J Exp Clin Med 2014;6(4):132–138

Contents lists available at ScienceDirect

Journal of Experimental and Clinical Medicine

journal homepage: http://www.jecm-online.com

ORIGINAL ARTICLE

Protective Effect of Diindolylmethane against *N*-Butyl-*N*-(4-hydroxybutyl) Nitrosamine-induced Bladder Carcinogenesis

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ARTICLE INFO

Article history:
Received: May 7, 2014
Revised: Jun 9, 2014
Accepted: Jun 12, 2014

KEY WORDS:
bladder cancer;
chemoprevention;
diindolylmethane;
N-butyl-*N*-(4-hydroxybutyl) nitrosamine

Background: Carcinogenesis involves three distinguishable stages, namely, initiation, promotion, and progression. Chemoprevention is the use of agents to inhibit, reverse, or retard tumorigenesis at the initiation stage itself. Diindolylmethane (DIM) is one such agent. The precursor of DIM, that is, indole-3-carbinol, has been found to have beneficial effects in the treatment of prostate and breast cancers. This study was designed to evaluate the protective role of DIM in bladder carcinogenesis. *N*-Butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) was used as a carcinogen to study the chemopreventive activity of DIM in bladder carcinogenesis.

Methods: Male Wistar rats ($n = 24$) were grouped into control and experimental groups and the study was terminated at the 32nd week. Group I rats were treated with 0.05% BBN for 8 weeks. Group II rats were treated with BBN + DIM [DIM (5 mg/kg) treatment was started 1 week prior to the BBN treatment, and it was administered for 8 weeks]. Group III rats were treated with DIM alone. Group IV rats were treated as control. The activity and levels of drug-metabolizing and liver enzymes were measured by standard spectrophotometric procedures. Tissues were stained with hematoxylin and eosin and subjected to histopathological examination.

Results: Male albino Wistar rats were divided into four groups of six animals each. Rats in Group I received BBN and served as experimental control. Group II rats were treated with BBN along with DIM for the initial 8-week period. During the same period, Group III rats were treated with DIM alone. Group IV rats served as the healthy control. At the end of 32 weeks, bladder tissues were collected and subjected to histopathological examination. Increase in the activities of cytochrome (CYP) P450, CYPb5, and CYP c reductase, nicotinamide adenine dinucleotide phosphate (reduced):quinone oxidoreductase 1, aspartate aminotransaminase, and alanine aminotransaminase and decrease in the activity of glutathione S-transferase were found in experimental rats when compared with control rats. Results of histopathological examination showed that DIM reduced BBN-induced initiation of carcinogenesis such as hyperplasia.

Conclusion: The results of this study show that DIM inhibited hyperplasia, a condition that reflects the development of initiation of carcinogenesis in an experimental model of nitrosamine-induced bladder carcinogenesis, and this effect of DIM can be attributed to its antioxidant activity and its ability to modulate xenobiotic enzymes.

1. Introduction

Bladder cancer is the most common type of cancer reported in developed nations.[1] Bladder cancer is the fourth most common cancer in men and eighth most common in women, with

Conflicts of interest: All contributing authors declare no conflicts of interest.

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http://dx.doi.org/10.1016/j.jecm.2014.06.008

transitional cell carcinoma comprising up to 90% of all primary bladder tumors.[2] It commonly manifests (in 70–80% of patients) as a nonmuscle invasive tumor. It is characterized by an overall good prognosis following transurethral resection in patients with low-grade tumors and intravesical chemotherapy and/or Bacillus Calmette–Guérin instillation in patients with high-grade tumors.[3] Almost 50% of the patients with high-grade bladder cancer have muscle invasive disease. Of these, 50% develop distant metastasis within 2 years and almost 50–60% of these patients die within 5 years despite treatment.[4] Indole compounds, which are found in cruciferous vegetables, are potent anticancer agents. Numerous studies have evaluated the effect of fruit and vegetable consumption in reducing the risk of developing bladder cancer, and most of these studies have reported a lower risk of bladder cancer in patients with high consumption.[5] The bioactive compounds indole-3-carbinol and its *in vivo* dimeric derivatives are found in cruciferous vegetables. 3,3′-Diindolylmethane (DIM) has the ability to deregulate multiple cellular signaling pathways leading to cancer.[6,7] *In vivo* studies using a mouse model for evaluating the chemopreventive effects of DIM in lung carcinogenesis were reported previously.[8] Infiltration of inflammatory cells was also inhibited by DIM administration, which also had anticancer properties against several types of cancers.[9–11] DIM inhibited the proliferation of human gastric cancer cell line SGC7901 *in vitro* by modulating cell apoptosis and arresting cancer cell cycle at the G_1 phase.[12] *N*-Butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) is an environmental tobacco-specific carcinogen that reproducibly causes high-grade, invasive cancers in the urinary bladder.[13] Most studies on bladder cancer have evaluated specific substances such as the various vitamins or specific foods (e.g., green tea or garlic), but there is a vast array of bioactive molecules in fruits and vegetables that are only beginning to be characterized. A meta-analysis of 38 studies on six dietary variables showed that the risk of bladder cancer is increased by diets low in fruits and vegetables.[14] Thus, there is a need for research on dietary phytochemicals, which may prevent cancer through antioxidation and gene–nutrient interactions, or which may be useful adjuncts in the prevention of cancer recurrence among successfully treated cancer survivors where prevention of cancer recurrence is vital. The preventive effect of DIM was not studied at the initiation stage of BBN-induced carcinogenesis by monitoring the levels and activity of enzymes and by analyzing the bladder pathology. Therefore, the aim of the study was to determine the preventive effects of DIM during the initiation stage of BBN-induced carcinogenesis using Phase I and II bladder enzymes and histopathology examination.

2. Methods

2.1. Animals and chemicals

Male albino Wistar rats were purchased from Central Animal House, King Institute of Preventive Medicine (Chennai, Tamil Nadu India) and were housed in polypropylene cages. The animals were kept in a room lighted for 12 hours each day and maintained at 20°C. The animals received standard pellet diet (Hindustan Lever Ltd, Mumbai, India) and water *ad libitum*. Animal care and experiments were in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals guideline (New Delhi, India). The Institutional Animal Ethical Committee at SRM University (Kattankulathur, Kancheepuram District, Tamil Nadu, India) has reviewed and approved the protocols detailed in the study. BBN was purchased from TCI Chemical Company (Tokyo, Japan). Other chemicals and reagents used were of analytical grade.

2.2. Experimental procedure

Rats were divided into four groups of six animals each. Group I rats were treated with 0.05% BBN for 8 weeks.[15] Group II rats were treated with BBN + DIM [DIM (5 mg/kg) treatment was started 1 week prior to the BBN treatment, and it was administered for 8 weeks].[16] Group III rats were treated with DIM alone. Group IV rats served as the healthy control. The rats were observed daily and their weights were measured every week. Food and water consumption was monitored every week. The experiment was terminated at Week 32 and all animals were sacrificed by cervical dislocation after ketamine anesthesia and the tissues were removed for subsequent analysis. Blood was collected in centrifuge tubes by retro-orbital blood collection technique using fine-walled Pasteur pipette. The activity of aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) was determined.[17]

2.3. Isolation of microsomal fractions

Microsomes were isolated by a standard procedure described previously.[18] The bladder tissues were excised and rinsed with cold 0.1 M KCl, and then dried and weighed. Bladder homogenate was prepared with an isotonic phosphate buffer (pH 7.4) using a homogenizer (treatment time: 1 minute). The homogenate was then centrifuged at 15,000*g* for 20 minutes in an ultracentrifuge using a fixed angle rotor to sediment the cell debris, unbroken cells, nuclei, and heavy mitochondria. Microsomes were pelleted at 100,000*g* for 90 minutes by subsequent centrifugation. The supernatant thus obtained (cytosolic fraction) was carefully decanted into small vials. The microsomal pellet was gently suspended in ice-cold 0.1 M KCl. Aliquots of this suspension were stored at −80°C and thawed prior to use. All procedures were carried out at temperatures between 0°C and 4°C. The protein content of microsomal fractions of bladder was determined using bovine serum albumin as the standard.[19]

2.4. Determination of CYP P450 activity

The total cytochrome P450 (CYP) content was determined spectroscopically.[20] Microsomes suspended in phosphate buffer (0.1 M, pH 7.0; 4 mg/mL) were reduced by adding a few milligrams of solid sodium dithionate. Then 1 mL of water saturated with carbon monoxide was added (CO was generated by treating concentrated sulfuric acid with anhydrous sodium formate), which leads to the formation of a CO adduct of CYP P450 that has an absorbance maximum at 490 nm. The change in absorbance at 450 nm was then converted to the concentration of CYP P450 using the extinction coefficient of 91 nM^{-1} cm^{-1}.

2.5. Determination of CYP b5 activity

To the microsomal suspension containing 4 mg of protein/mL in phosphate buffer, 1 mL of nicotinamide adenine dinucleotide + hydrogen (NADH; 0.4 mM) was added. The absorbance spectrum between 400 nm and 500 nm was read against the blank containing microsomal suspension alone. The level of cytochrome CYT b5 was calculated using the molar extinction coefficient of 185 nM^{-1} cm^{-1} between 424 nm and 409 nm and was expressed as nmoles/milligram protein.

2.6. Determination of nicotinamide adenine dinucleotide phosphate (reduced)–CYP c reductase

The activity of nicotinamide adenine dinucleotide phosphate (reduced)–CYP c (NADPH–CYPc) reductase was assayed as described previously.[21] The assay mixture containing 2.5 mL of

0.3 M phosphate buffer (pH 7.6), 0.2 mL of 1 mM potassium cyanide, 0.1 mL of 0.05 mM CYP c was mixed gently. After 3 minutes, 0.1 mL of 0.045 mM NADPH was added and the change in optical density was recorded at 30-second intervals for 3 minutes at 550 nm. The activity of NADPH–CYP P450 reductase was expressed as nmoles of CYP c reduced/minute/milligram protein.

2.7. Determination of glutathione S-transferase

Activity of glutathione S-transferase (GST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate.[22] The reaction mixture contained 1.7 mL of 100 mmol/L phosphate buffer (pH 6.5), 0.1 mL of 30 mmol/L CDNB. After preincubating the reaction mixture at 37°C for 5 minutes, the reaction was started by adding 0.1 mL of diluted cytosol and the absorbance was followed for 5 minutes at 340 nm. The reaction mixture without the enzyme was used as blank. The activity of GST is expressed as nmoles of glutathione–CDNB conjugate formed/minute/milligram protein.

2.8. Determination of NAD(P)H:quinine oxidoreductase

Activity of NQO1 was determined using 2,6-dichlorophenolindophenol (DCPIP) as the substrate, and is calculated by measuring the conversion of NADH into NAD^+.[23] The decrease in NADH was measured at 600 nm for over 1 minute. The NQO1 activity was measured in 800-μL reactions containing bladder cytosol, 200 μM NADH, 40 μM DCPIP, and Tris–HCl buffer (25 mmol/L Tris–HCl, pH 7.4, 0.7 mg/mL bovine serum albumin). Parallel reactions were performed in the presence of 20 μM dicumarol, which inhibits the activity of NQO1. The rate of dicumarol-sensitive NQO1 activity was determined as the difference between the uninhibited and the dicumarol-inhibited rates and was normalized to total cytosolic protein. The concentration of cytosolic protein was measured according to the Lowry method.

2.9. Histopathology examination

After formalin fixation (10% phosphate buffered, pH = 7.4) and dehydration process, paraffin-embedded urinary bladder sections (5 μm) were stained with hematoxylin and eosin. Histopathological analysis for the urinary bladder sections was evaluated at 40× magnifications.

2.10. Statistical analysis

Data were analyzed using analysis of variance and the group mean ± standard error of the mean was compared with Tukey's multiple range test using SPSS version 19 (SPSS Inc., Chicago, IL, USA). Results were considered statistically significant if $p \leq 0.05$.

3. Results

Table 1 presents the changes in body weight of control and experimental animals after either with or without designed treatments. The result demonstrated that all rats in this study had an increase in body weight. Compared with healthy control rats, however, body weight gain in rats treated with BBN was significantly less. Furthermore, there was a significant increase in body weight in rats treated with DIM + BBN when compared with BBN-treated rats. The DIM-treated rats showed no statistically significant changes in body weight gain when compared with healthy control rats.

Figure 1 illustrates the contents of CYP P450 in rats without any treatments or treated with BBN, DIM + BBN, or DIM only. The level of CYP P450 in BBN-treated rats was significantly increased when compared with rats in control. Compared with BBN-treated rats, the level of CYP P450 in DIM + BBN-treated rats was significantly reduced. The DIM-treated rats displayed no significant changes in the contents of CYP P450 when compared with healthy control rats. Figure 2 shows the level of CYT b5 in control and experimental rats. An increase in the level of CYT b5 was found in BBN-treated rats

Table 1 Effect of DIM on the changes in the body weight of animals treated with or without BBN.

Treatment	Initial body weight (g)	Final body weight (g)	Changes in body weight (g)
Control	112.8 ± 2.6	217.5 ± 2.0	105.3 ± 3.4*
BBN treated	132.2 ± 3.4	144.4 ± 1.5	12.4 ± 4.8
BBN + DIM	120.6 ± 1.6	185.6 ± 2.6	65.0 ± 2.4
DIM	110.6 ± 2.0	236.2 ± 2.0	126.4 ± 3.2*

* Values are expressed as mean ± standard error of the mean. Values that are not sharing a common symbol are different significantly at the $p \leq 0.05$ level by Tukey's *post hoc* one-way analysis of variance.
BBN = *N*-(4-hydroxybutyl) nitrosamine; DIM = diindolylmethane.

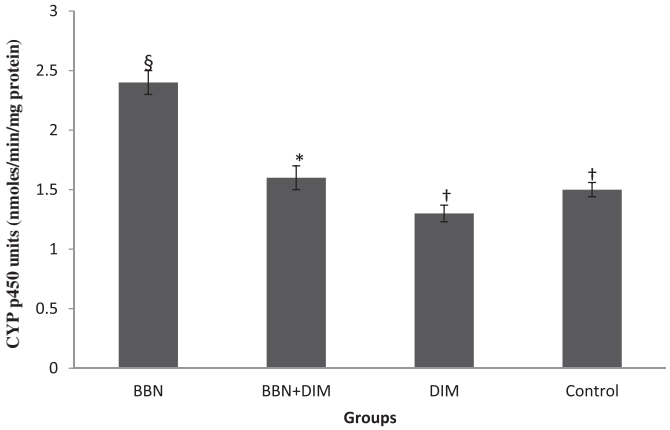

Figure 1 Effect of diindolylmethane (DIM) on the levels of cytochrome P450 in control and experimental rat bladder. Values are expressed as mean ± standard error of the mean. Values that are not sharing a common symbol are different significantly at the $p \leq 0.05$ level by Tukey's *post hoc* one-way analysis of variance. BBN = *N*-(4-hydroxybutyl) nitrosamine; units = nmoles of P450 levels were quantitated from the ferrous form complexed with CO/minute/mg protein.

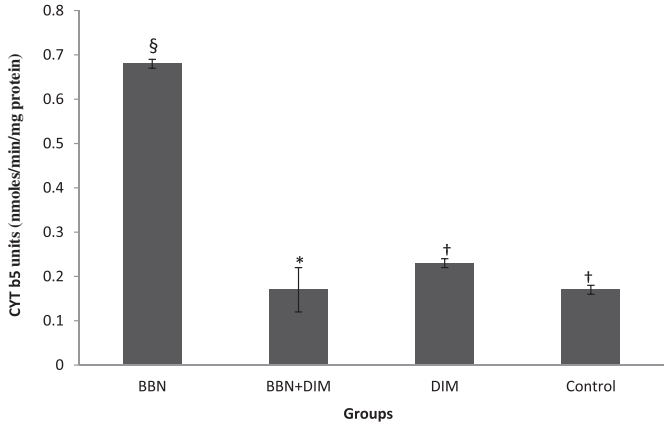

Figure 2 Effect of diindolylmethane (DIM) on the levels of cytochrome b5 in control and experimental rat bladder. Values are expressed as mean ± standard error of the mean. Values that are not sharing a common symbol are different significantly at the $p \leq 0.05$ level by Tukey's *post hoc* one-way analysis of variance. BBN = *N*-(4-hydroxybutyl) nitrosamine; units = nmoles of CYT b5 content were quantified from the ferrous form of the cytochrome/minute/mg protein.

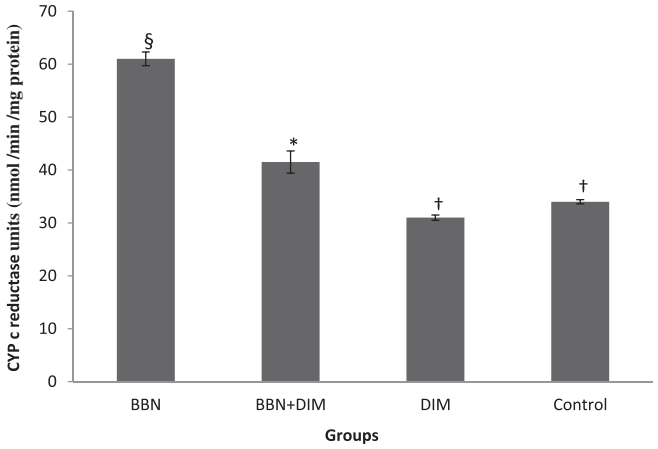

Figure 3 Effect of diindolylmethane (DIM) on the activity of cytochrome c reductase in control and experimental rat bladder. Values are expressed as mean ± standard error of the mean. Values that are not sharing a common symbol are significantly different at the $p \leq 0.05$ level by Tukey's *post hoc* one-way analysis of variance. BBN = *N*-(4-hydroxybutyl) nitrosamine; units = nicotinamide adenine dinucleotide phosphate (reduced) cytochrome c reductase quantified from nmoles of cytochrome C reduced/min/mg protein.

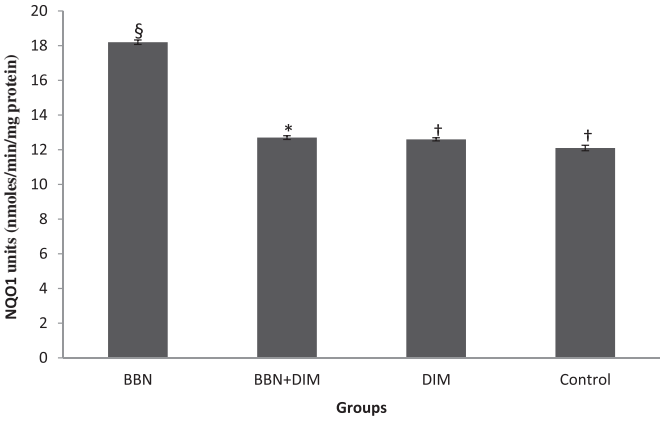

Figure 5 Effect of diindolylmethane (DIM) on the activity of NQO1 in control and experimental rat bladder. Values are expressed as mean ± standard error of the mean. Values that are not sharing a common symbol are significantly different at the $p \leq 0.05$ level by Tukey's *post hoc* one-way analysis of variance. BBN = *N*-(4-hydroxybutyl) nitrosamine; units = nmoles conversion of nicotinamide adenine dinucleotide + hydrogen to NAD^+ using 2,6-dichlorophenolindophenol/min/mg protein.

when compared with healthy control rats. There was a decrease in the level of CYT b5 in DIM + BBN-treated rats when compared with BBN-treated rats. The DIM-alone-treated rats showed no significant changes in the level of CYT b5 when compared with healthy control rats. Figure 3 illustrates the activity of CYP c reductase expressed in control and experimental rats. An increase in activity of CYP c reductase was found in BBN-treated rats when compared with healthy control rats. There was a reduction in the activity of CYP c reductase in DIM + BBN-treated rats when compared with BBN-treated rats. The DIM-treated rats showed no significant changes in the activity of CYP c reductase when compared with healthy control rats. Figure 4 shows the activity of NQO1 in control and experimental rats. An increased activity of NQO1 was found in BBN-treated rats when compared with control rats. There was a decreased activity of NQO1 in DIM + BBN-treated rats when compared with BBN-treated rats. The DIM-alone-treated rats showed no significant changes in the level of NQO1 when compared with healthy control rats. Figure 5 shows the activity of GST in control and experimental rats. Reduced activity of GST was found in BBN-treated rats when compared with healthy control rats. There was increased activity of GST in DIM + BBN-treated rats when compared with BBN-treated rats. The DIM-alone-treated rats showed no significant changes in the activity of GST when compared with healthy control rats.

Figure 6 shows the activity of AST in control and experimental rats. An increased activity of AST was noticed in BBN-treated rats when compared with healthy control rats. Diminished activity of AST in DIM + BBN-treated rats was observed when compared with BBN-treated rats. The DIM-treated rats showed significant increase in the activity of AST when compared with healthy control rats. Figure 7 shows the activity of ALT in control and experimental rats. An increased activity of ALT was found in BBN-treated rats when compared with healthy control rats. There was a decreased activity of ALT in DIM + BBN-treated rats when compared with BBN-treated rats. The DIM-treated rats showed no significant changes in the activity of ALT when compared with healthy control rats.

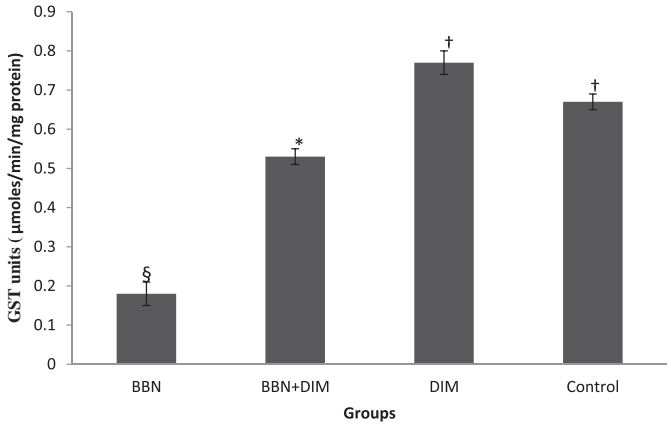

Figure 4 Effect of diindolylmethane (DIM) on the activity of glutathione S-transferase (GST) in control and experimental rat bladder. Values are expressed as mean ± standard error of the mean. Values that are not sharing a common symbol are significantly different at the $p \leq 0.05$ level by Tukey's *post hoc* one-way analysis of variance. BBN = *N*-(4-hydroxybutyl) nitrosamine; units = µmoles of 1-chloro-2,4-dinitrobenzene–glutathione conjugates/minutes/mg protein.

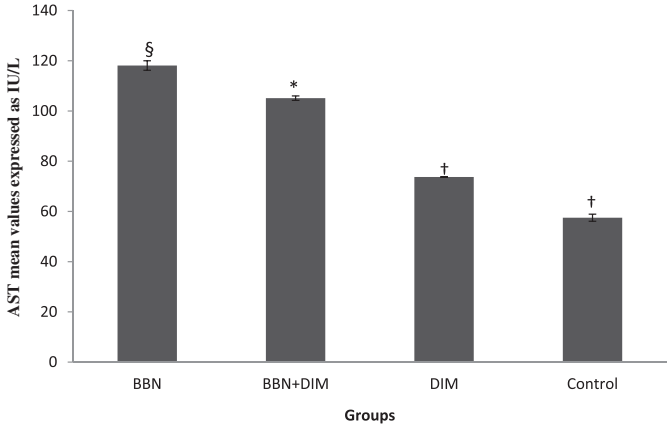

Figure 6 Effect of diindolylmethane (DIM) on the activity of serum aspartate amino-transaminase (AST) in control and experimental rats. Values are expressed as mean ± standard error of the mean. Values that are not sharing a common symbol are significantly different at the $p \leq 0.05$ level by Tukey's *post hoc* one-way analysis of variance. BBN = *N*-(4-hydroxybutyl) nitrosamine.

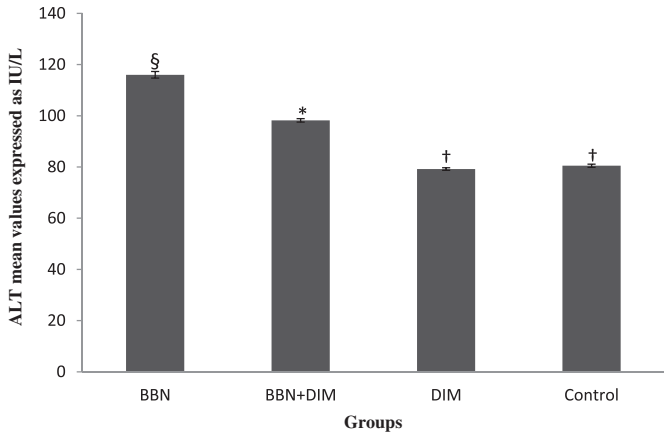

Figure 7 Effect of diindolylmethane (DIM) on the activity of serum alanine aminotransaminase (ALT) in control and experimental rats. Values are expressed as mean ± standard error of the mean. Values that are not sharing a common symbol are significantly different at the $p \leq 0.05$ level by Tukey's *post hoc* one-way analysis of variance.

Figure 8 demonstrates the histopathology results, indicating the protective effect of DIM in BBN-induced initiation of carcinogenesis. The urinary bladder sections from BBN-treated experimental rats showed the papilloma (Figure 8A). The urinary bladder sections derived from BBN + DIM-treated rats displayed the decreased hyperplasia (Figure 8B). The urinary bladder sections from DIM-treated experimental rats showed no abnormality (Figure 8C). Figure 8D shows the normal architecture of the urinary bladder in the sections from control healthy rats.

4. Discussion

The present results demonstrate the chemopreventive effects of DIM as evidenced from its activity in lowering the levels of drug-metabolizing enzymes. This study used BBN as an initiator of hyperplasia to induce bladder carcinogenesis in experimental rats. The BBN was used to evaluate the effects of orally ingested bioactive substances such as DIM in rats for chemoprevention.[24] There was a significant reduction in body weight in BBN-treated rats due to the cancer cachexia, resulting in progressive loss of body weight, which is mainly due to wasting of host body compartment such as skeletal muscle and adipose tissue. Our results showed higher NQO1 in BBN-initiated bladder carcinogenesis. The rise in NQO1 levels in the bladder tumor was reported.[25] The NQO1 enzymes are important cellular protectants against carcinogens and oxidants.[26] Our results are in line with that of Paonessa et al who demonstrated that tobacco-related carcinogens (polycyclic aromatic hydrocarbon or PAH) and bladder carcinogens induce the activity of NQO1 in the bladder.[27] Results of epidemiologic studies have shown that dietary isothiocyanates and cruciferous vegetable intake are inversely associated with bladder cancer risk in humans. The CYP4B1 in the bladder tissues from rats and rabbits activates bladder carcinogens such as benzidine, 2-naphthylamine, and *N*-nitrosodibutylamine.[28,29] These findings indicate that CYP4B1 might contribute to the metabolism of BBN in rat bladder and account for the higher levels of CYP P450, CYT

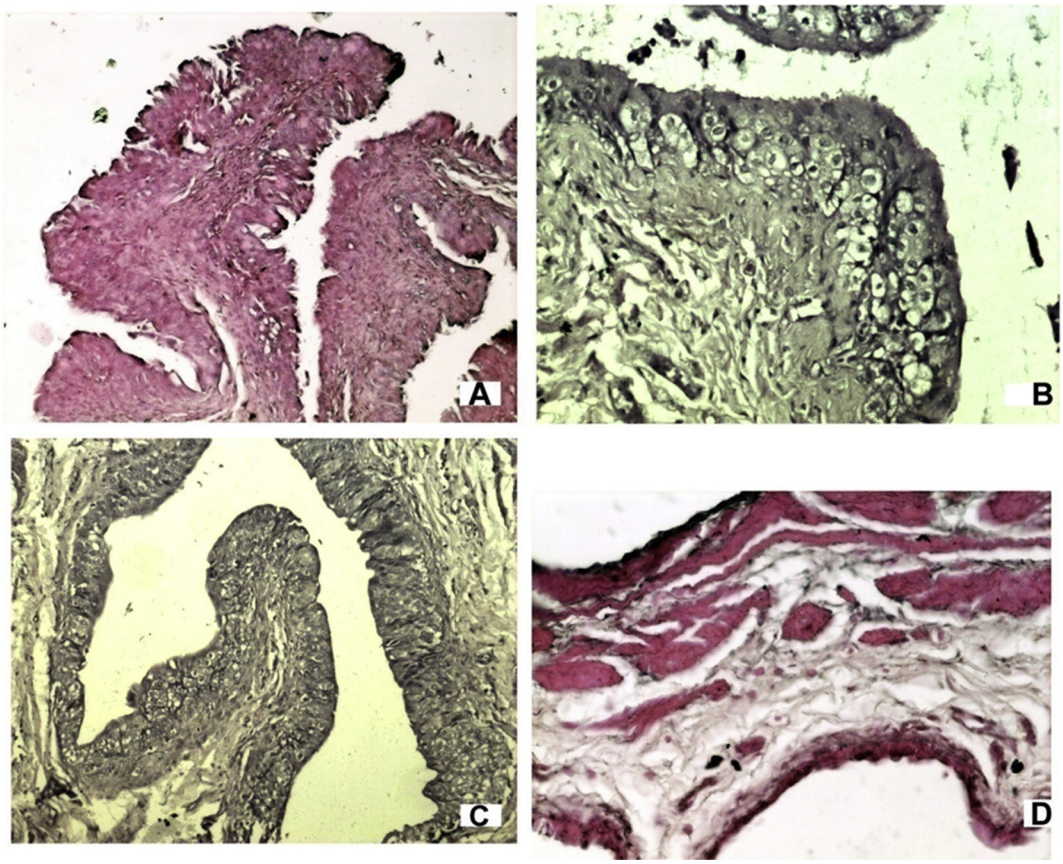

Figure 8 Histopathological changes in the control and experimental groups. (A) BBN-treated rat bladder shows hyperplasic papilloma. (B) BBN + DIM-treated rat bladder shows decreased changes in hyperplasia. (C) DIM-alone-treated rat bladder shows no abnormality. (D) Control rat bladder shows normal architecture. BBN = *N*-(4-hydroxybutyl) nitrosamine; DIM = diindolylmethane.

b5, and CYP c reductase. Administration of DIM modulated the levels of CYP P450, CYT b5, and CYP c reductase. It was reported that DIM blocks carcinogenesis by enabling normal cells to mount a stringent antioxidant response. The DIM has been shown to reduce oxidative stress and stimulate the expression of antioxidant response element-driven gene, suggesting the antioxidant function of indole compounds.[30] Several experimental studies have shown that DIM inhibited oncogenesis and cancer cell growth, and induced apoptosis in cancer cells *in vitro* and *in vivo*, suggesting that DIM could serve as a potent agent for the prevention of tumor progression and/or treatment of cancers. Previous *in vivo* experiments with rodents indicated that indoles and isothiocyanates, two major groups of glucosinolate breakdown products, attenuate the effects of PAHs and nitrosamines by inhibiting the activity of CYP P450 isoenzymes, respectively. The potential of chemoprevention in the reduction of mortality associated with common epithelial cancers was well documented.[31] DIM inhibited Akt activation, migration, and cellular proliferation in tumor-derived breast cancer cells.[32] DIM contributes to chemoprotection by multiple mechanisms involving modulation of xenobiotic metabolizing enzyme system in the liver and extrahepatic tissues, and by diminishing the bioavailability of genotoxic and active metabolites of hazardous xenobiotic compounds including dietary carcinogens (such as heterocyclic amines) in experimental animals. Because preventing diseases including cancer initiation could be achieved by protecting cells and tissues against oxidative stress-mediated damages, an effective mechanism of defense against such damages would be through the induction of cellular Phase I and II enzymes as well as antioxidant enzymes such as uridine diphosphate glucose glucuronosyltransferase, GST, NQO1, and SOD1.[33] Several studies point to noteworthy quantitative differences concerning P450 isoenzymes following DIM treatment. The DIM induced a G_1 arrest in human prostate cancer cells and human breast cancer cells.[34,35] It is widely accepted that metabolic activation of nitrosamines by Phase I enzymes to reactive electrophiles is required for their cytotoxic, mutagenic, and carcinogenic activities. Wu et al[36] found that DIM inhibits the human CYP1A1 and 1A2, and rat CYP2B1. Overall, these results point toward the ability of DIM in inhibiting catalytic activities of a number of CYP isoforms, which may form the basis of its anticarcinogenic mechanism of action. The DIM induces cell death in gastric cancer cells by activating the Hippo signaling pathway.[37] Sun et al, studied the inhibitory effects of DIM in bladder cancer cell lines in STAT pathway.[38] DIM acts as a cancer epigenetic-modifying agent and may contribute to the future clinical development.[39] Therefore, based on the results of this study (i.e., modulation of drug-metabolizing enzymes and histopathological analysis), chemopreventive measures such as the prevention of BBN-initiated bladder carcinogenesis in rats were demonstrated by DIM.

Acknowledgments

This work was supported by a grant from the Department of Science and Technology, Government of India under the Fast Track Scheme for Young Scientists. The infrastructure was kindly provided by SRM University (Tamil Nadu, India).

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