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Research Article

Pharmacology for drug discovery

Histomorphological Progression of Diethylnitrosamine (DEN) Induced Hepatocellular Carcinoma Model in Rat

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Abstract: Hepatocellular carcinoma (HCC) commonly known as hepatoma is a frequently occurring liver cancer that occupies 75% of all types of liver cancers. It affects the hepatocellular cells which is the major cell of the liver. Some models can be developed for HCC in rats but it is time-consuming. Nitrosodiethylamine is a very popular carcinogenic drug, very useful to develop experimental animal models. The present study focuses on the establishment of an animal model through a simple approach of experimental protocol along with the duration of the experiment that leads to creating a scope for future studies associated with metastasis as well as anti-metastasis studies. For the present study 2 groups were formed where each group containing 16 rats. Phenobarbital was given for 7 days to each group after diluting with water at a concentration of 0.3g/L before administering the first DEN. The control group received the same number of vehicles for 8 weeks. 50mg/kg of DEN was received intraperitoneally by the animal of the 2nd group twice a week for eight weeks. Bodyweight, the weight of the liver, and all the blood parameters were analysed like alpha-fetoprotein, SGPT, SGOT, and ALP, and a comparison was made with the control group. After evaluating all biological and histological data it can be concluded that the outcome of the present study signifies a simple experimental protocol along with an appropriate experimental duration animal model which leads to help in the study of the mechanism of metastasis and anti-metastasis agent in near future. The current work also represents a straightforward experimental methodology that aids in the investigation of metastatic mechanisms and anti-metastasis agents, with phenobarbital serving as a good promoting agent.

Keywords: Hepatocellular Carcinoma (HCC), DMN, DEN, Phenobarbital, SGPT, SGOT, ALP, Anti-Metastasis Agents

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1. INTRODUCTION

Hepatocellular carcinoma (HCC) is a liver malignancy that develops from hepatocytes. It is known as the primary reason behind death due to cancer. Hepatocellular tumors include benign, malignant, and dysplastic lesions.[1] Based on 2019 WHO classification of liver tumors, HCC is divided into eight subclasses: Steatohepatitis, Clear cell HCC, Macrotrabecular HCC, Scirrhous HCC, Chromophobe HCC, Fibrolamellar HCC (FL-HCC), Neutrophil-rich HCC, lymphoepithelioma (LEL) carcinomas.[2] Past 5 years, HCC has had a survival rate of 18% for advanced-stage patients when no treatments are available and also for those who were initially not treated well.[3] The present study indicates the mechanism of the HCC and how it can be developed by inducing DEN. It is also noted that phenobarbital is a good promoting agent. In the future, this study may help to determine the proper mechanism of some anticancer drugs. This model may help in the several types of research works associated with hepatocellular carcinoma.

1.1 Risk Factors

Geographical areas affect the etiology of HCC. HCC is mainly caused by HBV infection in countries like Sub-Saharan Africa, Alaska, and Asia, where HCC is common, but cirrhosis due to alcohol or chronic viral infection is the major cause of HCC in low-risk areas or countries.[4] Nearly 90-95% of these HCC tumors result from chronic infections caused by hepatitis B & hepatitis C virus as well as toxins like aflatoxins, alcohol, or pyrrolizidine alkaloids also cause HCC. In addition, diabetes and obesity are the factors independently associated with HCC development.[5] HCC correlates with metabolic as well as disorders such as 1-antitrypsin disease, Wilson's disease, tyrosinemia, glycogen storage disease (type I & II), hemochromatosis, and porphyria. Additionally, cigarette smoking increases the risk of HCC development. It has become apparent in recent years that NASH (Non-Alcoholic Steatohepatitis), especially in the West, is the fastest-growing cause of HCC.[6]

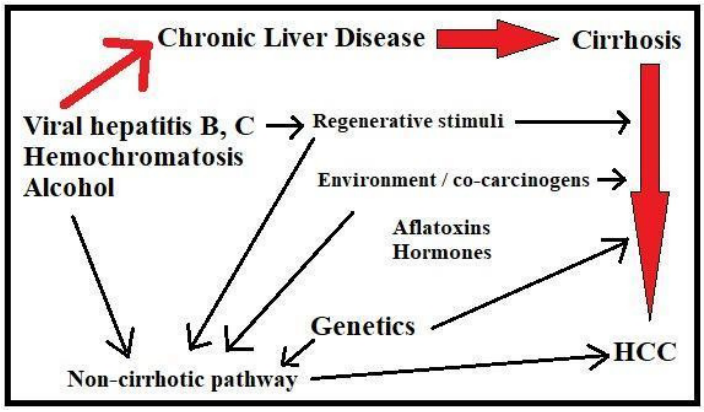


Fig 1: Pathobiology of hepatocellular carcinoma[7]

1.2 Epidemiology

A total of 841,080 new cases of liver cancer were recorded in 2018, making liver cancer the sixth most frequent type of cancer globally as well as the fourth most fatal malignancy worldwide.[8] Men were diagnosed with HCC at a higher rate than women (2.4:1), especially in Middle and Western Africa, Eastern and Southern Asia, Micronesia/Polynesia, and Melanesia. The age-adjusted liver cancer incidence rate among Alaska Natives and American Indians has increased from 1.6 to 4.6 per 100,000 people and hence, they have a higher age-adjusted risk of liver cancer as compared to Whites, Blacks, and Hispanics.[9] Those with HBV genotype A or genotype D may have a lower occurrence of HCC in India.[10]

1.3 Symptoms

The majority of the population who developed HCC already bears the symptoms of cirrhosis or chronic liver disease. Cancer patients either have deteriorating symptoms or are asymptomatic at the detection time. In addition to liver disease-associated symptoms like jaundice, unintentional weight loss, loss of appetite, and abdominal swelling, HCC can also include some non-specific symptoms like nausea, abdominal pain, feeling tired, and vomiting. (Fig 1).[11]

1.4 Treatment Strategies

Hepatocellular carcinoma treatment varies depending on the stage of the disease, the patient's capacity to undergo surgery, and whether or not a liver transplant is available. The ideal treatment depends on the severity of the case.[5] In Barcelona clinic liver cancer (BCLC) staging (Fig 2), curative options are provided for preliminary stage HCC patients while palliative treatments are available for patients with HCC at intermediate-stage as well as advanced-stage.[3] These are some following treatment strategies that are used to treat hepatocellular carcinoma.

- ***Surgical therapy of hepatocellular carcinoma*** [5, 9, 12]

❖ Surgical Resection of Liver
❖ Liver Transplant Surgery

- ***Non-surgical treatment of hepatocellular carcinoma*** [9, 13-17]

❖ Transarterial Chemoembolization
❖ Transarterial Radioembolization
❖ Ablation:
✔ Radiofrequency ablation
✔ Cryotherapy
✔ Percutaneous ethanol injection (PEI)
❖ Systemic Therapy

- ***Nanotechnology-Based Treatment***

Nowadays, nanotechnology has become a popular strategy for the diagnosis and treatment of cancer as well as for drugs targeting various types of tumors. The size of Nanomedicine is in the range of 1 to 100 nm and it contains nanomaterials that can be used for the detection, treatment as well as inhibition of cancer. Delivering anticancer agents via nanocarriers can increase their accumulation in tumor tissues as well as further reduce resistance to the drug.[18]

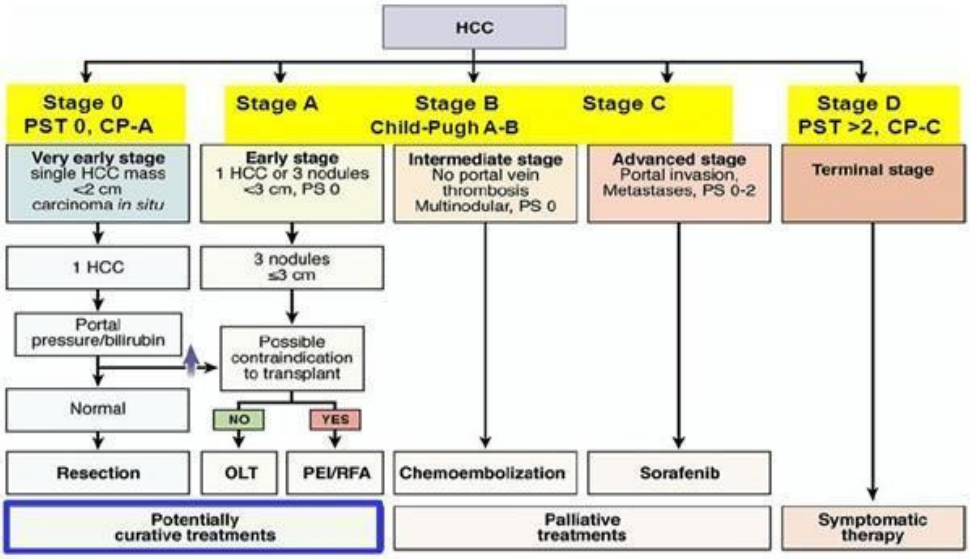


Fig 2: BCLC Staging System[8]

2. INTRODUCTION OF DEN

2.1 Chemical Name

N-nitrosodiethylamine; Diethylnitrosamine; 55-18-5; Diethylnitrosoamine; NDEA; N-Ethyl-N-nitrosoethanamine (Fig 3).

2.2 *Molecular Formula*: $C_4H_{10}N_2O$

2.3 *Molecular Weight*: 102.137 g/mol

2.4 Mechanism of action of DEN

In experimental animal models, diethylnitrosamine (DEN), also known as N-Nitrosodiethylamine, is commonly utilized as a carcinogen. DEN causes different cancers in mice, including those of the liver, gastrointestinal system, skin, respiratory tract, and hematopoietic cells, when given orally or by intraperitoneal injection. Because DEN does not cause cancer on its own, it must be bioactivated in the liver by cytochrome P450 (CYP) enzymes, resulting in DNA adducts formed through an alkylation mechanism. The DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT), also known as O6-alkylguanine-DNA alkyltra, can remove these alkylation adducts.[19]

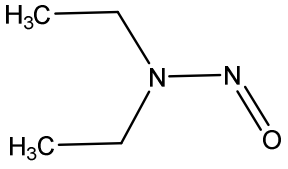


Fig 3: Structure of DEN

3. PATHOGENIC MECHANISM OF LIVER DAMAGE

High doses of DEN like nitrosamines which are highly carcinogens in nature can induce hepatic carcinoma in rodents after continuous oral or parenteral administration.[20-21] The irreversible carcinogenic effect can be seen by administering DEN at 10-90 mg/kg body weight.[22-23] In the case of rats, mice, rabbits, dogs, and guinea pigs 25mg/kg of DMN acts as a potent carcinogen, mutagen, hepatotoxin as well as immunotoxin. DMN gets metabolized into the liver by the cytochrome P450 enzyme and forms highly reactive metabolites which are further responsible for the formation of some methylated macromolecules like N7 methylguanine, O4 methyl thymidine, N3 methyladenine, and O6 methylguanine in DNA. These modified bases in DNA or RNA can cause mispairing of bases due to the transversions of guanine to thymine at apurinic sites. 3-methylcholanthrene and phenobarbital induce dealkylation of DMN and DEN producing reactive carcinogenic metabolites in rats and mice.[24-25] It has been proven that the number of induced liver neoplasms is directly proportional to the dose rate. Thus, non-neoplastic liver abnormalities like shrinkage of hepatic cells and hyperplastic nodules can be caused by low dose rates. The experiment with DEN has proven that it's highly reproducible to induce liver damage in a dose-dependent manner. Repeated administration of DMN for three weeks can cause total loss of proteins in the liver by increasing DNA content.[26] DEN indirectly causes oxidative stress in hepatocarcinogenesis. Another hepatotoxin Phenobarbital is used in combination with DEN to act on the

toll-like receptor 4 (TLR4) signaling to develop more hepatic inflammation, fibrosis, and ultimately setting of hepatocellular carcinomas. In this study, HCC was induced by DEN intraperitoneal injection and the dose was 50 mg/kg body weight. Here Phenobarbital was used as a promoter.[27]

4. MATERIALS AND METHODS

4.1 Materials

The list of chemicals is mentioned below in Table 1:

Table 1: List of the Chemicals

Name of the Chemicals	Name of the Industry
Diethylnitrosamine (DEN)	TCI Chemicals (India) Pvt. Ltd.
Phenobarbital (Gardenal 30)	Abbott Healthcare Pvt. Ltd.
Formaldehyde (95%)	Bengal Labs Pvt. Ltd.
Propofol (Propovan 10 mg/ml)	Bharat Serum
NaCl (0.9% w/v)	Nirlife Healthcare

4.2 Experimental Animals

Laboratory breed male Albino Wistar rats (110-160 g) were taken and they were housed. They were kept under laboratory conditions at 21 ± 2°C, relative humidity 60±3%, and 12:12 photoperiodic conditions.[28] During the experiment period, a standard diet along with water ad libitum was fed to them. All the experiments on animals were conducted as per the protocols under the approval of the Institutional Animal Ethics Committee (Protocol No: 1458/PO/E/11/CPCSEA; Ref: NCPT/IAEC-02/2018). All conditions were followed according to CPCSEA norms and "WHO guidelines for the care and use of animals in scientific research".

4.3 Experimental Design

Experimental groups: 2 groups were created where 16 animals in each group were placed.

- Control Group (Normal Control): Treated with Phenobarbital + Saline
- Positive Control (DEN induced rats): Treated with Phenobarbital + DEN

4.4 Methodology

Phenobarbital was diluted with drinking water at a concentration of 0.3 g/l seven days before of first DEN administration, it was administered to each group of animals as well as given simultaneously during the whole experiment to reduce the time of the experiment as well as to cause the enzyme induction.[27-28] The control group received the same number of vehicles for 8 weeks. 50 mg/kg of DEN was received by the 2nd group of animals intraperitoneally twice a week continuously for 8 weeks. Animals were sacrificed at different time points. At the end of the 2nd, 4th, and 8th week, 4 animals were sacrificed respectively at these periods from both groups after DEN treatment.[28-29] Before sacrifice, the animals were anesthetized by treated with propofol for collecting the blood samples from the orbital venous. Propofol was administered intraperitoneally.[30-31] The serum was collected and sent to a pathology lab for testing. After that, animals were sacrificed with cervical dislocation, and livers were quickly removed, weighed, and preserved in a 10% formalin solution. There was also the creation of histopathology slide preparation.[31]

4.5 Preparation of Histopathology Slide

First, the animals were decapitated and livers were isolated and kept in formalin fixative. 10% neutral buffered formalin is made from stock solutions. To make histological fixative from this, a 10% solution is needed.[27-28] One part of the stock formalin is mixed with nine-part water (preferably distilled water). The liver tissues were made and then stained with hematoxylin and eosin dye for photo microscopically determination of necrosis, steatosis, and fatty changes of hepatic cells.[29]

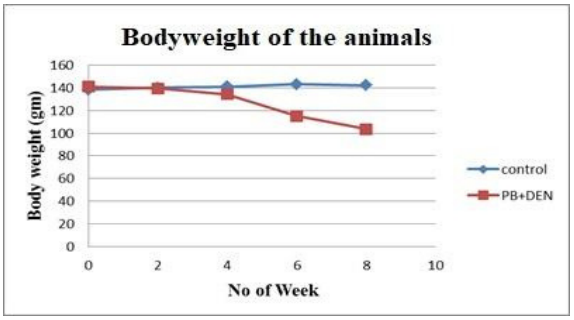
5. STATISTICAL ANALYSIS

The data obtained were analysed using GraphPad Prism software (Version 8.4.0). The student's (paired) "t" test was performed for analysis of comparison. The data were presented as mean ± standard deviation (SD). Probability value (P) of less than 0.05 was considered statistically significant. In statistical analysis, one-way ANOVA was also performed along with the student's t-test.[28-29]

6. RESULTS

6.1 Bodyweight (Mean ± SEM)

At the end of the 2nd, 4th, 6th, and 8th week, 4 animals were taken from each group and the bodyweight was calculated (Fig 4).



N= 4 no of animals. All the values are very significant p<0.05. Significant differences between means were evaluated by Student's t-test. Fig 4 signifies that the bodyweight was increased in PB+DEN treated group, whereas in the control group the bodyweight was increased.

Fig 4: Bodyweight of the animals

6.2 Weight of the liver (Mean ± SEM)

At the end of the 2nd, 4th, and 8th week, 4 animals were selected from each group and they are sacrificed by cervical dislocation. The liver was separated from each of the animals and weighed (Table 2).

Table 2: Weight of the liver

Groups Weeks	Control	PB+DEN
2nd	2.8 ± 1.2	3.4 ± 1.7
4th	3.2 ± 1.5	4.3 ± 1.6
8th	3.1 ±1.7	7.2± 1.5

N= 4 no of animals. All the values are very significant p<0.05. Significant differences between means were evaluated by Student's t-test. Table 2 signifies that the weight of the liver was increasing day by day in the PB+DEN group. In the 8th week, the weight of the liver is the highest of all due to the fatty liver. In the control group, the weight of the liver was quite normal.

6.3 Parameters of the Blood

6.3.1 At the end of the 2nd week (Mean ± SEM)

Table 3: Parameter of the blood (2nd week)

Group	AFP	SGPT	SGOT	ALP	TC	Lymphocyte
Control	33.25 ± 1.750	33.25 ± 1.750	36.50 ± 1.708	140.8 ± 2.529	2525 ± 85.39	31.25 ± 1.109
PB+DEN	92.00 ± 1.780	55.25 ± 1.750	57.75 ± 2.428	217.8 ± 1.652	4350 ± 155.5	43.25 ± 1.750

N= 4 no of animals. All the values are very significant p<0.05. Significant differences between means were evaluated by Student's t-test. Table 3 signifies that all of the parameters of the blood were found in the control and PB+DEN treated group at the 2nd week. In PB+DEN treated group all the values were increased.

6.3.2 At the end of the 4th week (Mean ± SEM)

Table 4: Parameter of the blood (4th week)

Group	AFP	SGPT	SGOT	ALP	TC	Lymphocyte
Control	55.75 ± 1.377	58.50 ± 1.848	43.25 ± 1.750	228.0 ± 1.826	2825 ± 110.9	34.00 ± 1.080
PB+DEN	263.8 ± 2.428	139.3 ± 1.750	127.8 ± 1.750	428.3 ± 1.887	10100 ± 168.3	55.00 ± 1.683

N= 4 no of animals. All the values are very significant p<0.05. Significant differences between means were evaluated by Student's t-test. Table 4 signifies that all the values were increased rapidly in PB+DEN treated group as compared with the control group. In the control group, all of the blood parameters were slightly increased. An increase in the blood parameters signifies the growth of hepatocellular carcinoma.

6.3.3 At the end of the 8th week (Mean ± SEM)

Table 5: Parameter of the blood (8th week)

Group	AFP	SGPT	SGOT	ALP	TC	Lymphocyte
Control	85.75 ± 2.323	87.50 ± 1.848	59.75 ± 1.109	328.0 ± 2.483	3200 ± 108.0	36.25 ± 1.109
PB+DEN	354.3 ± 2.056	389.5 ± 3.428	426.8 ± 2.689	616.0 ± 2.345	16100 ± 91.29	66.25 ± 1.377

N= 4 no of animals. All the values are very significant p<0.05. Significant differences between means were evaluated by Student's t-test. Table 5 signifies that all of the blood parameters were rapidly increased in PB+DEN treated group at the 8th week. In the control group, the parameters of the blood were also increased in lesser as compared with the PB+DEN treated group. It signifies that the carcinomas were growing by the changing of the parameters of the blood.

6.3.4. Graphical depiction of 8th week

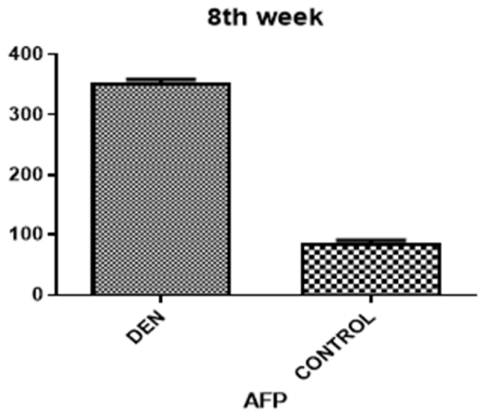


Fig 5: Graphical depiction of AFP at 8th week

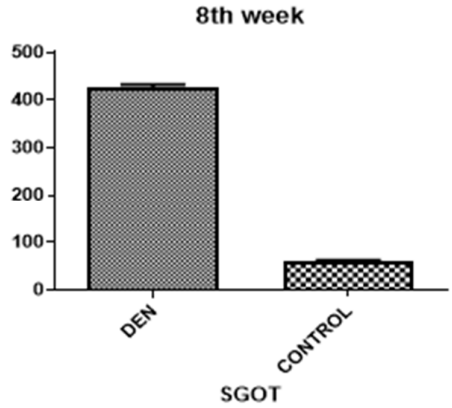


Fig 6: Graphical depiction of SGOT at 8th week

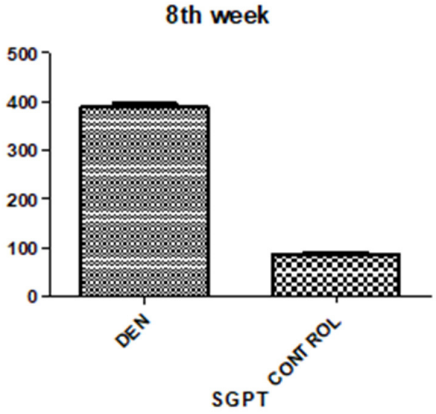


Fig 7: Graphical depiction of SGPT at 8th week

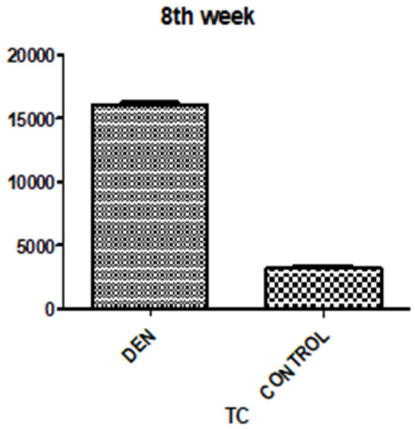


Fig 8: Graphical depiction of TC at 8th week

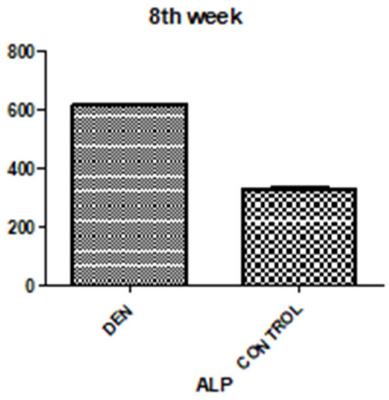


Fig 9: Graphical depiction of ALP at 8th week

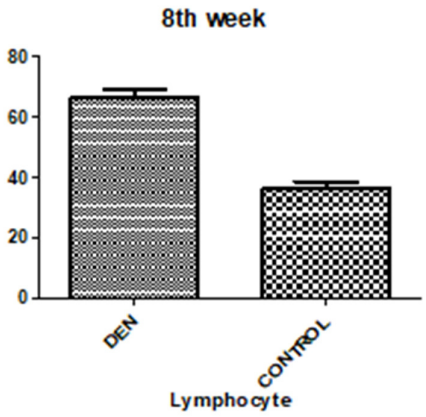


Fig 10: Graphical depiction of Lymphocyte at 8th week

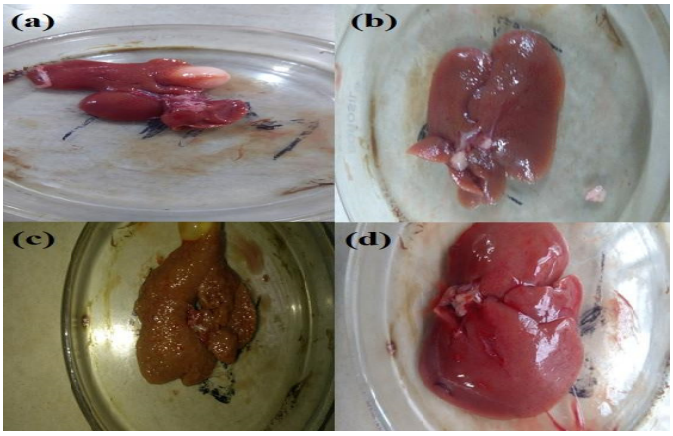


Fig :11 Indicates the condition of the liver. It also signifies how the hepatocellular carcinoma progressed. Fig 11(a), Fig 11(b), and Fig 11(c) indicate the stages of hepatocellular carcinoma in the 2nd, 4th, and 8th weeks.

Fig 11: (a) Liver of the rat at 2nd week of DEN treatment, (b) Liver of the rat at 4th week of DEN treatment, (c) Liver of the rat at 8th week of DEN treatment, and (d) Liver of the rat (control group)

6.4 Histopathological Study

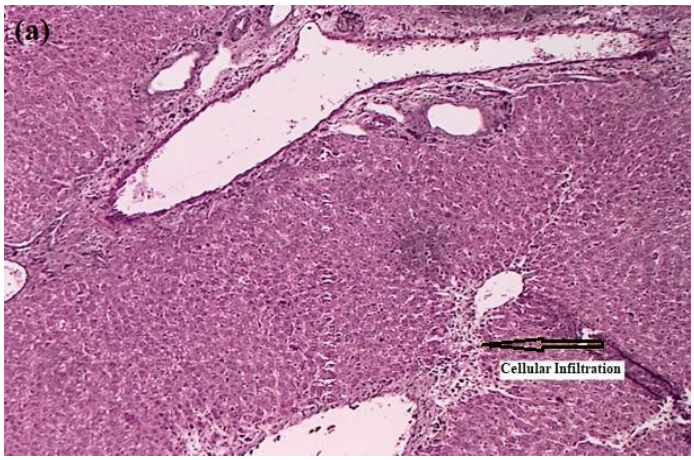


Fig 12(a): 10x magnification: (a) In the 2nd week of DEN treatment, the non-specific injury occurred such as cellular swelling, cellular infiltration

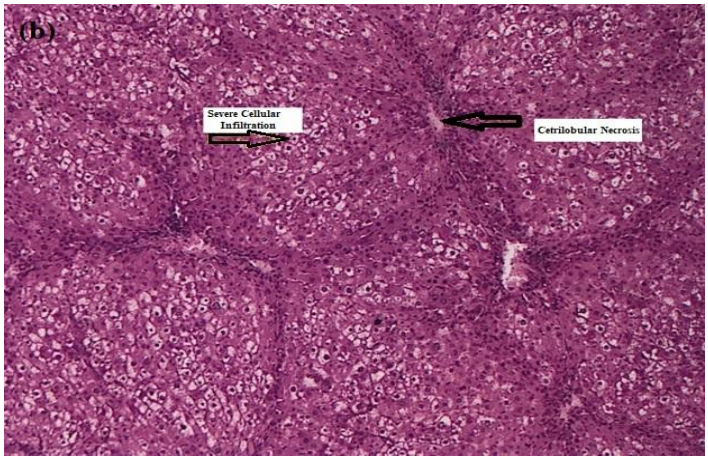


Fig 12(b): 10x magnification: (b) In the 4th week of DEN treatment, it was found signs of chronic damage such as severe cellular infiltration, centrilobular necrosis

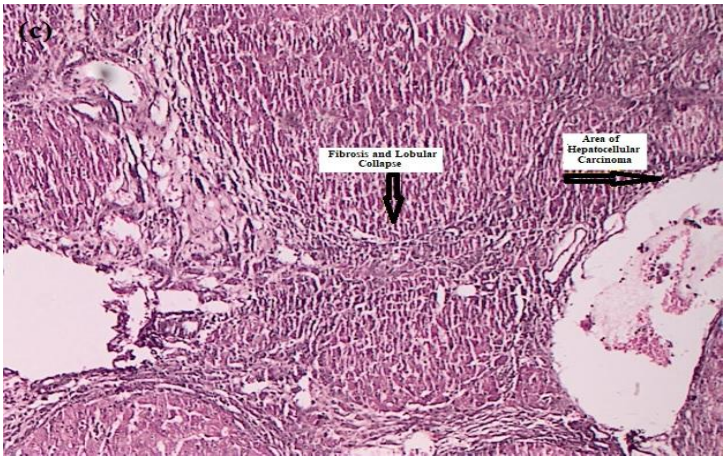


Fig 12(c): 10x magnification: (c) At 8th weeks of DEN treatment.

Well-differentiated Hepatocellular Carcinoma (HCC) was seen. It may be difficult to distinguish from normal hepatic parenchyma or hepatocellular adenomas. Three main architectural growth patterns were identified: trabecular (most common), solid and tubular, here trabecular pattern was shown, and severe fibrosis and lobular collapse were also detected

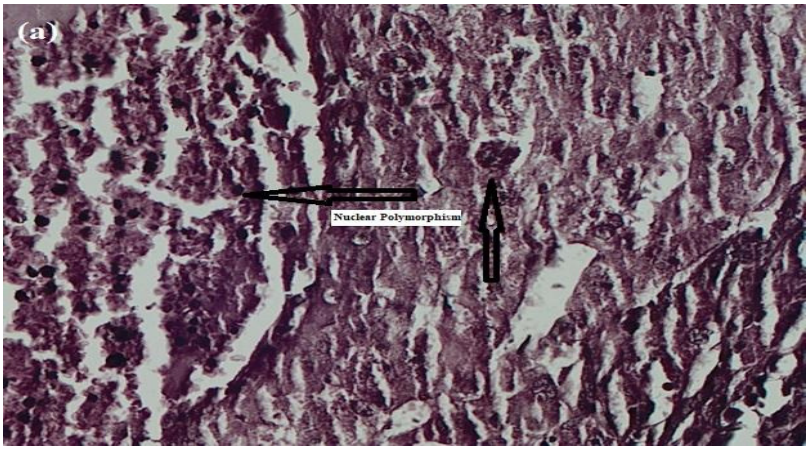


Fig 13(a): 40x magnification: (a) At 8th weeks of DEN, HCC with remarkable nuclear polymorphism, which is an important property of HCC or hepatocellular adenomas

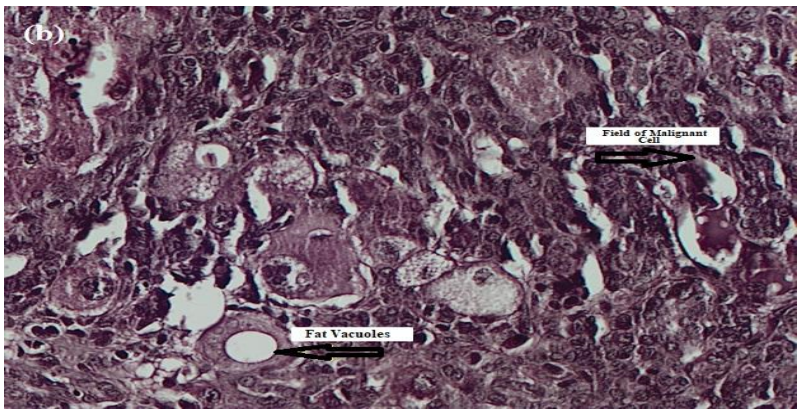


Fig 13(b): 40x magnification: (b) The neoplastic cells in hepatocellular carcinoma

As well as in adenomas, can synthesize and store various components of hepatocytes such as lipids, and alpha-fetoprotein. This finding can be of huge diagnostic importance in difficult cases. The presence of fat in well-differentiated tumors is very common. Large fat vacuoles (macro steatosis), very small fat droplets (micro steatosis)

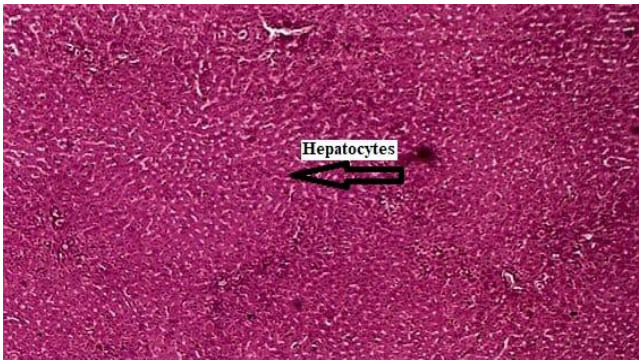


Fig 14 10x magnification: Control group, normal cellular architecture was present

7. DISCUSSION

The most frequent form of hepatic cancer is hepatocellular carcinoma (HCC), often referred to as hepatoma, which constitutes about 75% of all types of liver malignancy.[31-32] HCC begins in the hepatocellular cells, which are the major form of cells of the liver.[33] The majority of the HCC cases are caused either by hepatitis B or hepatitis C infections or by alcoholism-induced liver cirrhosis.[34-35] The compound that is most useful as a carcinogen is N-nitrosodiethylamine (DEN). The target organ where DEN causes malignant tumors varies by species.[35-36] The ability of DEN to alkylate DNA structures accounts for its carcinogenic potential.[36-37] The results indicate that the adaptation suggested via the present study, utilizing DEN 50 mg/kg twice-weekly related to the phenobarbital use at 0.3 g/L concentration with animal drinking water, 7 days before the first usage and subsequently all through the experiment.[37-38] It has contributed to the time needed for the progress of hepatocellular carcinoma and cirrhosis.[38] Hepatic toxicity is caused by the use of enzyme inducers, resulting in chemical-induced damage to the liver (Fig 11).[39-40] In this design, hepatocellular carcinoma (HCC) is determined in the 8th week (Fig 13a and Fig 13b), implying that in terms of survival after 8 weeks, all of the animals died within thirteen weeks, with some being dead by the 5th and 7th week. As per the study report, animals of the second group (DEN + phenobarbital) had lost bodyweight gradually for 4 weeks (Fig 4), followed by a rapid decline in bodyweight after the 4th week (Fig 4).[40-41] Abdominal edema was noticed in some animals, which is an important sign of chronic liver diseases such as HCC and cirrhosis, and reduced food consumption in animals was also seen after the 4th week (Fig 12b)[41]. After analysis, every parameter of blood such as SGPT, Alpha-fetoprotein, ALP, and SGOT are compared with the control group (Table 3 - Table 5 and Fig 5 – Fig 10).[42] It was discovered that every parameter of the group receiving DEN treatment was elevated corresponding to the week of the experiment, indicating the existence of hepatocellular carcinoma in rats (Table 5).[41-43] In the 8th week, many nodules were detected on the liver surface, with some measuring between 0.5 mm and 1.1 cm in diameter (Fig 12c, Fig 13a, and Fig 13b). In the DEN-treated animal group, liver weight was significantly greater as compared to the control group (Table 2).[43] The highest liver weight of the DEN treated group was recorded in the 8th week. In the 2nd week (Table 2), histopathological studies showed non-specific damages like cellular infiltration and cellular swelling.[43-44] Cellular infiltration was primarily discovered in 2nd week (Fig 12a). Signs of chronic damage, like severe cell infiltration and necrosis of the centrilobular tissue, were discovered in the 4th week (Fig 12b).[44] Lobular collapse and severe fibrosis were discovered in the 8th week (Fig 12c).[45] There was also substantial parenchymal loss, fat vacuoles, and nuclear polymorphism (Fig 13a and Fig 13b) whereas the cellular architecture of the control animal group was normal (Fig 14).[43-46] Hepatocellular carcinoma or adenoma was the cause of all of them.[47-48]

Highlights of the manuscript

- 50 mg/kg bodyweight DEN is induced to the development of HCC.
- 0.3g/L Phenobarbital was given for the progression of HCC.
- Phenobarbital is also a good promoting agent.
- The present study signifies a simple experimental protocol that helps in the study of the mechanism of metastasis and anti-metastasis agent.

8. CONCLUSION

It may be concluded that the pre-clinical models for hepatocellular carcinoma (HCC) used in this work are trustworthy and short-term pre-clinical models HCC. It is also suggested that DEN is the potentially carcinogenic chemical for the development of HCC and Phenobarbital is also a good promoting agent for the progression of HCC. The development of quick HCC was noticed along with fibrosis efficiently in rats with the administration of DEN and lobular collapse, extensive parenchymal loss, nuclear polymorphism, and fat vacuoles in histopathological evaluation. It is also found that the biochemical parameters are increased in DEN treated group as compared with the control group. So, it can be finally concluded that it is an animal model with a simple experimental protocol and an appropriate experimental duration that can help for further study of the mechanism of metastasis and anti-metastasis agents.

List of Abbreviations

AFB_1 - Aflatoxin B_1
AFP - Alpha fetoptotein
ALP - Alkanine phosphatase
ALT - Alanine aminotransferase
AST - Aspartate aminotransferase
AS rate - Age-standardised
BCLC - Barcelona Clinic Liver Cancer
PB - Phenobarbital
DEN - Diethylnitrosamine
DMN - Dimethylnitrosamine
ER - Estrogen Receptors
HCC - Hepatocellular carcinoma

HBV – Hepatitis B Virus
JAK - Janus Kinase
LPO - Lipid Peroxides
p53 - Tumor protein 53
Raf - Rapidly accelerated fibrosarcoma
RFA - Radiofrequency ablation
DNA - Deoxyribonucleic acid
RNA - Ribonucleic acid
SGPT - Serum glutamic pyruvic transaminase
SGOT - Serum glutamic-oxaloacetic transaminase
TACE Transcatheter arterial chemoembolization
TGF - α Transforming Growth Factor-alpha
TC - Total count
Wnt/β - Wnt signaling pathways

9. AUTHORS CONTRIBUTION STATEMENT

Mr. Kunal Mukherjee and Mr. Souvik Biswas designed the model, implemented the process, did the overall planning, and performed the statistical analysis. Mr. Dipan Roy checked the similarity index and formatted the manuscript according to the authors guideline. Ms. Payel Laha and Ms. Nabanita Pramanick reviewed the literature and wrote the manuscript accordingly. Mr. Arijit Das checked the spelling and language of the manuscript.

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12. CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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