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Anticancer effect of rosiglitazone in rats treated with N-nitrosodiethylamine via inhibition of DNA synthesis: an implication for hepatocellular carcinoma

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ABSTRACT

Rosiglitazone, peroxisome proliferator-activated receptor-γ (PPARγ) ligand, is a clinically tested drug used in the treatment of diabetes. Several reports have proved that rosiglitazone is involved in the regulation of glucose and lipid homeostasis, proliferation, inflammation and differentiation. The current study was conducted to exemplify the effect of rosiglitazone on experimental hepatic toxicity induced by N-diethylnitrosamine (DENA). The groups of rats were dosed as follows: Group I: normal control (2 ml/kg), Group II: rosiglitazone (40 mg/kg, b.w.), Group III: DENA (200 mg/kg, b.w.), Group IV: DENA+rosiglitazone (40 mg/kg, b.w.). All groups of animals were sacrificed after 22 weeks of treatment schedule and appraised for biochemical changes and alteration in antioxidant markers along with histopathological modulation in rat liver. Rosiglitazone significantly ($P<0.001$) altered the elevated levels of above serum markers along with the inhibition of free radical formation by scavenging the hydroxyl ions. It also restored the levels of lipid hydroperoxide (LPO) and significantly ($P<0.001$) modulated the levels of endogenous antioxidant enzymes in DENA mediatedhepatocellular carcinoma (HCC). Biochemical estimation of different hepatic markers, antioxidant enzymes and histopathological studies of liver tissues support its anti hepatocarcinogenic role in experimental

animals. In addition, *in vitro* cell line study clearly indicates that rosiglitazone acts as anticancer drug by inhibiting DNA synthesis.

Key words: Rosiglitazone, liver, hepatocarcinogenesis, PPAR gamma agonist

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major threat to human health, fifth most common cancer in the world and third leading cause of cancer-related deaths. Every year around 500,000 to 1,000,000 new cases of HCC are reported and annually approximately 750,000 people die from HCC.[1-3] In modern lifestyle, environmental factors and diet seem to be the major cause of cancer development. Changing the dietary consumption and composition can significantly alter the process of carcinogenesis.[4-5] N-diethylnitrosamine (DENA), a chemical carcinogen from N-nitroso family is known to be found in air, ground water, alcoholic beverages, processed meats, tobacco smoke, whiskey and foods like soybean, cheese, and smoked, salted and dried fish.[6] Some pharmaceutical drugs are also reported to produce DENA as a metabolite.[7] It is well documented that DENA and its metabolites generate reactive oxygen species and are responsible for its carcinogenic potential. Reactive oxygen species is known to induce DNA damage and tissue injury in animals.[8-9] The modern approach of chemoprevention for cancer control accepts alternative therapy options to reduce and minimize cancer related deaths. Many natural and synthetic compounds are known to have chemopreventive effect against carcinogenesis. Many preclinical and clinical trials are already going on for evaluating new chemical entities for their chemopreventive effects on severe malignancies.

Peroxisome proliferator-activated receptor-γ (PPARγ), an activated transcription factor from nuclear receptor superfamily of ligands, is known to play an active role in the metabolism of lipids, insulin sensitization of peripheral cells and anti-inflammatory response.[10-11] PPARγ ligand therapy is known to inhibit cancer cell proliferation along with diminution of liver fibrosis and it may play a potential role in tumor cell death in animal models.[12] In this class of drugs, pioglitazone, rosiglitazone, troglitazone, 15-deoxy-prostaglandin J2, and certain polyunsaturated fatty acids have been known for their ligand binding properties to exert the above mentioned effects.[13] PPARγ is expressed in organs like liver, lungs, small intestine, breast epithelium and adipose tissue.[10] PPARγ ligands have been evaluated for their cell differentiation and apoptotic role in the treatment of malignant disease proving their application as a potential chemopreventive agent against cancer development.[14] But still no studies have been conducted on biochemical estimation of enzyme alteration in cancer cell by PPARγ ligand compound/drugs. Therefore, the present protocol was designed to evaluate the role of rosiglitazone on DENA-induced hepatic cancer along with an estimation of various enzymes.

MATERIALS AND METHODS

Chemicals

Rosiglitazone was provided as a gift from Oscar lab, Baddi, Himachal Pradesh, India. Diethylnitrosamine (DENA) was procured from Fluka Chemicals, Switzerland. Other chemicals were of analytical grade and were purchased locally.

Animals

Sprague Dawley rats of weight 160–180 g and age of 7-8 weeks were procured from Indian Veterinary Research Institute Bareilly, U.P. (IVRI). The animals were maintained as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines at a temperature (25 ± 20 ^{o}C) and relative humidity (30-70%) with a 12:12 hr light-dark cycle in the departmental animal house facility under ambient conditions of the Siddhartha College of Pharmacy (SIP), Dehradun. The animals were kept on standard semi purified diet and water *ad libitum*. The project was approved by the Institutional Animal Ethics Committee (IAEC) of Siddhartha College of Pharmacy, Dehradun.

In vitro assessment of growth inhibition in HCC cell lines

Cell lines and cell culture condition

HCC cell lines Hep G2 and HuH-7 were established based on the reported method of Nakabayashi et al.[15] The cells were grown at 37°C in Dulbecco's Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (Life Technologies India Pvt. Ltd.), L-glutamine, penicillin-streptomycin (Himgiri Traders, Dehradun) and maintained in an incubator with 5% CO_2 and constant humidity. Sensitivity to rosiglitazone was studied in both the cell lines.

Preparation of different dilutions of the experimental drug

Rosiglitazone was prepared fresh before use by dissolving in dimethylsulfoxide (0.5 % of DMSO). The solution was serially diluted, mixed into complete media to obtain the desired concentration of the experimental drug in solution and then applied to the growing adherent cells. Vehicle concentration in the medium was maintained at 0.1% v/v for all concentrations. A dose ranging from 0.1 to 50 mM rosiglitazone was selected for this *in vitro* study (Plosker and Faulds, 1999).

Assessment of growth inhibition

Viable cell counting

Effect of rosiglitazone on cell growth was evaluated by direct cell counting method using a haemocytometer. 5×10^5 cells were seeded into 60 mm plates and after 24 hr, PPARγ ligand

rosiglitazone was added to culture media in 10, 25 and 50 mM concentration. After 48 hr, both floating and adherent cells were harvested and viable cells were counted by trypan blue dye exclusion. Relative rate of increment in cell counts in the presence of ligands compared with that of the control without drug was considered representative of cell growth.[16]

Assay for DNA synthesis

DNA synthesis was assessed by ^{3}H-thymidine incorporation. 10^5 cells were seeded into 24-well plates and after 24 hr culture in complete media, experimental drug in varying concentrations dissolved in media was added to the growing cells. After 24 hr treatment with drug, 1 µCi [methyl-3H]-thymidine (Himgiri Traders, Dehradun) was added to each well and incubated for further 6 hr. Afterwards, the cells were trypsinated and harvested onto a glassfibre filtermat by a cell harvester, dried for 1 hr and 3H-thymidine incorporation was measured using 1450 MicrobetaTM scintillation counter (Wallac Oy, Finland). Assays were performed in duplicate, the mean CPM values after normalization were analyzed for relative 3H-thymidine incorporation and repeated in triplate.[16]

Experimental design

Animals were randomly divided into four groups with 6 rats in each group. Group I, vehicle control animals were kept on pure drinking water and standard semi purified diet. Group II rats were on 40 mg/kg of rosiglitazone mixed in diet for total experimental period. Group III, DENA induced cancer rats (150 mg/kg body weight in drinking water). Group IV, Rosiglitazone (40 mg/kg mixed in / kg diet) was administered along with DENA (150 mg/kg in drinking water). Dose fixation study was performed to select the effective dose of rosiglitazone. After 16 weeks, animals were anesthetized with ether, serum was obtained by centrifugation after obtaining the blood from retro orbital plexus.

Rats were sacrificed and liver was excised, washed with saline (ice-cold) and further blotted to dryness. Liver was homogenated in phosphate buffer (0.1 M; pH 7.4), subjected to centrifugation for the clear supernatant component. The supernatant was then used for biochemical estimation of various parameters.

Biochemical Analysis

All the enzymes, namely, Alanine amino transferase (ALT), Aspartate amino transferase (AST), and Alkaline phosphatase (ALP), were assayed using standard kits (Siemens Healthcare Diagnostics Ltd, India). Total protein and total bilirubin were estimated through standard kits (Siemens Healthcare Diagnostics Ltd, India). Serum α-feto protein (AFP) was analyzed as per standard methodology.[17]

A portion of 10% of the liver homogenate was used for estimation of antioxidant enzymes such as lipid peroxidation, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and Glutathione transferase (GST) by using standard kits (Siemens Healthcare Diagnostics Ltd, India).

Histopathology

Liver tissue was reduced to small pieces and these pieces were fixed in 10% formalin, embedded in paraffin wax and 5- 6 micron sections were obtained, which were subsequently stained with hematoxylin and eosin for further study. All the sections of the tissues were examined under a microscope in order to see any evidence of altered architecture of the liver tissue due to DENA challenge as well as improved liver architecture from the test drug rosiglitazone. Tissue sections were examined under the microscope for histopathological changes and photographs were obtained. The photographic figures provide an evidence to improved architecture of the liver due to pretreatment with test drug and the models of our study (Dr. Lal Pathology Lab, New Delhi).

Statistical analysis

The results obtained were expressed as mean ± S.E.M., (n=6). One way ANOVA was applied to obtain statistical figures followed by least significant difference [LSD] test. $P<0.05$ was considered as significant.

RESULTS

Rosiglitazone induced growth inhibition

Trypan blue staining and direct counting of viable cells 48 hr after rosiglitazone treatment at the concentrations of 10, 25 and 50 mM showed a significant decrease in relative increment cell number in both cell lines (Fig.1).

Rosiglitazone inhibited DNA synthesis

Rosiglitazone induced inhibition of DNA synthesis was confirmed by 3H-thymidine incorporation in all the HCC cell lines after 30 hr drug treatment. There was a significant dose-dependent decrease in 3H-thymidine incorporation with all concentrations of rosiglitazone used starting from 1 mM in Hep G2, HuH-7. More than 95% inhibition was observed in both the cell lines at 50 mM rosiglitazone, about 80% at 25 mM and nearly 50% at 10 mM. Rosiglitazone induced inhibition of 3H-thymidine incorporation was found significant from 1 mM onwards in both cell lines Hep G2 and HuH-7 (Fig.2).

Effect of rosiglitazone on serum marker enzymes

Table 1 represents the effect of rosiglitazone on serum marker enzymes. Rosiglitazone treatment significantly (P<0.01) decreased the levels of AST, ALT, ALP, total bilirubin and AFP in serum and increased the total protein level when compared to DENA control. However, the levels of these enzymes and proteins were almost the same as that of the vehicle control group in the rosiglitazone control group.

Effect of rosiglitazone on antioxidant enzyme level

Table 2 represents the effect of rosiglitazone on DENA-induced lipid peroxidation. DENA significantly (P<0.05) increases the lipid peroxidation levels and it was significantly inhibited (P<0.05) in animals when armored with rosiglitazone. The antioxidant enzyme potential was reduced in the hepatic cells of animals administered with DENA. However, rosiglitazone+DENA treated animals showed significant (P<0.05) restoration of SOD, catalase, GPx and GST activities.

Histology

Histological examination (Fig 3 A) of vehicle control animals reveals normal hepatic cells with well outlined central vein. However, the changes in histological nature of the liver tissue in DENA control group were characterized by hepatic steatosis, centrilobular necrosis, macrovasicular fatty changes and disturbed portal vein architecture. (Fig 3 B). Individual rosiglitazone group (Fig 3 C) is similar to the normal control group. Rats treated with rosiglitazone + DENA exhibited almost normal architecture and absence of centrilobular necrosis and hepatic steatosis. (Fig 3 D).

DISCUSSION

The present protocol demonstrates the anticancer activity of rosiglitazone by its ability to reverse the levels of different hepatic marker and antioxidant enzymes, which were previously altered by DENA.

Estimation of serum AST and ALT levels suggests the normal or altered function of the liver. Serum levels of AST and ALT in group IV animals were altered when treated with rosiglitazone, intoxicated with DENA. The cytoplasm of liver cells is rich in ALT and AST.. Damage to hepatic cells results in leakage of liver specific enzymes (ALT and AST) into the plasma. Cellular damage and functional integrity of the liver cell membrane can easily be evaluated if there are increased levels of ALT and AST in serum.[17] The hepatoprotective nature

of a compound/drug can be assessed by its potential to protect or restore the normal liver functional mechanism that has been previously induced by a carcinogen like DENA.

Altered levels of ALP are indicative of pathological changes in biliary flow while high concentration of bilirubin in serum suggests increased degeneration rate of red blood cells.[18] DENA induces elevation of ALP and bilirubin in serum, two known markers for toxicity. Rosiglitazone shows effective control of the ALP and bilirubin levels in rosiglitazone+DENA group towards an early improvement in the secretory mechanism of hepatocytes.

The lower level of total protein is indicative of reduction in the biosynthesis of proteins due to destruction and dissociation of polyribosomes on the endoplasmic reticulum caused by toxicity of DENA.[19] Rosiglitazone restricted the protein synthesis by protecting the polyribosomes. The change in biochemical parameters of these enzymes has been further authenticated by histopathological studies. It is now well established by Liang-qi Cao et al. that among the altered proteins, septin 2 (SEPT2) was found to exhibit oncogenic function. PPARγ can alter the expression of SEPT2, which subsequently blocks the promoting effects of SEPT2 on HCC cell proliferation, invasion and its altered effect on cell apoptosis. Moreover, it has been further suggested that SEPT2 promoted HCC cell growth via upregulation of matrix metalloproteinase (MMP)-2 and -9, while simultaneously inhibiting the cleavage of caspase-3, -7, and -9. Hence, the expression and blocking of the oncogenic function of SEPT2 can retard the onset of cancer in HCC.[20]

Serum α-feto protein is a serum protein that is detected in elevated concentrations in conditions like HCC. It is a serum protein similar in size, structure and amino acid composition to serum albumin, but AFP is detectable only in minute amounts in the serum of normal adults. Elevated serum concentrations of this protein can be achieved in the adults by exposure to hepatocarcinogenic agents. AFP is a specific tumor marker for hepatocarcinoma. Its altered concentration in serum confirms the presence of HCC and represents the response of therapy to a particular drug in treatment of liver malignancies. The present research shows that the levels of serum AFP in DENA treated rats was significantly high as compared to vehicle control group, thus providing evidence for the occurrence of premalignant liver changes in DENA treated rats. The elevation of serum AFP in HCC was well documented by other researchers.[21,22] Co-treatment with rosiglitazone significantly reduced serum AFP.

Free radicals are known to initiate the process of lipid peroxidation, one major mechanism of corpuscle damage.[23] Administration of DENA is known for generating malondialdehyde and 4-hydroxy nonenal, the two byproducts of lipid peroxidation that may react

with various molecules leading to oxidative stress and carcinogenesis.[24] If not properly controlled then this change may initiate uncontrolled production of free radicals, which cannot be easily cleared by antioxidant enzymes produced by corpuscles.[25] SOD and catalase are known for their detoxifying mechanisms by converting superoxide anion and hydrogen peroxide into oxygen and water, respectively. GPx is known for its neutralizing property of reactive peroxides to alcohol and water. GST is used in detoxification of electrophilic compounds (phase 2 mechanism). There are many reports which suggest that GST plays significant and important role in protecting cells from reactive oxygen species. These reactive oxygen species are known for their catalytic role in damaging the lipid layer of cell membrane leading to the process of lipid peroxidation.[26,27]

Reduction in antioxidant enzymes such as SOD, catalase, GPx and GST was observed in hepatic carcinoma cells in the present research. Anything known to inhibit the generation of excessive free radicals can inhibit the process of carcinogenic activity in the body. Such studies lend support to our results that the activity of these antioxidant enzymes was brought back to almost normal levels in rosiglitazone-treated rats and, therefore, counter the initiation of carcinogenesis by DENA.

Thus, it may be concluded from the above discussion that rosiglitazone may play a significantly important role in restoring the elevated levels of marker enzymes and suppress the generation of free radical processes by scavenging hydroxyl ions. Furthermore, it modulates the levels of lipid peroxidation and markedly increases the endogenous antioxidant enzyme levels in DENA induced hepatocellular carcinogenesis. The upregulation of phosphatase and tensin homologue deleted on chromosome 10 gene (PTEN), which is involved in the inhibition of cell growth and the induction of cell apoptosis by rosiglitazone, is well documented suggesting the beneficial role of rosiglitazone in liver cancer therapy.[28] These results suggest the preventive influence of rosiglitazone on liver carcinogenesis in rats induced by DENA. Moreover, PPARγ, a low affinity dietary lipid receptor,[11] is abundantly expressed in adipose tissue and cells of hepatobiliary origin.[27-30] Functional PPARγ on human hepatocytes have been identified.[31,32] After binding to a ligand, it forms a heterodimer with the cis-retinoid acid RXR receptor, binds to a PPRE and activates transcription of selected genes.[33] Treatment of human hepatic cancerous cells with PPARγ ligands (15d-PGJ2 and troglitazone) arrested cell cycle and induced apoptosis.[16,31,34,35] Researchers also proved that PPARγ ligands showed anti-tumor effect mediated in part through accumulation of p21WAF1/Cip1, p27Kip1, and p18INK4c. [31,32,33] These investigations are further consistent with observations of the investigators that

pharmacological PPARγ ligands induce growth arrest, differentiation and apoptosis of several other cancer cells *in vitro* as well as in nude mice and increase the possibility that PPARγ may play a role as a tumor suppressor. [14,30,36,37,38] Several population based studies have tried to established the link between thiazolidinedione on cancer[39] but the relation was never clearly established with protein expression. Moreover, the researchers identified loss-of-function mutations in the PPARγ gene in certain human cancers.[38] PPARγ compounds are known to inhibit the human cell growth by inhibiting the process of angiogenesis.[40] Rosiglitazone PPARγ ligand is known to induce the apoptosis in human lung cancer cell.[41] From the available literature, it is evident that PPARγ deficiency induces susceptibility to tumorigenesis while rosiglitazone treatment is effective in reducing tumor size. In addition, it also induces antimetastatis effects.[42]

In our experiment, rosiglitazone induced growth inhibition in all the HCC cell lines. Compared to the rate of cell-count increment in drug-free control, rosiglitazone caused a significant inhibition in cell growth. Inhibition of cell proliferation was also evident in ^{3}H-thymidine incorporation assays, thus demonstrating rosiglitazone induced inhibition of DNA synthesis in HCC cell lines.[43,44] A similar dose response in inhibition of thymidine incorporation was also reported on colon cancer cells with troglitazone.[45] Hence, reduction in thymidine incorporation clearly establishes the mechanism for rosiglitazone by DNA synthesis inhibition. To support our claim on rosiglitazone, Chang et al have demonstrated in a recently reported study that rosiglitazone best works on liver cancer and colorectal cancer in type 2 diabetes mellitus.[46] Thus, it can be concluded from above results that rosiglitazone may play a significant role in the treatment of liver cancer.

Conflict of interest

No Conflicts declared

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Table 1: Effect of rosiglitazone on hepatic marker enzymes in DENA induced hepatotoxicity in rats

Group	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Total protein (mg/dL)	Total bilirubin (mg/dL)	AFP (ng/ml)
Vehicle control	103.72±6.18	31.82±1.93	126.16±8.25	7.91±0.71	1.62±0.55	21.03±1.42
Rosiglitazone alone	99.62±1.28	28.01±1.20	129.41±7.19	7.11±0.33	1.25±0.02	18.13±1.31
DENA	318.38±24.10[a]	102.42±7.25[a]	431.91±11.28[a]	4.17±0.61[a]	5.83±0.35[a]	304.75±3.56[a]
Rosiglitazone+DENA	196.03±17.61[a]	46.51±2.72[a]	172.81±7.01[a]	6.62±1.28[a]	2.19±0.17[a]	39.53±2.91[a]

Values are expressed as mean±S.E.M. (n = 6); Statistical significance [a]$P< 0.05$; Group III compared with with group I (Vehicle control); Group IV Comparisons are made with group 3 (DENA-induced).

Table 2: Effect of rosiglitazone on antioxidant enzymes in DENA induced hepatotoxicity in rats

Group	LPO (µM/mg Protein)	Catalase (nmol/min/ml)	SOD (U/ml)	GPx (µmol)	GST (U/min/mg Protein)
Vehicle control	6.10±0.51	0.87±0.14	1.12±0.82	7.10±0.36	0.15±0.01
Rosiglitazone alone	6.04±0.36	0.81±0.45	1.15±0.26	6.95±0.49	0.18±0.11
DENA	12.28±1.70[a]	0.47±0.31[a]	0.82±0.57[a]	3.37±0.34[a]	0.06 ±0.09[a]
Rosiglitazone+DENA	7.81±0.19[a]	0.76±0.47[a]	1.31±0.81[a]	7.02±1.81[a]	0.14±0.13[a]

Values are expressed as mean±S.E.M. (n = 6); Statistical significance [a]$P< 0.05$; Group III compared with with group I (Vehicle control); Group IV Comparisons are made with group 3 (DENA-induced).

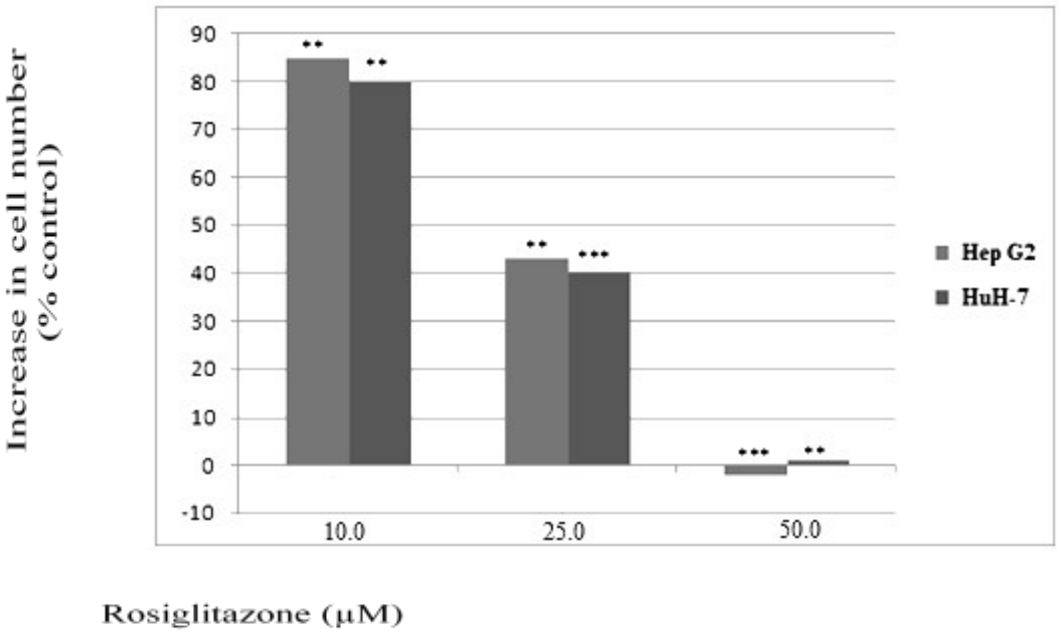

Fig.1. Effect of rosiglitazone on proliferation of hepatocellular carcinoma cell lines. Cells were incubated in presence or absence (control) of rosiglitazone and after 48 h, total number of viable cells was counted by trypan blue dye exclusion. Increment of cell number with drugs is expressed compared with that of control. All the values are given as Mean ± S.E.M.

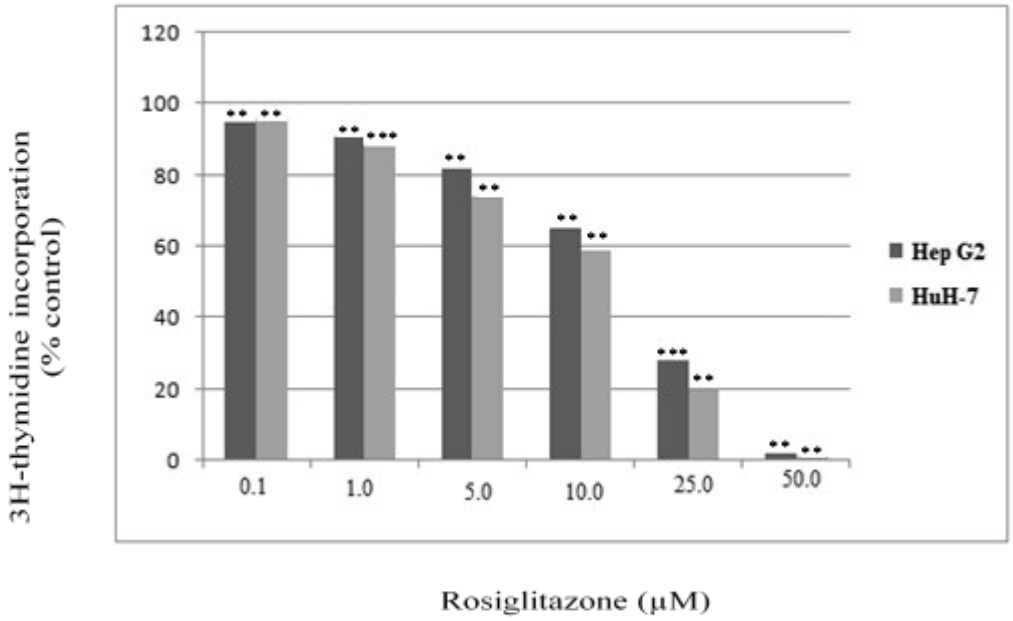

Fig.2. Rosiglitazone induced inhibition of DNA synthesis in hepatocellular carcinoma cell lines. Cells were treated with ligands in 0.1 to 50 mM concentration and after 24 h, a standard ^{3}H-thymidine incorporation assay was performed. All the values are given as mean ± S.E.M.

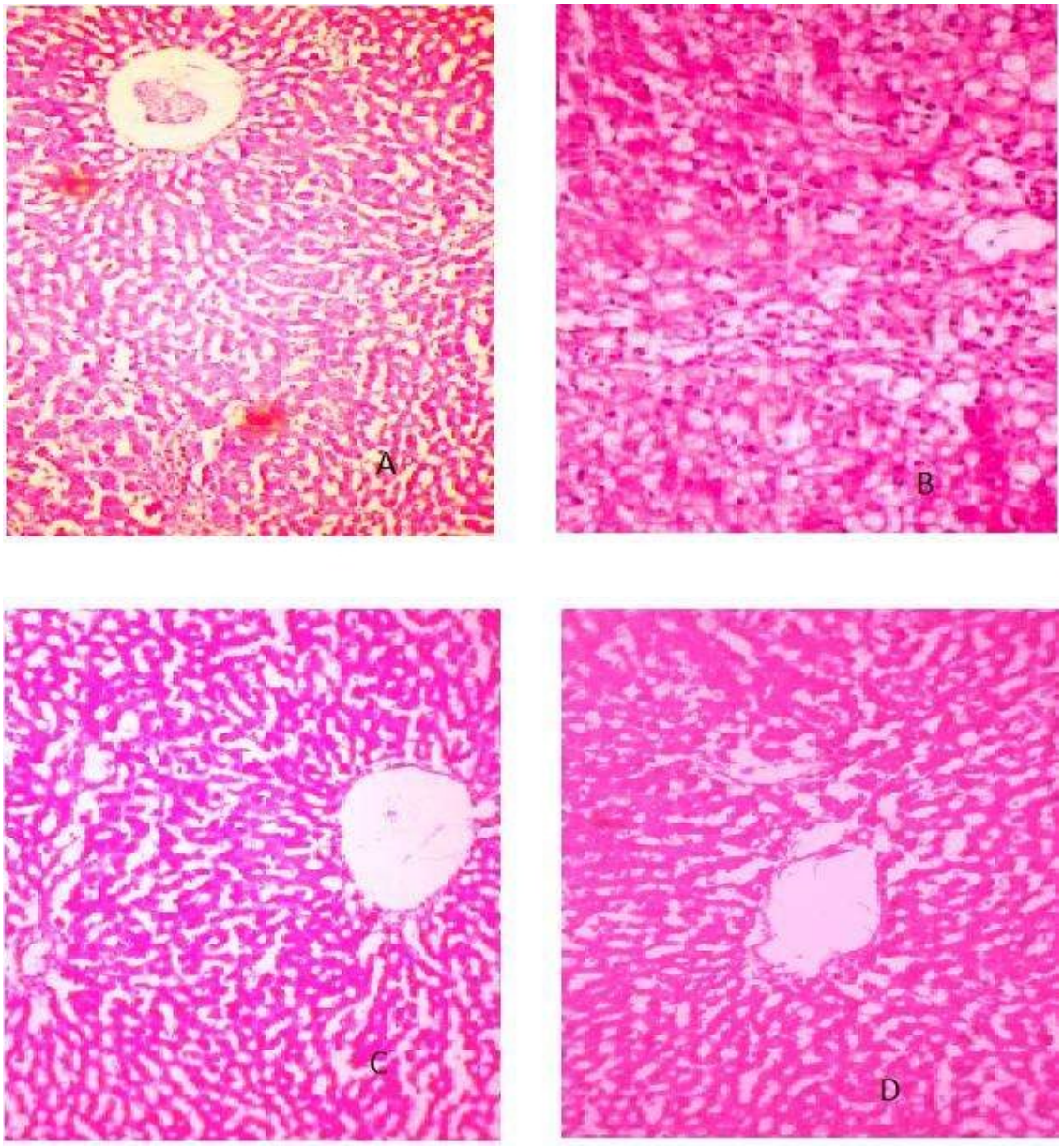

Fig.3. Representative photo micrographs of histopathological changes showing the effect of Rosiglitazone on DENA induced hepatotoxicity in rat (magnification 10×). (A) Liver of vehicle control group showing Normal hepatic cell with well brought out central vein. (B) Liver of rats treated with DENA exhibited hepatic steatosis, centrilobular necrosis, macrovaesiular fatty changes and disturbed portal vein architecture. (C) Liver of rats treated with rosiglitazone alone. (D) Liver of rats treated with Rosiglitazone+DENA shown almost normal architecture and absence of centrilobular necrosis, hepatic steatosis.