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Boron inhibits the proliferating cell nuclear antigen index, molybdenum containing proteins and ameliorates oxidative stress in hepatocellular carcinoma

Hina Zafar [1], Shakir Ali *

Department of Biochemistry, Faculty of Science, Jamia Hamdard, Hamdard Nagar, New Delhi 110062, India

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ABSTRACT

Hepatocellular carcinoma (HCC) is a common malignancy and the main cause of mortality in patients with chronic liver diseases. This study reports the inhibitory effect of boron on HCC induced in rats by administering thioacetamide (TAA) (0.03%) in drinking water for 400 days. Boron (4 mg/kg body weight) was administered orally after induction of carcinoma. Treatment was continued for 122 days, and cell proliferation, histology and biochemistry of treated and control group of rats were studied. Proliferating cell nuclear antigen (PCNA), and [^{3}H]-thymidine incorporation, which increased in rats exposed to carcinogen, significantly decreased after boron treatment. PCNA index decreased from 80 in HCC rats to 32 after boron treatment. In the control group, it was 20. Boron caused a dose-dependent decrease in carcinogen-induced [^{3}H]-thymidine uptake by the rat hepatocyte. It could partially reverse the activity of selected biochemical indicators of hepatic damage, oxidative stress, selenium and serum retinol, which are depleted in liver cancer, and improved overall health of animal. The study implicates the elevated levels of mammalian molybdenum Fe–S containing flavin hydroxylases, which increase the free radical production and oxidative stress, consequently causing increased hepatic cell proliferation in HCC, and reports boron to ameliorate these changes in liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is the main cause of mortality in patients with chronic liver diseases, and one of the leading causes of cancer deaths worldwide. Recognized etiologic or predisposing factors include chronic injury to the liver caused by alcohol, hepatitis B and C, hepatotoxins, and the cirrhosis of liver [1]. Liver cirrhosis has been found to cause a 200-fold increased risk of liver cancer [2]. Mild but prolonged liver injury by chemicals needing bioactivation produces focal lesions, which are believed to be the precursor of liver cancer [3]. Prevention of hepatocyte focal lesions due to mild but prolonged injury caused by a variety of reasons might protect the cancer of the liver.

Boron, which has been reported for its efficacy in conditions such as the Ehrlich ascites carcinoma in mice [4], might be a potential candidate for the treatment of liver cancer. Boron is already used in boron neutron capture therapy, which is a binary form of cancer radiation therapy that uses a neutron beam that interacts with boron injected intravenously into a patient. It has potential ability to selectively kill tumor cells embedded within normal tissue, and has been proposed to be an effective treatment for malignant hepatic tumors [5]. The beneficial effect of boron in the pathology of liver has also been studied in acute liver failure [6], which is important because the process of cancer development can be interrupted at many levels including the initial stages of hepatic injury. Boron is a non-metallic element, abundant in nature, though only in compounds and in combination with sodium and oxygen. In nature, boron exists as borax (Bx) ($Na_2B_4O_7{\cdot}10H_2O$) and boric acid (H_3BO_3), which are completely absorbed by the oral route [7]. Boron does not show mutagenic or carcinogenic effect [8], and is safe at physiological concentrations. This study investigates the effect of boron post-treatment on HCC in rat model.

Materials and methods

Anti-PCNA antibody (PC-10 monoclonal antibody) and LSAB (labeled streptavidin–biotin) kit were purchased from Dako, Copenhagen, Denmark. [Methyl-^{3}H]-thymidine (specific activity 73.0 Ci/

Abbreviations: AO, aldehyde oxidase; ALP, alkaline phosphate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bx, borax; CAT, catalase; GGT, gamma glutamyl transpeptidase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione S-transferase; G6PD, glucose 6-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HPLC, high performance liquid chromatography; i.p., intraperitoneal; LPO, lipid peroxidation; Mo Fe–S FH, molybdenum Fe–S flavin hydroxylases; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; SOD, superoxide dismutase; TAA, thioacetamide; XO, xanthine oxidase.

* Corresponding author.
E-mail addresses: ali.alishakir@gmail.com, sali@jamiahamdard.ac.in (S. Ali).
[1] Present address: Department of Pharmacology & Neuroscience, University of North Texas Health Science Center, Fort Worth, TX 76107, USA.

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mmol) was purchased from GE Healthcare Ltd., a part of Amersham Corporation (UK). Selenium (Se) stock standard was purchased from Fluka Chemika, Switzerland. All other chemicals were of highest purity grade and purchased from Merck Pvt. Ltd. and SRL, India.

Female rats (Wistar strain) used in this study were obtained from and kept in the Central Animal House Facility of the institute. Rats weighing between 150 and 200 g were housed in polypropylene cages at room temperature, 60 ± 15% relative humidity, and a 12-h light–dark cycle. Animals were provided standard laboratory chow and purified water *ad libitum*. The study was approved by the Institutional Animal Ethics Committee (Proposal approval number 261). Ethical guidelines for the care and use of laboratory animals in experiments were followed in letter and spirit.

Induction of liver cancer and experimental protocol

Rats were divided into two groups, I and II. Group I was administered normal saline for 400 days, and Group II received thioacetamide (TAA) (0.03% in drinking water) for 400 days to induce HCC according to the protocol described by Dasgupta et al. [9]. After the completion of treatment with saline or TAA, animals were left untreated for one week, and thereafter, group I was divided into two subgroups, IA and IB. Subgroup IA, the normal control (NC) group, was continued with normal saline solution, and subgroup IB was given borax (Bx) (4 mg/kg body weight, orally) for 122 consecutive days. Group II was also divided into subgroups: IIA and IIB. While any sort of treatment was discontinued in subgroup IIA, the hepatocellular carcinoma or HCC group, subgroup IIB was treated with Bx (4 mg/kg body weight, orally) daily for a similar duration of time (122 days), and was designated as TAA + Bx. Treatment protocol for induction of liver cancer is shown in Fig. 1. Rats from each group were sacrificed at the same time point at the end of the experiment.

Evaluation of HCC

Gross morphological examination of the liver and its histology (Fig. 2), immunohistochemistry for proliferating cell nuclear antigen (PCNA) (Fig. 3), and liver weight to body weight ratios (Table 1) were used to assess the effect of chronic TAA administration on the liver. For histopathology, few μm thick sections of the left lobe of excised liver were cut and fixed in 10% neutral buffered formalin. The fixed tissue samples were processed, cut into 5–6 μm thick sections, and stained in hematoxylin-eosin (H&E). For immunohistochemistry of PCNA, avidin–biotin complex method was used as described in the kit to demonstrate PCNA expression in about 4 μm thick sections of liver. Tissue was de-waxed with xylene and hydrated through a graded alcohol series. Endogenous peroxidase activity was blocked by incubating the slides with 0.3% hydrogen peroxide in methanol for 30 min. Slides were then placed in citric buffer (pH 6.0) and heated in a microwave oven (800 W) for 10 min to expose antigens. After washing with phosphate buffer saline (PBS), the slides were incubated with primary antibodies (PC-10 monoclonal antibody) with the dilution of 1:100, and incubated overnight at 4 °C. The LSAB kit was used for immunostaining the secondary antibodies. Then secondary antibodies were conjugated with biotin. Thereafter, sections were incubated at room temperature for 45 min with streptavidin-horseradish peroxidase. Between all the above steps, slides were washed with PBS. The reaction product was visualized with 3,3′-diaminobenzidine to produce a brown staining wherever primary and secondary antibodies were attached. Finally, the sections were counterstained with H&E and scanned. All sections were scanned and analyzed by a pathologist who was not aware of the treatment group.

Biochemical analysis

For biochemical analysis, either the serum or hepatic tissue obtained from the control or treated groups of rats was used. Serum was prepared from the blood collected from eye vein, the retro-orbital blood. Briefly, the blood was allowed to clot at room temperature, and serum was separated by centrifugation at 1200*g* for 10 min. Preparation of the tissue homogenate and subcellular fractionation and other biochemical estimations were done as per the standardized protocol described in one of our earlier publications [10]. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma glutamyl transpeptidase (GGT) were determined from the serum. ALT, AST and ALP were estimated using diagnostic kit based on the spectroscopic procedure. GGT was measured according to the method described by Orlowski and Meister [11]. Serum was also used to determine the level of retinol by chromatic method [12] on a Shimadzu (model LC-10ATvp) HPLC (High Performance Liquid Chromatography) equipped with a binary gradient, a multiple wavelength detector (SPD-10Avp), C-18 column (alpha Bond C18 125A 10 μm 300 × 4.60 mm) and SPINCHROM software. The analytical recovery of retinol from serum was between 85% and 95%.

Selenium was determined on an atomic absorption spectrometer (AAS) (AAS ZEE nit 65) equipped with a graphite furnace, graphite wall type tube and auto sampler (MPE60 Analytik Jena AG). Samples were analyzed without digestion. Briefly, serum (100 μl) was mixed for a min, and, thereafter, diluent (0.6% HNO_3) was added before properly mixing the solution, pouring it into the sample cup for analysis. The serum samples and Se standard were measured by AAS at 196 nm. Temperature program was as follows: drying I (90 °C for 34 s), drying II (105 °C for 25 s), drying III (110 °C for 12.5 s), pyrolysis (250 °C for 12.8 s), atomization (2100 °C for 5.3 s), and cleanout step (2300 °C for 4.4 s). Injection volume was 20 μl. Se standards were prepared in 0.6% HNO_3 and run in the range of 100–1000 ppb (μg/l). The calibration graph for Se was plotted using the standard by different volumes.

All other biochemical estimations were performed on the hepatic tissue. Hepatic lipid peroxidation (LPO) [13] and glutathione (GSH) [14] were measured from the rat liver homogenate. The

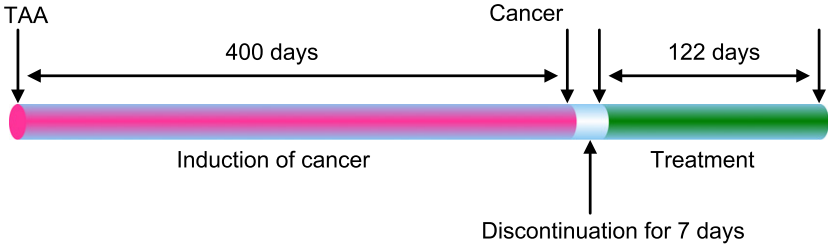

Fig. 1. Experimental protocol to induce hepatocellular carcinoma in rat and its treatment with boron. Thioacetamide (TAA) was administered to rats in drinking water (0.03%) for 400 days. Treatment was discontinued, and the animals were left untreated for one week before administering boron for 122 consecutive days.

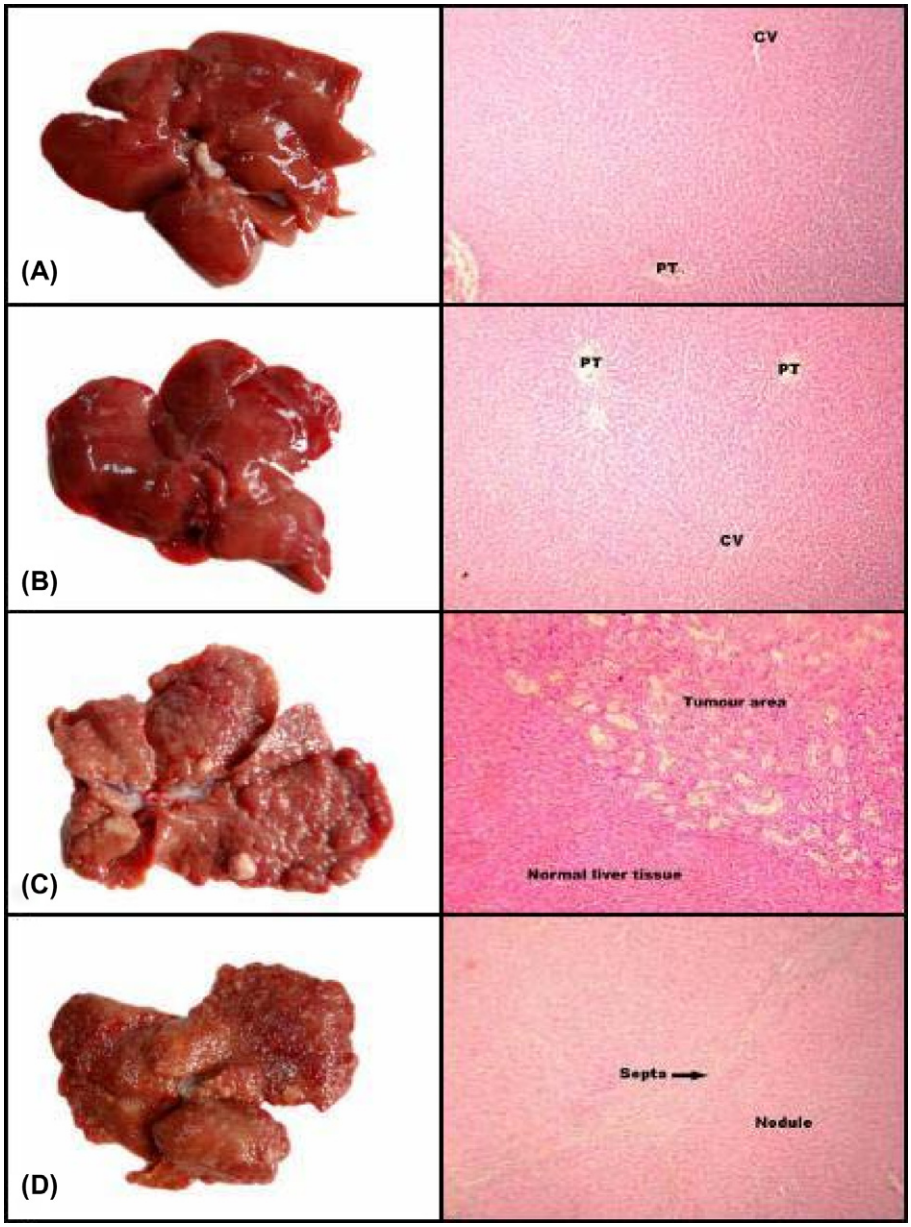

Fig. 2. Gross morphological examination and respective histopathological microphotographs of the liver from (A) normal control, (B) boron alone treated, (C) carcinogen (thioacetamide) treated rats, and (D) rats treated with boron after the induction of carcinoma. PT: portal triad, CV: central vein, H&E: hematoxylin & eosin; H&E × 100.

activities of hepatic glutathione reductase (GR) [15], glutathione peroxidase (GPx) [16], glutathione S-transferase (GST) [17], glucose 6-phosphate dehydrogenase (G6PD) [18], catalase (CAT) [19], superoxide dismutase (SOD) [20], and xanthine oxidase (XO) [21] were determined from the post-mitochondrial supernatant. Aldehyde oxidase (AO) [22] was measured from the cytosol, obtained after subcellular fractionation by differential centrifugation.

Cell proliferation assay

For cell proliferation assay, rats were randomly divided into six groups as follows: Group I (normal saline), Group II (boron, 8 mg/kg body weight/day, administered orally for three consecutive days), Group III (boron administered orally followed by a single intraperitoneal injection of TAA, 400 mg/kg body weight, freshly dissolved in 0.9% NaCl), and Groups IV, V, and VI (each administered with boron at a dose level of 2, 4 or 8 mg/kg body weight/day, orally, for three days consecutively followed by TAA on day 3). TAA was administered 1 h after the last dose of boron was administered to group III, IV, V, or VI. After 16 h of TAA administration, all groups were injected with [^{3}H]-thymidine (25 μCi/0.2 ml saline/100 g body weight, i.p.). Animals were sacrificed by survival dislocation 2 h after thymidine injection. Livers from each group were excised quickly, washed in ice-cold saline (0.9% NaCl), and processed for quantization of hepatic DNA synthesis according to the method described by Smart et al. [23]. Briefly, 20% tissue lysate was prepared in cold water, and precipitated with an equal volume of ice cold 10% tricholoroacertic acid (TCA) before centrifuging at 5000 rpm at 4 °C for 10 min. Supernatant was discarded and precipitate was dissolved in 5 ml of ice-cold TCA (5%), and centrifuged again at the same rpm, temperature and time as mentioned above. After centrifugation, supernatant was discarded and pellet was dissolved in 5 ml ice-cold perchloric acid (10%) and kept for 18 h at 4 °C. After that, all tubes were centrifuged at 5000 rpm at 4 °C for

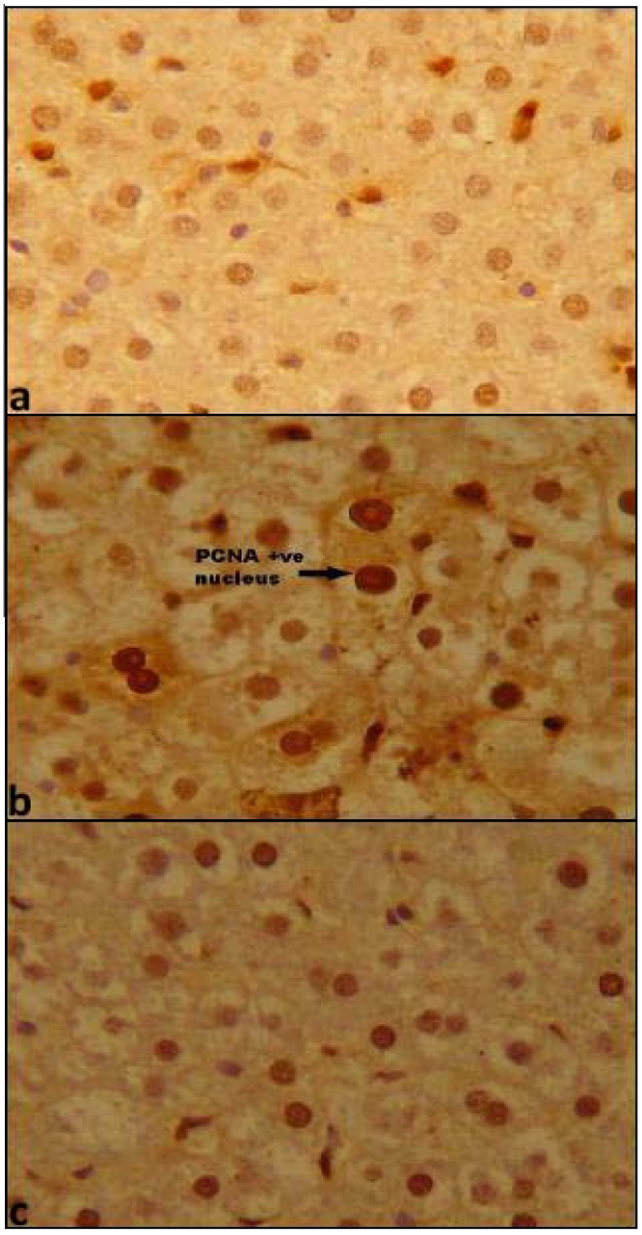

Fig. 3. Immunohistochemistry of hepatic PCNA of (A) normal control, (B) boron alone treated, (C) hepatocellular carcinoma and (D) boron treated rats. Hepatic parenchyma shows largely negative staining in control group (PCNA index = 20). Predominance of strongly positive hepatocytic nuclei (PCNA index = 80) can be seen in rats with HCC. Boron treated group showed a scattered and mild positivity for PCNA in the hepatocytes (PCNA Index = 32). PCNA index indicates the number of PCNA positive cells/100 cells.

10 min, and the pellet was mixed with 5 ml ice-cold perchloric acid (5%) and centrifuged at 5000 rpm for 10 min at 4 °C. Precipitate obtained after the final centrifugation was incubated with 5 ml of warm perchloric acid (10%) in boiling water bath for 30 min. Thereafter, tubes were kept at room temperature for 20 min, and then the sample was filtered through a Whatman-50 filter paper to get clear brown solution, which was used for DNA estimation and counting radioactivity.

Radioactivity was counted in β scintillation counter (Beckman Coulter, USA) by adding 200 μl of the brown filtrate to the scintillation vial containing 5 ml of scintillation fluid. The amount of DNA in the filtrate was estimated by diphenylamine method described by Giles and Myers [24]. Amount of [^{3}H]-thymidine incorporated into DNA was expressed as disintegration per minute (dpm)/μg DNA. Scintillation fluid was prepared immediately before use. For scintillation fluid, 2,5-diphenyloxazole (PPO) (3.25 g) and 1,4-di-2-5-phenyloxazolyl benzene (POPOP) (0.065 g) were dissolved in a mixture containing 1,4-dioxin (250 ml), toluene (250 ml) and methanol (99.9%) (150 ml). The mixture was kept in magnetic stirrer for 15 min before adding napthalene (52 g). The resulting solution was stored in dark bottle.

Statistical analysis

One-way ANOVA (analysis of variance) was applied to determine significant differences in data of various groups. Subsequently, Student–Newman–Keuls test was applied for analyzing the significance between different treatment groups. Value for each experimental parameter represented Mean ± S.E.M ($n = 6$), and the level of significance was chosen at $p < 0.001$, $p < 0.01$, or $p < 0.05$.

Results

Effect of boron on gross organ morphology and histopathology in hepatocellular cancer

Gross morphological features of the liver and respective representative histopathological microphotographs of control (NC), HCC, and boron treated groups of rats are shown in Fig. 2. In HCC, the liver was large and studded with irregular hard nodules and many tumorous growths (Fig. 2**C**). Its size increased considerably, showing an almost fourfold increase in the liver weight to body weight ratio when compared to the control (Table 1). In the boron treated rats (Fig. 2D), nodules were diffused and tumorous growths were absent. The liver weight to body weight ratio also decreased considerably (Table 1). The values in rats treated with boron alone (Fig. 2B) were not significantly different from the normal control group (Fig. 2A).

Histopathological changes in the hepatic tissue architecture in HCC rats include loss of normal lobular architecture, and increased *N*/*C* ratio [25]. Representative sections of the liver from HCC rats (Fig. 2C) showed a completed distorted hepatic architecture, cancerous changes (irregular hard nodules), and signs of inflammation. Inflammation was less in boron treated rats, although microphotograph revealed cirrhotic changes (nodules were diffused) (Fig. 2D). In HCC rats, the hepatic tissue architecture was

Table 1
Liver weight to body weight ratio in hepatocellular carcinoma in rats treated with boron.

Parameters	NC	B	HCC	HCC + B
Liver weight (g)	9 ± 1	8 ± 2	21 ± 3[a]	16 ± 0.1[b]
Body weight (g)	274 ± 13	266 ± 10	180 ± 12[a]	218 ± 13[b]
Liver weight to body weight ratio	3 ± 0.1	3 ± 0.1	12 ± 0.1[a]	7 ± 0.2[c]

Each value represents Mean ± S.E.M ($n = 6$). [a]$p < 0.001$, when compared with the saline treated group (normal control). [b]$p < 0.05$ and [c]$p < 0.001$, when compared with HCC. NC: normal control, B: treated with boron alone, HCC: hepatocellular carcinoma; HCC + B: HCC treated with boron. Boron was administered for 122 consecutive days as per the treatment protocol described in the methods section.

cancerous, and only cirrhotic changes were found in treated rats (Table 2). Bile duct proliferation, neoplastic changes, fibrosis, and regenerative activity were all considerably less in treated rats. Portal triad inflammation was not there in any of the microscopic field, although mild triad inflammation was present in HCC. Neither control nor treated group showed hepatocyte degeneration.

Effect of boron on the liver weight to body weight ratio in hepatocellular carcinoma

The liver weight to body weight ratio (Table 1) increased in rats treated with the carcinogenesis protocol. It increased from 3 ± 0.1 (NC) to 12 ± 0.1 (HCC), and decreased significantly ($p < 0.001$) after the boron treatment (7 ± 0.2).

PCNA expression in boron treated and control group of rats

The expression of PCNA has been reported to increase in cancer. In the experimental animal model of hepatocellular carcinoma in this study, the PCNA index increased to 80 (Fig. 3), but decreased significantly in boron treated rats. The index, which is calculated as the number of PCNA positive cells per 100 cells, was 32 in the boron treated rats. In the normal control group of animals, it was 20.

Effect of boron on serum biochemical markers of hepatic injury

The activity level of serum biochemical markers of hepatic injury, GGT, AST, ALT and ALP increased in HCC rats, and decreased in animals treated with boron (Table 3). GGT, which is measured as nmol of *p*-nitroaniline formed/min/mg protein, increased from 254 ± 16 nmol (control) to 448 ± 15 nmol in HCC rats, and was significantly brought down to 307 ± 19 nmol after the boron treatment. AST, ALT, and ALP activity, measured in Units/ml, also increased similarly in HCC rats to 259 ± 9 (*vs.* the respective control value of 136 ± 7), 66 ± 3 (*vs.* the respective control value of 29 ± 3), and 12 ± 1 (*vs.* the respective control value of 7 ± 1), respectively, and decreased significantly by boron to 201 ± 10, 48 ± 4, and 9 ± 0.4 Units/ml. There was no significant change in activity of any of these enzymes in rats receiving boron alone, and all values were well within the control limits. The activities of AST, ALT, and ALP in rats receiving boron alone were 136 ± 8, 29 ± 2, and 7 ± 1 Units/ml, respectively.

Effect of boron on mammalian molybdenum Fe–S containing proteins in liver cancer

The activity of AO and XO, the molybdenum containing Fe–S flavin hydroxylases in mammalian cells, which contribute to liver injury by generating reactive oxygen species (ROS) while catalyzing the reaction, increased in HCC, but decreased after the boron treatment (Fig. 4A and B).

Table 2
Liver histology in hepatocellular carcinoma in boron treated and control group of rats.

Liver histology	NC	B	HCC	HCC + B
Hepatic architecture	WNL	WNL	Cancerous	Cirrhotic
Bile duct proliferation	Nil	Nil	Adenocarcinoma	+
Neoplastic changes	Nil	Nil	++++	+
Fibrosis	Nil	Nil	+++	+
Regenerative activity	Nil	Nil	++	+
Portal triad inflammation	Nil	Nil	+	Nil

++++: Highly severe, +++: Severe, ++: Moderate, +: Mild, WNL: within normal limits, NC: normal control, B: treated with boron alone, HCC: hepatocellular carcinoma; HCC + B: HCC treated with boron. Boron was administered for 122 consecutive days as per the treatment protocol described in the methods section.

Table 3
Serum biochemical markers of hepatic injury in liver cancer in boron treated and control group of rats.

Groups	GGT	AST	ALT	ALP
NC	254 ± 16	136 ± 7	29 ± 4	7 ± 1
B	256 ± 13	136 ± 8	29 ± 2	7 ± 1
HCC	448 ± 15[a]	259 ± 9[a]	66 ± 3[a]	12 ± 1[a]
HCC + B	307 ± 19[b]	201 ± 10[b]	48 ± 4[b]	9 ± 0.4[b]

Data represent Mean ± S.E.M ($n = 6$). [a]$p < 0.001$, when compared with the saline treated group (normal control). [b]$p < 0.001$, when compared with the HCC group. GGT: gamma-glutamyl transpeptidase (nmol of p-nitroaniline formed/min/mg protein), AST: aspartate transaminase (Units/ml), ALT: alanine transaminase (Units/ml), ALP: alkaline phosphatase (Units/ml), NC: normal control, B: treated with boron alone, HCC: hepatocellular carcinoma; HCC + B: HCC treated with boron. Boron was administered for 122 consecutive days as per the treatment protocol described in the methods section.

Effect of boron on hepatic biomarkers of oxidative stress

LPO and glutathione, the biochemical markers of oxidative stress, changed significantly in hepatocellular carcinoma. While there was a significant increase in LPO, the level of glutathione decreased in hepatic tissue homogenate prepared from the liver excised from HCC rats (Table 4). LPO, which was measured as nmol of malondialdehyde/mg protein, increased from 142 to 425 nmol/mg protein, and glutathione decreased from 301 to 104 μmol/g tissue. After the boron treatment, LPO decreased to 305 nmol/mg protein and glutathione increased to 172 μmol/g tissue, which was a significant change, indicating amelioration of oxidative stress in liver cancer. The enzymes involved in glutathione metabolism, GST and GR were also measured (Table 5). GST, which increased to 180% in HCC rats, decreased significantly after the boron treatment (118%). On the other hand, GR, which registered a decrease of 36% in cancerous rats, increased to 52% after boron treatment. G6PD increased significantly ($p < 0.001$) in liver cancer, and decreased after the boron treatment (Fig. 4C). The activity of peroxide metabolizing enzymes, GPx, CAT and SOD, which are accepted indicators of the generation of free radicals, decreased in HCC (Table 6). In HCC rats, the activity of GPx, CAT, and SOD decreased to 50%, 31%, and 30%, respectively, and increased significantly ($p < 0.001$) after boron treatment. Se (Fig. 4D), which is required for the activity of GPx, was also measured and found to decrease in HCC rats, and increase after the boron treatment.

Effect of boron on serum retinol in HCC rats

As shown in Table 7, serum retinol decreased significantly in HCC rats. The concentration of retinol in the serum, when compared to the control (25 ± 1 μg/dl), was 14 ± 1 μg/dl. Serum retinol increased to 19 ± 1 μg% after the boron treatment. In HCC rats, serum retinol was 57% of the control value, and increased to 75% after the treatment.

Effect of boron on the uptake of radioactive thymidine by the rat liver exposed to carcinogen

Boron showed a dose-dependent inhibition on carcinogen (TAA)-induced uptake of [^{3}H] thymidine by the rat liver cells (Fig. 5).

Discussion

Cell proliferation is the most central and key phenotypic property of cancer. In hepatocellular carcinoma, hepatocyte prolifera-

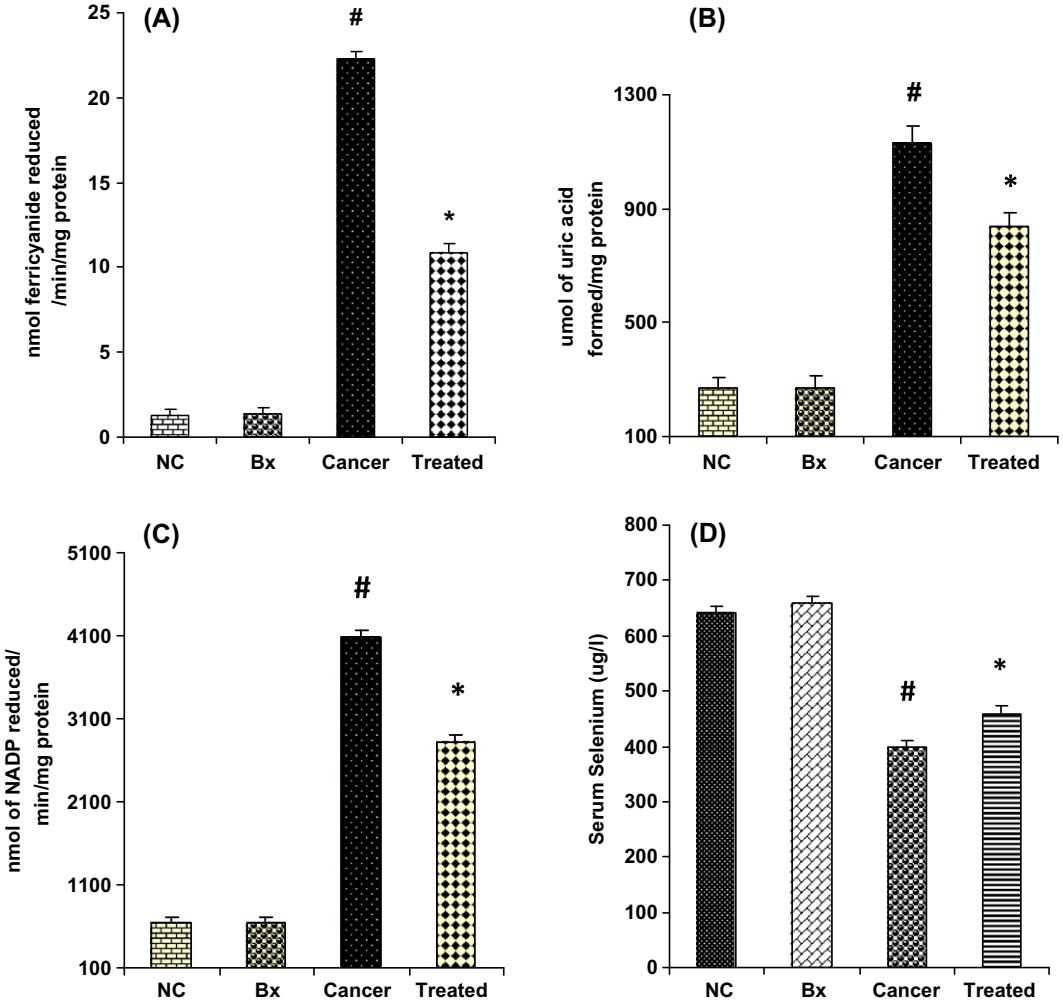

Fig. 4. Effect of boron on rat hepatic (A) aldehyde oxidase, (B) xanthine oxidase, (C) glucose 6-phosphate dehydrogenase, and (D) serum selenium in thioacetamide-induced hepatocellular carcinoma (HCC). Data represent Mean ± S.E.M ($n = 6$). $^{*}p < 0.001$, when compared with HCC group, and $^{\#}p < 0.001$, when compared with the control group. NC: normal control; Bx: boron alone; Treated: HCC treated with boron.

Table 4
Effect of boron on oxidative stress markers in hepatocellular carcinoma.

Groups	Lipid peroxidation	Glutathione
NC	142 ± 7	301 ± 9
B	143 ± 7	302 ± 9
HCC	425 ± 10[a]	104 ± 9[a]
HCC + B	305 ± 9[b]	172 ± 7[b]

Data represent Mean ± S.E.M ($n = 6$). [a]$p < 0.001$, when compared with the saline treated group (normal control), and [b]$p < 0.001$, when compared with the cancerous group. Hepatic lipid peroxidation was measured as nmol malondialdehyde produced per mg protein, and the amount of glutathione in the liver was calculated as μmol/g tissue. NC: normal control, B: treated with boron alone, HCC: hepatocellular carcinoma; HCC + B: HCC treated with boron. Boron was administered for 122 consecutive days as per the treatment protocol described in the methods section.

Table 5
Effect of boron treatment on enzymes involved in glutathione metabolism.

Groups	Glutathione S-transferase	Glutathione reductase
NC	361 ± 3	434 ± 7
B	359 ± 3	430 ± 5
HCC	650 ± 8[a]	154 ± 7[a]
HCC + B	427 ± 6[b]	228 ± 9[c]

Data represent Mean ± S.E.M ($n = 6$). [a]$p < 0.001$, when compared with the saline treated group (normal control). [b]$p < 0.01$, [c]$p < 0.001$, when compared with the cancerous group. The activity of glutathione S-transferase and glutathione reductase were determined by measuring, respectively, the amount of CDNB conjugate (nmol) formed/min/mg protein and NADPH (nmol) oxidized/min/mg protein in rat liver. CDNB: 1-chloro-2,4-dinitrobenzene; NADPH: nicotinamide adenine dinucleotide phosphate (reduced form); NC: normal control, B: treated with boron alone, HCC: hepatocellular carcinoma; HCC + B: HCC treated with boron. Boron was administered for 122 consecutive days as per the treatment protocol described in the methods section.

tion is central at both the early and late stages [26]. This study reports a series of protective effects of boron on hepatic cancer, as determined by measuring the liver cell proliferation and various biochemical parameters in rats treated with a chemical carcinogenesis protocol and borax (Fig. 1). Treatment with boron considerably improved the distorted morphology of the cancerous liver, the liver weight and liver weight to body weight ratio, and several biochemical changes associated with liver cancer.

Hepatocyte focal lesions are believed to be the precursor for liver cancer [3]. Mild but prolonged injury by carcinogens such as thioacetamide, which need bio-activation to inflict damage, disturbs the hepatic tissue architecture, producing focal lesions in a multistep process involving DNA damage and cell proliferation. The process, neoplasia, can be interrupted at many levels. In one of the studies, boron has been shown to protect the liver against

Table 6
Effect of boron treatment on peroxide metabolizing enzymes in hepatocellular carcinoma.

Groups	Catalase	Glutathione peroxidase	Superoxide dismutase
NC	2048 ± 69	755 ± 10	6 ± 0.2
B	2049 ± 55	752 ± 9	6 ± 0.1
HCC	641 ± 72[a]	378 ± 16[a]	2 ± 0.2[a]
HCC + B	945 ± 56[b]	462 ± 14[b]	3 ± 0.2[b]

Data represent Mean ± S.E.M ($n = 6$). [a]$p < 0.001$, when compared with the saline treated group (normal control). [b]$p < 0.001$, when compared with HCC rats. The activity of catalase and glutathione peroxidase was calculated, respectively, as nmol H_2O_2 consumed and nmol NADPH oxidized/min/mg of rat liver protein. Superoxide dismutase was calculated as units/mg sample. NADPH: nicotinamide adenine dinucleotide phosphate (reduced form); NC: normal control, B: treated with boron alone, HCC: hepatocellular carcinoma; HCC + B: HCC treated with boron. Boron was administered for 122 consecutive days as per the treatment protocol described in the methods section.

Table 7
Serum retinol in liver cancer and boron treated rats.

Groups	Retinol	Percent change
NC	25 ± 1	–
B	23 ± 1	94%
HCC	14 ± 1	57%
HCC + B	19 ± 1	75%

Data represent Mean ± S.E.M ($n = 5$). Percent change indicates increase or decrease with respect to the saline treated (normal control) group. Concentration of retinol in the serum was measured in μg/dl. NC: normal control, B: treated with boron alone, HCC: hepatocellular carcinoma; HCC + B: HCC treated with boron. Boron was administered for 122 consecutive days as per the treatment protocol described in the methods section.

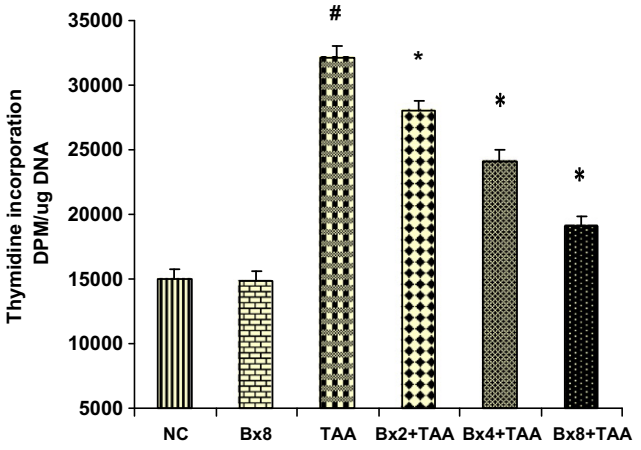

Fig. 5. Effect of boron on carcinogen (thioacetamide)-induced hepatic cell proliferation in rat. Hepatic cell proliferation was measured by [^{3}H]-thymidine incorporation method. Data represent Mean ± S.E.M ($n = 6$). $^*p < 0.001$, when compared with TAA alone treated group, and $^\#p < 0.001$, when compared with the control group. Bx2, Bx4, and Bx8 represent the dose of boron, which was 2, 4, and 8 mg/kg body weight, respectively. NC: normal control; TAA: rats treated with a single dose of thioacetamide (TAA, 400 mg/kg body weight, i.p.) to induce hepatic cell proliferation.

the damage produced by thioacetamide in the initial stages of hepatic injury [6]. In this study on HCC, which is characterized by and differentiated from other hepatic lesions by means of typical morphologic features such as the internal heterogeneity, intra-tumoral septa and/or scar, capsule, daughter nodules and vascular invasion, we found boron to ameliorate cancerous lesions in rat. In boron treated rats, hepatic nodules were diffused and tumorous growths were absent (Fig. 2). Treatment with boron improved the liver weight to body weight ratio, which has been reported to increase in liver cancer due to impaired nutrient absorption and metabolic utilization [27,28].

The increase in the liver weight observed in HCC has been attributed to hepatocyte proliferation [28]. We hypothesized boron to exert its effect by inhibiting liver cell proliferation. Cell proliferation and apoptosis are two different but associated processes in cancer [29]. The proliferation index (PI) and apoptosis index (AI) reflect the biological characters of tumor cells [30]. Proliferation of tumor cells triggers amplification of initiated cell population and induce tumor development. As soon as PI overcomes AI, cancer develops in some degree. The tumor suppressor protein p53, which can control cyclin-dependent kinases to regulate DNA replication, involve PCNA interaction by p21 protein pathway [30]. PCNA is a protein acting as a cofactor of DNA polymerase δ in eukaryotes. It increases the processivity of leading strand synthesis during DNA replication. PCNA is only synthesized and expressed in proliferating cells, where it interacts with multiple proteins that play key role in DNA synthesis, repair, cell cycle regulation, chromatin remodeling, and apoptosis [31]. Overexpression of PCNA with high frequency is often used as a reliable marker for the assessment of tumor progress, premalignant evolution and clinical prognosis of patients with various malignancies. In a study by Gallardo-Williams [32], reduced expression of PCNA has been reported to decrease the amount of mitoses in prostate adenocarcinoma in boric acid treated mice. Mitotic figures reflect DNA synthesis and proliferative activity. The greater is the number of mitoses, the more will be the intensity of cell division and tumor growth. Boron is clearly shown to down-regulate the expression of PCNA, and, hence, the cell division and tumor growth in HCC (Fig. 3). The inhibitory effect of boron on liver cancer was further evident by decreased activity of GGT, AST, ALT, and ALP, the serum biochemical markers of hepatic injury in liver cancer [33–36]. While the GGT is an early enzyme marker for liver cancer [33], AST/ALT ratio has been reported to increase in it, with AST being more closely related to tumor growth than ALT, which reflects parenchyma damage [35]. ALP has been reported in earlier studies to decrease with chemotherapy, when carcinoma has responded with diminution in liver size [36].

The serum biochemical markers of hepatic injury are released into the serum as a result of change in membrane permeability. One of the mechanisms that could prevent the leakage of these enzymes into the blood could be the stabilization of the membrane structure by boron. Benderdour et al. have already reported boron to preserve the cell function and control inflammation by inhibiting the release of lysosomal enzymes from the liver and PMN neutrophils [37]. This role of boron assumes significance because cancer commonly starts with the deterioration of membrane, which, in the presence of boron is stabilized, thereby inhibiting the process of tumor initiation.

One of the mechanisms that may change the membrane permeability involves free radicals. Generation of free radicals including the reactive oxygen species is of common occurrence in cells and tissues. However, production of free radicals beyond the capacity of the cell to scavenge them inflicts damage. Free radicals produced in excess primarily attack the membrane lipids, causing their peroxidation, and hence change membrane permeability. Increase in free radical generation and circulating lipid peroxidation products has been reported in carcinogenesis [38,39]. In this study, we found boron to considerably reduce lipid peroxidation in liver cancer, indicating a mechanism of alleviating cancer by inhibiting the damage caused by free radicals. We proposed molybdenum Fe–S containing flavin hydroxylases (Mo Fe–S FH) as an endogenous source of free radicals in liver cancer. Mo Fe–S FH is a group of enzymes that produce reactive oxygen species while catalyzing the reaction [10,40]. This study correlates the activity of Mo Fe–S FH with lipid peroxidation in liver cancer. Treatment with boron de-

creased both, the activity of Mo Fe–S FH and elevated levels of lipid peroxidation products (Fig. 4B), indicating the modulation of oxidative stress by boron as a possible mechanism of inhibiting cancer. Oxidative stress has been correlated in earlier studies with cell proliferation [41], which is hypothesized in this study to be inhibited by boron. We propose boron to inhibit initial stages in the development of cancer by inhibiting oxidative stress and consequently liver cell proliferation. The reactive oxygen species derived from Mo Fe–S FH, especially xanthine oxidase, have been reported to participate in the recruitment of inflammatory cells by activating a neutrophil chemotactic factor, increasing the leukocyte adhesion and cytokine production by monocytes [42], and inducing the accumulation of leukocytes in liver microvasculature [43]. Similarly, aldehyde oxidase, the other mammalian Mo Fe–S FH, contributes to liver damage, and a strong correlation has been observed between reduced AO expression and tumor stage [44,45]. AO produces, in addition to the reactive oxygen species, acetic acid or retinoic acid upon oxidation of acetaldehyde or retinaldehyde. This study reports boron to replenish the depleted level of serum retinol in HCC. Retinol and its derivative (retinoid) have antioxidant activity and promote cell differentiation. Retinoid manifests anti-tumor role by suppressing tumor promotion and progression, and its depletion is often observed during pre-malignancy and cancer development [46]. The finding has implication in liver fibrosis, where hepatic satellite cells undergo a process of activation, and transform from quiescent vitamin A storing cells into proliferative and fibrogenic myofibroblasts characterized by a gradual loss of intracellular vitamin A, eventually promoting chronic injury [47,48]. Boron might inhibit the proliferation of fibrogenic myofibroblasts, and may be useful in liver fibrosis.

An impaired anti-oxidative system in cancer apparently favors the accumulation of free radicals. Decrease in the activity of antioxidant enzymes, GPx, CAT, and SOD is one of the reasons of higher susceptibility of tumor cells to treatments likely to involve oxygen radicals [49–51]. This study reports boron to significantly increase the activity of these enzymes in HCC, and also increase serum selenium. Selenium is involved in strengthening the antioxidant defense of the body [51,52]. Its level has been found to decrease in HCC patients [46]; selenium supplementation is known to considerably reduce the risk of cancer mortality and incidence [53,54]. Boron might act by inducing the expression of antioxidant enzymes including those that contain selenium, for example, GPx, and might reduce tissue damage by hastening the destruction of reactive oxygen species by increasing the activity of key antioxidant enzymes [55]. Boron is also reported to replenish the depleted level of glutathione in HCC. Glutathione, the antioxidant tripeptide, and the enzymes involved in glutathione metabolism, GST, GR, and G6PD, which have been reported to perturb in liver cancer, significantly improved after the boron treatment. Boron significantly reduced the elevated level of GST in HCC. GST is closely related with cancer, and is commonly recognized as a tumor marker [56]. It is a key enzyme in xenobiotic metabolism and biosynthesis of leukotrienes, cytosolic phospholipase A21 (cPLA21) and cyclooxygenase-2 (COX-2)-controlled prostaglandin signaling cascade in liver cancer. Reduction in prostaglandin synthesis might be a therapeutic approach to disrupt inflammation, dysplasia and malignant transformation processes. The finding is consistent with the literature where boron has been proposed as a physiological regulator of normal inflammatory response [55], and reported to inhibit cyclooxygenase and lipoxygenase, the enzymes involved in inflammatory cascade, and reduce inflammation [57,58]. The anti-inflammatory role of boron is pertinent to its anti-cancer effect, because reducing cyclooxygenase and lipoxygenase activity decreases the synthesis of prostaglandin E2 and other unfavorable eicosanoids such as leukotrienes. This study further correlated the activity of GST with GR, which is inversely related to GST, and reported to decrease in liver cancer [59]. GR mediates reduction of oxidized glutathione (GSSG) to its reduced form (GSH) in a reaction catalyzed by G6PD. Treatment with boron significantly increased the activity of GR and decreased GST, when compared with the HCC rats. The activity of G6PD also decreased in the treated group. Increased G6PD might be related to enhanced glucose metabolism, as cancer cells constitutively up-regulate glucose metabolism and synthesize ATP mainly through glycolysis, a metabolic state that is linked to high glucose uptake and local acidification owing to lactate production.

Uncontrolled proliferation of tumor cells is hallmark of cancer. We investigated the effect of boron on hepatocyte proliferation in rats exposed to a single dose of TAA, the carcinogen used to induce hepatocellular carcinoma in this study. Boron caused a dose-dependent decrease in carcinogen-induced uptake of radioactive thymidine by the rat liver cells. TAA has been previously used to promote DNA synthesis and mitosis in the liver [60]. The proliferation peaks at 36 h after a single injection of TAA [61]. As already discussed, cell proliferation is a phenotypic event in cancer, and a dose-dependent decrease in TAA-induced uptake of radioactive thymidine in the presence of boron suggests antitumor promoting potential of boron in experimental animal model.

Conclusions

This study concludes potential therapeutic role of boron in hepatocellular cancer. Boron is shown to inhibit elevated expression level of PCNA, and inhibit the uptake of [^{3}H]-thymidine by the rat liver cells exposed to carcinogen. It could partially reverse the activity of selected biochemical markers of hepatic injury, oxidative stress, selenium, and serum retinol, which are depleted in liver cancer. The study correlates the level of mammalian molybdenum Fe–S containing flavin hydroxylases with hepatocarcinoma, and provides an insight into the mechanism of action of boron in liver cancer.

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