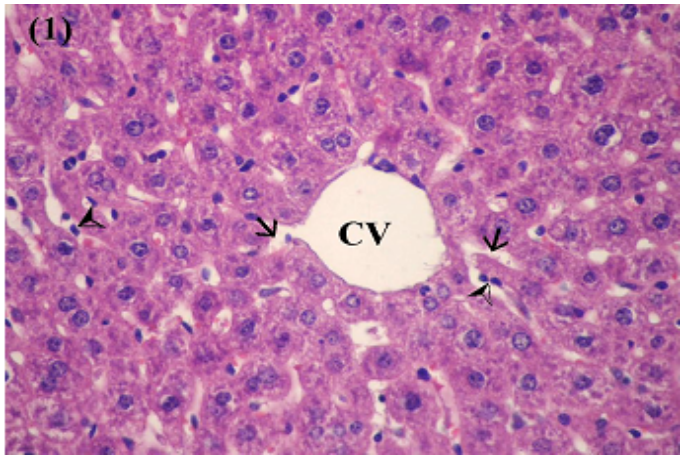


Fig. 1: Paraffin section of control mice liver showing cords of hepatocytes radiated from the central vein (CV). Kuffer cells (➢) are associated with sinusoids (↑). (H&E-bar = 50).

Six weeks after DEN injection, liver sections showed evidence features of necrosis varied from spotty necrosis with intralobular lymphocytic infiltration surrounded by intact hepatocytes to areas of confluent necrosis. Enlargement and darkening of nuclei with clumping of chromatin and cholestasis were also observed (Fig 2 a & b).

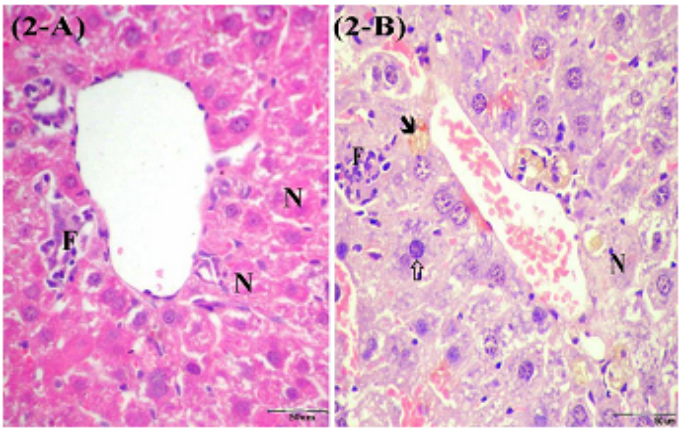


Fig. 2: Paraffin section of mice liver after 6 weeks of receiving DEN showing:
(A) Spotty and random coagulative necrosis of hepatocytes (N), and intralobular lymphocytic infiltration (F). (H&E-bar = 50)
(B) Enlargement & darkening of hepatocytes nuclei with clumping chromatin (⇧) , confluent necrosis (N), cholestasis (➚) and mild intralobular lymphocytic infiltration (F). (H&E-bar = 50)

After 12 weeks of receiving DEN, liver sections revealed evidence of dilatation and proliferation of bile ducts as well as dilatation of hepatic sinusoids with kupffer cell hyperplasia. Focal apoptosis with condensed eosinophilic cytoplasm and pyknotic nuclei were also seen beside the presence of disruption in endothelial lining of dilated vessel (Fig 3 a & b). A progressive increase in number of cells having large nuclei and open chromatin pattern with eosinophilic staining of their cytoplasm were observed. Few cells with intranuclear vacuoles and periportal lymphocytic infiltration were also seen (Fig3c& d).

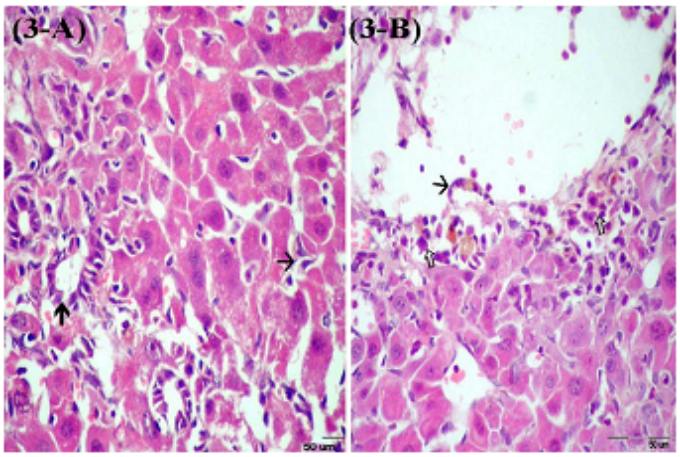
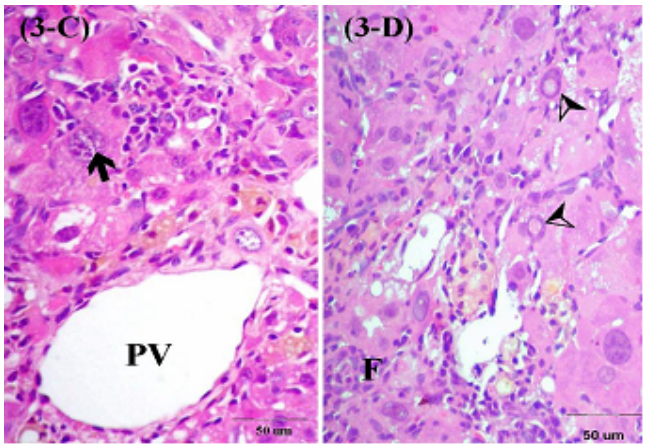


Fig. 3: Paraffin section of mice liver after 12 weeks of receiving DEN showing:
(A) Bile ducts dilatation and proliferation (➚) , dilatation of hepatic sinusoids with kupffer cell hyperplasia (→).(H&E-bar = 50)
(B) Focal apoptosis at perivenous area with pyknotic nuclei and condensed eosinphilic cytoplasm (⇧). Note endothelial disruption (→). (H&E-bar = 50)



(C) Hepatocyte with large nuclei, prominent and multiple nucleoli (↑). (H&E-bar = 50).
(D) Hepatocytes with large prominent esinophilic cytoplasm, hepatocytes with increase of nuclear cytoplasmic ratio, intranuclear vacules (➢), and moderate lymphocytic infiltration (F). (H&E-bar = 50).

After 18 weeks of DEN injection, liver sections showed disruption of hepatic architecture to the degree of complete disorganization with increase in inflammatory cells (Fig 4a). Liver nodules of solid HCC consisting of relatively giant hepatocytes with large nuclei and eosinophilic cytoplasm surrounded by fibrotic stroma were seen at higher magnification (Fig.4b). Finally, HCC features appeared as sheets of malignant hepatocytes, multinodular areas of coagulative necrosis and nodules of ghosts necrotic malignant hepatocytes (Fig.4 c & d).

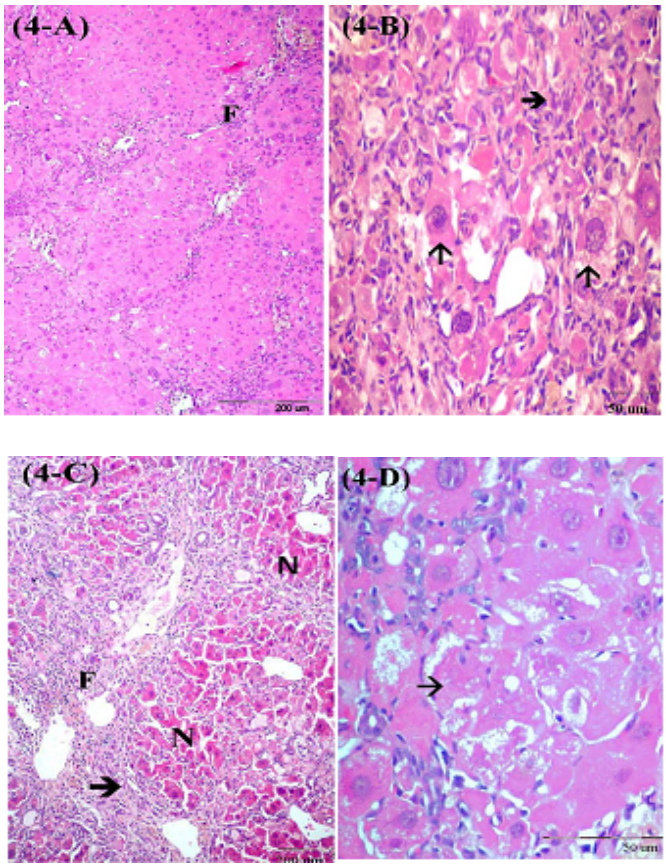


Fig. 4: Paraffin section of mice liver after 18 weeks of receiving DEN showing: (A)Loss of hepatic architecture with inflammatory cell infiltration (F). (H&E-bar =200). (B) Nodule of giant hepatocytes with large nuclei, abundant eosionophilc cytoplasm (↑) surrounded by fibrotic inflamed stroma (➔).(H&E-bar = 50). (C) Multinodular pattern of HCC with area of coagulative necrosis (N) separated by thick fibrous septa (➔)with moderate lymphocytic infiltration (F). (H&E – bar=200). (D) Nodule of ghosts necrotic malignant hepatocytes (➔). (H&E-bar = 50).

Masson's trichrome stain

Normal liver sections stained with trichrome showed a thin rim of collagen around central veins, portal tracts and sinusoids (Fig 5). Six weeks after DEN injection revealed a slight increase in collagen deposition in liver sections especially around the central veins and portal tracts (Fig.6).

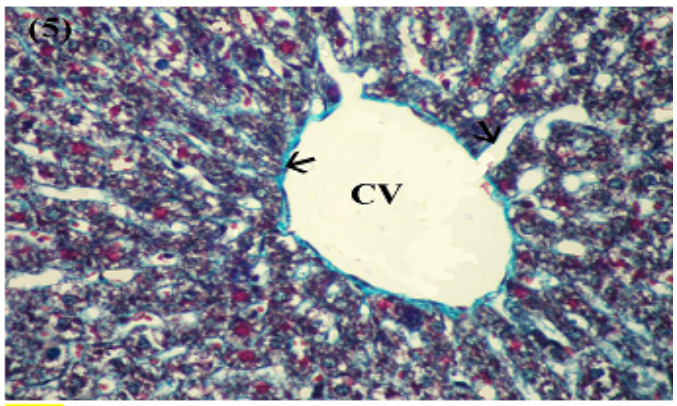


Fig. 5: Paraffin section of control mice liver showing a thin rim of collagen around central vein and sinusoids (↑). (Masson's trichrome – bar=50).

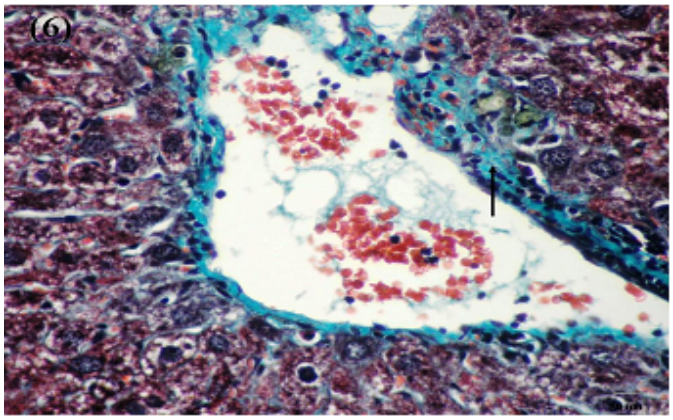


Fig. 6: Paraffin section of mice liver after 6 weeks of receiving DEN showing a marked increase in collagen (↑) perivascularly (immature short collagen fibers). (Masson's trichrome –bar=50)

After twelve weeks of treatment the collagen deposition markedly increased to be heavily accumulated perivascularly and within the portal tracts (Fig.7), till it reached its maximum level around and within the nodules in HCC specimens after 18 weeks of DEN injection (Fig 8 a&b).

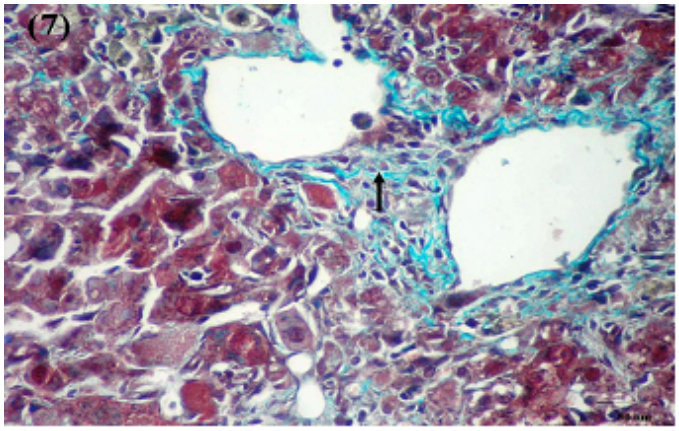


Fig. 7: Paraffin section of mice liver after 12 weeks of receiving DEN showing a marked increase in collagen (↑) perivas perivascularly (immature short collagen fibers). (Masson's trichrome –bar=50).

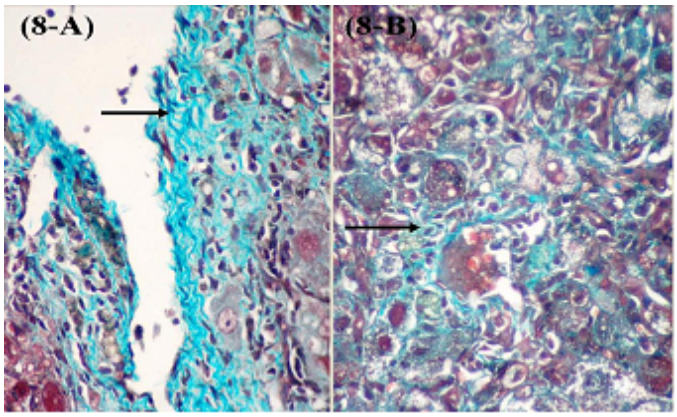


Fig. 8: Paraffin section of mice liver after 18 weeks of receiving DEN showing: (A) Heavily collagen deposition around portal tract (→). (Masson's trichrome-bar= 50). (B) Accumulation of collagen deposition around tumor cells (→) (Masson's trichrome - bar= 50)

Immunohistochemical results

Ki 67 immunoreactivity was localized in the nucleolus and nucleus mainly in the nuclear membrane. Its expression was estimated as the percentage of cells positively stained by the antibody. In control liver section there was a limited number of ki 67 positive cells as shown in figure (9) with mean labeling index 4±.547723.

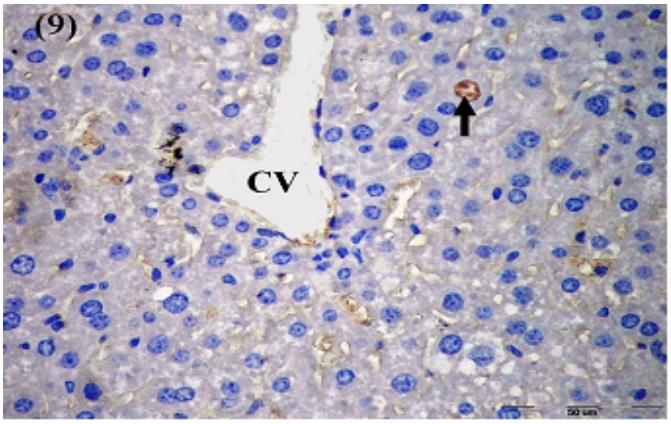


Fig. 9: Paraffin section of control mice liver showing a single Ki 67 positive cell (↑). (IHC of Ki 67- bar=50)

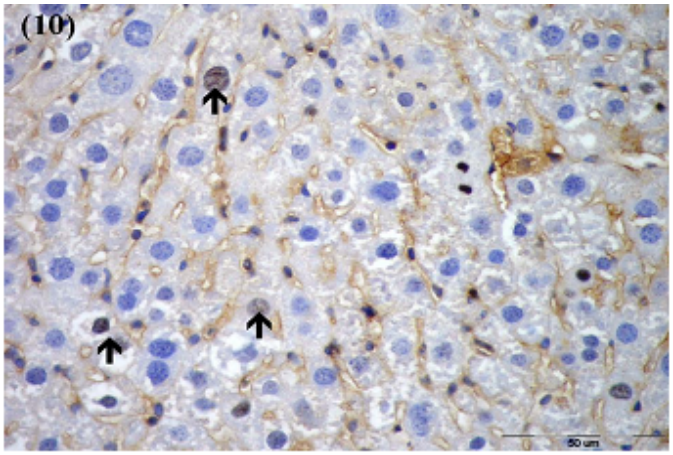


Fig. 10: Paraffin section of mice liver after 6 weeks of receiving DEN showing increase in number of Ki 67 positive cells as compared to normal one (↑). (IHC of Ki 67- bar=50)

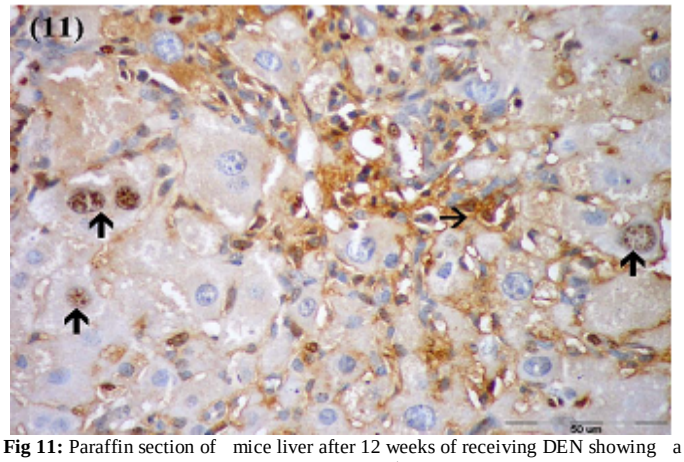


Fig 11: Paraffin section of mice liver after 12 weeks of receiving DEN showing a progressive increase of Ki 67 positive cells (↑) with strong immunostaining of lymphocytes is seen (→). (IHC of Ki 67- bar=50)

The number of Ki 67 positive cells was increased significantly ($p<.05$) in DEN treated group. After 6 weeks of DEN injection (Fig 10), the mean labeling index was 10±2.915476. After 12 and 18 weeks Ki 67 expression reached to high positive cells number with strong immunostaining of lymphocytes (Fig 11) with mean labeling index 14.4 ±3.911521 and 21.4 ± 6.228965 respectively. Some cells with cytoplasmic staining were found (Fig 12). These results were summarized and illustrated in table (1) and graph (1).

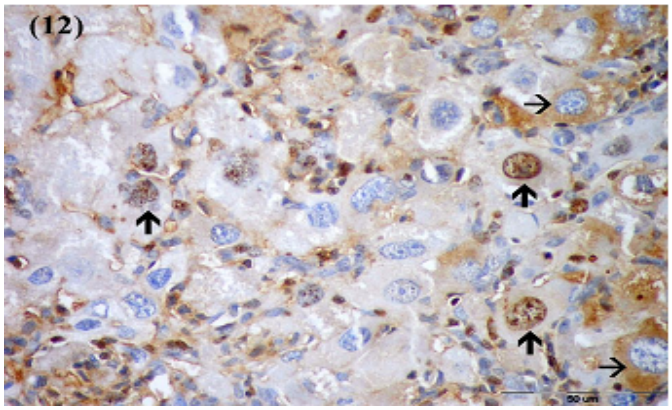
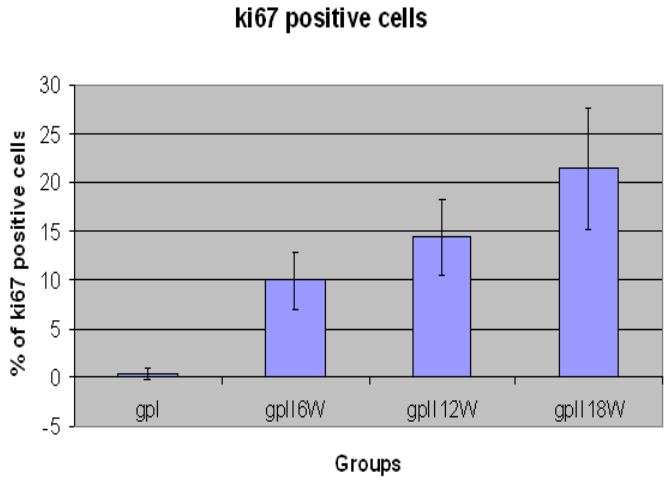


Fig. 12: Paraffin section of mice liver after 18 weeks of receiving DEN showing Ki 67 positive nuclei with strong intensity of immunostaining in tumor cells (↑). Note the cells with cytoplasmic staining (→). (IHC of Ki 67- bar=50).

Table: 1: The expression of ki 67 positive cells in control (Group I) and DEN treated group (Group II).

Groups / Mouse No	Group I (control group)	Group II (Treated group)		
		6 Weeks	12 Weeks	18 Weeks
1	0	12	18	30
2	1	7	17	14
3	0	8	15	20
4	1	9	14	18
5	0	14	8	25
Min	0	7	8	14
Max	1	14	18	30
Mean	0.4	10	14.4	21.4
SD	0.547723	2.915476	3.911521	6.228965
P value		0.001418	0.001419	0.001073



Graph. 1: Illustrating the number of ki-67 positive cells presented in table (1).

DISCUSSION

The prognosis of hepatocellular carcinoma (HCC) still remains dismal, although many advances in its clinical study have been made. So it is important for tumor control to identify the factors that predispose patients to death. Thus, the pathological and biological prognostic factors of HCC have been studied quite extensively (Qin and Tang, 2002). DEN plays an important role in induction of hepatic carcinogenesis via increased generation of ROS and decreased antioxidant enzymes in liver (Sivaramakrishnan *et al*., 2008). DEN metabolism is catalysed by enzymes of the mixed-function as cytochrome P-450. Cytochrome P-450-dependent monooxidase system and its metabolic activation is responsible for the onset of the toxic effects. Intermediate reactive compounds originating from DEN bioactivation have little affinity for the catalytic sites of conjugating enzymes and therefore, they may form covalent bonds with important cell constituents, thus inducing the onset of necrosis mutations and cancer (Schmitt *et al*., 1993). The histopathological results obtained in the current study, showed that DEN induced necrosis varied from vacuolar degeneration, spotty necrosis, confluent necrosis, nodules of ghosts necrotic malignant hepatocytes, and nodules of solid HCC containing giant hepatocytes with large nuclei. This liver necrosis which has been found in many of the animals causes collapse of the parenchymal framework of the liver, which can resemble fibrotic changes. Also progressive increase in numbers of cells acquiring large nuclei having open chromatin pattern with eosinophilic staining of their cytoplasm was observed. Inflammatory cell infiltration and focal apoptosis at perivenous area were also noticed. Few cells showing intranuclear vacuoles have been , kupffer cell hyperplasia and cholestasis have been observed.

In agreement, Chattopadhyay *et al*. (2005) demonstrated gross structural alterations in rats liver treated with DEN, with predominantly basophilic, eosinophilic and clear cell foci. They showed extensive vacuolation in the cytoplasm encircling the nucleus with masses of acidophilic materials and some nuclei in the cells were large and hyperchromatic with prominent and centrally located nucleoli. Similar findings were achieved by Li *et al*. (2007) who showed that treatment with DEN induced DNA damage, mutations and induction of HCC.

The current results were in accordance with many studies; Sadik *et al* (2008) showed HCC with enlarged hyperchromatic nuclei and scattered mitosis induced by DEN in rats. It was also revealed by Al-Rejaie *et al*. (2009) that DEN caused severe histopathological lesions in liver tissues; where central veins surrounded by extensive necrosis and inflammatory cell infiltration, clusters of hepatocyte necrosis, and portal tract with bile duct proliferation and marked atypia. Also Gang *et al*. (2009) showed that DEN injection at dose 50 mg/kg for 16 weeks resulted in several gray nodes in the liver with hemorrhage and necrosis on the surface. Their results revealed that most of the cancer nodules were surrounded by sclerotic tissue. In our study, cholestasis was observed in different liver sections. Reinehr *et al* (2005) reported that acute cholestasis is associated with oxidative stress. This can be explained by Miyoshi *et al*. (1999) whom reported that bile acids activate the mitochondrial apoptotic machinery, a characteristic of death receptor-mediated apoptosis in hepatocytes. Thus after bile duct ligation in mice, delayed hepatocyte apoptosis occurs mediated by induction and translocation of Bax, a pro-apoptotic Bcl2 family protein, to mitochondria.

Overproduction of extracellular matrix (ECM), including collagen, is a prepathological state that occurs as a consequence of severe liver damage so that Tanaka *et al*. (1991) showed that the activated hepatic stellate cells (HSCs) behave like myofibroblasts, and initiate vigorous collagen synthesis. In accordance, the study of George and Chandrakasan (1996) showed enhanced collagen deposition which may reflect increased HSCs activity and accumulation of connective tissue proteins, especially collagen, has been reported in dimethylnitrosamine (DMN)-induced liver injury. Hepatic stellate cells are primarily responsible for the increased collagen synthesis in the injured liver (Albanis and Friedman, 2001). As the liver become fibrotic, there are both quantitative and qualitative changes in composition of the hepatic ECM. The total content of collagen and non collagenous components increases 3-5 fold, accompanied by the shift in the type of ECM in subendothelial space from the normal low density basement membrane like matrix to interstitial type matrix containing fibril-forming collagens (Grigorescu , 2006)

In accordance, our results showed that collagen deposition of liver tissue in DEN treated group was markedly increased at 6, 12 and 18 weeks respectively as compared with control group and this increase was associated with the degree of tissue damage.

On the other hand, It has been reported by Liu *et al*. (2006) that growth factors, reactive oxygen species, and products of lipid peroxidation, are regarded as triggers that activate HSCs, and activates the transcription of collagen genes.

With regard to the Ki 67, Ito *et al*. (1999) indicated that the expression of Ki 67 and its levels are critical in evaluating multistage chemical carcinogenesis in rat liver, and its presence during all active phases of the cell cycle other than in resting cells makes it as an excellent marker for neoplasia.

The present study showed a distinct nuclear staining for Ki 67 in liver cells of all sacrificed mice treated with DEN. The number of Ki 67 positive cells were increased significantly ($P<0.05$) of DEN treatment at 6, 12, 18 weeks with labeling index of 10 ± 2.915476, 14.4 ± 3.911521 and 21.4 ± 6.228965 respectively as compared to control group. We also noticed that some cells with dysplastic changes were negative for Ki 67 expression.

Our results were coincided with that of Pizem *et al*. (2001) who stated that PCNA and Ki 67 were useful for proliferative activity assessment of hepatocytes and their expressions were higher in HCC than in non-neoplastic liver. They showed a statistically significant correlation between PCNA and Ki 67 proliferative indexes in HCC as well as positive correlation between tumor grade and Ki 67 index. In this context, Umemura *et al*. (2003) reported that high dose treatment with DEN induced cell proliferation associated with the DNA damage, mutations and induction of HCC.

Also the study of Guzman *et al*. (2005) showed that immunostaining of HCC lesions for Ki 67 was associated with higher mitotic activity. Tumor size and Ki 67 expression have been found to be risk factors of early recurrence after surgical resection. Koskinas *et al*. (2005) showed that cells with large dysplastic changes were negative for Ki 67 expression. So the Ki 67 was significantly higher in the non-malignant liver tissue of patients with cirrhosis and HCC as compared with cirrhotic specimens without HCC. They concluded that the expression of Ki 67 was highly significant related with the tumor grade. On the other hand, study of Yeh *et al*. (2000) on the model of human HCC with histological heterogeneity showed that dedifferentiation of human HCC induced telomerase activation and Ki 67 expression. On contrast, other clinical studies have demonstrated that hepatocyte proliferation decreased with the severity of cirrhosis, indicating that liver regeneration was suppressed in cirrhosis compared to non-cirrhotic injured livers (Clouston *et al*., 2005 and Lunz *et al*., 2005). In the present study, the cytoplasmic staining of Ki 67 which was observed in some sections at 18 week in DEN treated group was more interesting result. Faratian *et al*. (2009) reported that also cytoplasmic and membranous expression of Ki-67 has a prognostic value in breast cancer. Their explanations include cross-reactivity with other protein(s), technical artifact, or relocalization of Ki 67 within the cell.

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