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ORIGINAL ARTICLE

Diindolylmethane and Lupeol Modulates Apoptosis and Cell Proliferation in N-Butyl-N-(4-Hydroxybutyl) Nitrosamine Initiated and Dimethylarsinic Acid Promoted rat Bladder Carcinogenesis

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Abstract Bladder cancer has been shown to resist programmed cell death with altered expression of both pro-apoptotic and anti-apoptotic proteins. To study is to investigate the apoptotic properties of Diindolylmethane (DIM) and Lupeol on N-Butyl-N-(4-hydroxybutyl) Nitrosamine (BBN) initiated and Dimethylarsinic Acid (DMA) promoted urinary bladder cancer. Sixty male Wistar rats were divided into 6 groups. Group I: Control. Group II: Rats were experimentally developed bladder carcinogenesis with BBN and DMA. Group III and IV: DIM and lupeol were administered after BBN treatment for 28 weeks. Group V and VI: DIM and lupeol alone treatment for 36 weeks. All the experimental rats were maintained and euthanized after 36 weeks protocol. Urinary bladder tissues were collected and processed for further investigations. Apoptotis and cell proliferative marker such as Bax, Bcl-2, caspase-3, caspase-9 and PCNA were quantified using immunohistochemical analysis. The Immunohistochemical expression of Bax, Bcl-2, caspase-3, caspase-9 and PCNA were aberrant in BBN + DMA treated tumor group. Administration of DIM and lupeol inhibited the progression of bladder cancer, induced the expression of apoptotic Bax, caspase-3, caspase-9 and inhibited the expression of anti-apoptotic Bcl-2, PCNA in the urinary bladder of rats. Administration of diindolylmethane and lupeol treatment induces apoptosis and cellular proliferation by its anti-carcinogenic properties. From our results DIM and lupeol would be the agent or adjunct for the treatment of bladder carcinogenesis.

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Introduction

Transitional cell carcinoma of the bladder (TCC) is the fifth most common solid malignancy [1]. The molecular factors such as cell proliferation and apoptosis that trigger each of these traits are critical to improve the treatment of cancer. It was highlighted from the clinical part that heterogeneity of bladder cancer in terms of both histological origin and clinical behavior shows clinical parameters such as tumor grade and stage are not yet enough to accurately predict biological behavior or to guide treatment reliably [2]. A significant degree of tumor heterogeneity remains even within prognostic subgroups though apoptotic parameters provide a certain degree of tumor biological potential [3].

Apoptosis is a fundamental form of physiological cell death driven by a distinct cellular mechanism characterized by morphological alterations, chromatin condensation, DNA cleavage, and generation of apoptotic bodies [4]. Caspase-3 is a proteolytic effector molecule that acts downstream in the apoptosis pathway, resulting in cellular disassembly. Cleavage of specific substrates by caspases leads eventually to biochemical and morphological changes that constitute apoptosis. Bcl-2 protein was reported to be over expressed in a variety of human cancers, functions as a suppressor of apoptosis, resulting in the survival of malignant cells [5]. Caspase-9 is the apoptotic initiator protease of the intrinsic or mitochondrial apoptotic pathway, which is activated at multi-protein activation platforms. It is an important therapeutic target for the cancer [6]. It was reported that ectopic expression of other Bcl-2 family proteins such as Bax induces

mitochondrial apoptosis and its expression is reduced in several types of cancers [7].

The molecular biomarkers have been largely excluded from current management algorithms for urologic malignancies. Presently, risk associations are beginning to be included in management bladder cancer [8] but risk groups and validated prognostic molecular biomarkers that can help clinicians to identify patients in need of early aggressive management are lacking.

PCNA, a 36-kDa nuclear protein with functions as an auxiliary protein for DNA polymerase, serves as an important proliferative marker in carcinogenesis [9, 10]. PCNA is known to express during the cell cycle and its rate of synthesis has a direct correlation with the proliferative rate of cells [11]. A substantially elevated expression of PCNA in bladder tumors of BBN animals is indicative of accelerated proliferation of tumor cells.

Glucobrassicin, a predominant glucosinolate, is converted upon mastication of vegetables into indole-3-carbinol (I3C), which then undergoes acid condensation in the stomach, predominantly to 3,3′-diindolylmethane (DIM). Studies in pancreatic cancer cells showed that induction of these responses by DIM leads to constitutive activation of death receptor and the extrinsic apoptosis pathway but the role of DIM in bladder cancer apoptosis is not yet well studied [12]. Lupeol is found in vegetables such as white cabbage, pepper, cucumber, tomato and in fruits. Lupeol has been attracted interest in context to chemoprevention attributable in large part to its antioxidant, apoptosis inducing, antiproliferative, antimutagenic and antiinflammatory properties [13–15]. Hence, the importance of developing novel therapeutic approaches is warranted and inhibitory effect of DIM and lupeol in the bladder carcinogenesis in rats was studied. Hence, the importance of developing novel therapeutic approaches are warranted and inhibitory effect of DIM and lupeol in the bladder carcinogenesis in rats are yet to be studied.

Materials and Methods

Animals and Chemicals

Male Albino Wistar rats were purchased from Central Animal House, Indian Institute of Science, Bengaluru, India. All animals were housed in polypropylene cages. The animals were kept in a room lighted 12 h each day and maintained at 20 °C, standard pellet diet (Hindustan lever Ltd.,) and water was provided *adlibitum*. Animal experiments were followed under the guidelines of CPCSEA (India). The study was approved by the Institutional Animal Ethical Committee, SRM University. OH-BBN and Dimethylarsinic acid was purchased from Tokyo Chemical Industry, Japan. Lupeol (Cat No. L5632) was purchased from Sigma Aldrich, USA. HRP-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, USA. Rabbit monoclonal Bax, Bcl-2, caspase-3, caspase-9 and PCNA antibody was purchased from Thermo Fisher Scientific, USA and Cell Signaling Technology, USA.

Experimental Design

Sixty rats were procured and divided into six groups of 10 animals each. Group I rats were served as healthy control. Group II rats were treated with BBN (150 mg/gavage/twice a week) for 8 weeks and the rats were given 100 ppm concentrations of Dimethylarsinic acid (DMA) in the drinking water for 28 weeks [16, 17]. Group III rats were treated with BBN; after cessation of BBN treatment, rats were given 100 ppm DMA in the drinking water with oral coadministration of Diindolylmethane 5 mg/kg body weight/day for 28 weeks [18]. Group IV rats were treated with BBN; after cessation of BBN treatment, rats were given 100 ppm DMA in the drinking water with oral coadministration of Lupeol 50 mg/kg body weight/day for 28 weeks [19]. Group V rats were treated with Diindolylmethane alone (5 mg/kg body weight/day) for 36 weeks. Group VI rats were treated with Lupeol alone (50 mg/kg body weight/day) for 36 weeks. All the experimental rats were maintained and euthanized at 36th week and bladder tissue samples were collected for subsequent analysis.

Immunohistochemistry

Proteins that play a major role in each of these processes have therefore assumed significance as end points for chemoprevention. Immunolocalisation of key proteins involved in proliferation Proliferating Cell Nuclear Antigen (PCNA), and apoptosis (Bax, Bcl-2, caspase-3 and caspase-9). Briefly, sections of paraffin embedded tissues were deparaffinized in xylene, rehydrated through graded ethanol solutions, and washed in phosphate-buffered saline (PBS). Antigen retrieval was carried out by heating the sections in 0.01 M citrate buffer (pH 6.0) for 30 min in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3 % H_2O_2 in PBS for 5 min. Nonspecific binding sites were blocked using 1 % bovine serum albumin for 20 min. Then, sections were incubated overnight at 4 °C with 1:300 dilutions of rabbit monoclonal antibodies against Bax, Bcl-2, caspase-3, caspase-9 and PCNA. After several washes with PBS, they were incubated with 1:2000 dilutions of goat anti-rabbit HRP conjugate secondary antibody for 2 h at room temperature. After rinsing with PBS, the slides were incubated with the chromogen 3,3′-diaminobenzidine for 3 min then counterstained with hematoxylin. Specimens were observed using a Nikon microscope and images were captured with camera. Scoring was done [20], according to the intensity of the

nucleic or cytoplasmic staining (3+ = strong staining, more than 50 % of cells were stained; 2+ = moderate staining, between 20 and 50 % of cells were stained; 1+ = mild staining, between 1 and 20 % of cells were stained; 0 = weak or negative, less than 1 % of cell staining).

Results

Immunohistochemistry Grading of Control and Experimental Groups

Table 1 showed the immunohistochemical grading of proapoptotic Bax, caspase 3, caspase 9, antiapoptotic Bcl-2 and proliferative PCNA in control and experimental groups. The percentage of positive cells was scored as: 3 + strong staining, more than 50 % of cells were stained; 2 + moderate staining, between 20 and 50 % of cells were stained; 1 + mild staining, between 1 and 20 % of cells were stained; 0 = weak or negative less than 1 % of cell staining.

Expression of PCNA Cell Proliferation in Control and Experimental Groups

To assess the in vivo effect of diindolylmethane and lupeol treatment on the proliferation index in the urothelium of experimental rats, the tissue samples were analyzed with PCNA immunostaining. The urothelium of control, diindolylmethane and lupeol alone treatment showed mild expression of PCNA protein (Fig. 1a, e and f). BBN + DMA treated tumor rats showed strong expression of PCNA in the epithelium of the rat urinary bladder (Fig. 1b). Diindolylmethane and lupeol with BBN + DMA treated rats showed mild expression of PCNA protein (1C & 1D). Thus, diindolylmethane and lupeol inhibits the proliferation in the bladder by its antiproliferative properties. The immunoexpression pattern of PCNA marker and the score of positively stained cells in control and experimental animals in each group are shown in Table 1.

Expression of Proapototic Bax Protein in Control and Experimental Groups

The apoptotic and proliferative features of the bladder tumors were evaluated to determine the effect of diindolylmethane and lupeol treatment. The bladders from control, diindolylmethane and lupeol alone treated rats showed strongly expressed proapoptotic Bax protein (Fig. 2a, e and f). However, in the BBN + DMA tumor group, lesions showed weak or no expression of Bax protein (Fig. 2b). Diindolylmethane and lupeol with BBN + DMA treated rats showed moderate expression of Bax protein (Fig. 2d). Diindolylmethane and lupeol treatment suppresses the tumor growth by inducing the expression of Bax protein in the urinary bladder of the rats. The immunoexpression pattern of Bax marker and the score of positively stained cells in control and experimental animals in each group are shown in Table 1.

Expression of Antiapoptotic Bcl-2 Protein in Control and Experimental Groups

The expression of antiapoptotic Bcl-2 were evaluated in the bladders from control, diindolylmethane and lupeol alone treated rats showed weak or no expression of antiapoptotic Bcl-2 protein (Fig. 3a, e and f). BBN + DMA treated tumor group showed strong expression of Bcl-2 protein (Fig. 3b). Diindolylmethane and lupeol with BBN + DMA treated rats showed mild expression of Bcl-2 protein (Fig. 3c and d). Diindolylmethane and lupeol treatment potentially inhibited the progression of bladder cancer and Bcl-2 protein expression in the urinary bladder of the rats. The immunoexpression pattern of Bcl-2 marker and the

Table 1 Immunohistochemistry grading of control and experimental animals

Markers	Bax				Bcl-2				Caspase-3				Caspase-9				PCNA			
Groups	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+
G-I	0	0	3	7	7	3	0	0	0	0	2	8	0	0	3	7	8	2	0	0
G-II	8	2	0	0	0	0	2	8	3	7	0	0	2	8	0	0	0	0	2	8
G-III	0	1	3	6	8	1	1	0	0	1	1	8	0	1	1	8	7	3	0	0
G-IV	0	2	2	6	7	2	1	0	0	1	2	7	0	1	2	7	7	2	1	0
G-V	0	0	2	8	9	1	0	0	0	0	1	9	0	0	2	8	9	1	0	0
G-VI	0	0	2	8	8	2	0	0	0	0	2	8	0	0	2	8	8	2	0	0

G-I Control, *G-II* BBN + DMA, *G-III* BBN + DMA + Diindolylmethane, *G-IV* BBN + DMA + Lupeol, *G-V* Diindolylmethane Alone, *G-VI* Lupeol Alone

The total no. of animals per group ($n = 10$). The percentage of positive cells was scored as: 3 + strong staining, more than 50 % of cells were stained; 2 + moderate staining, between 20 and 50 % of cells were stained; 1 + mild staining, between 1 and 20 % of cells were stained; 0 = weak or negative less than 1 % of cell staining

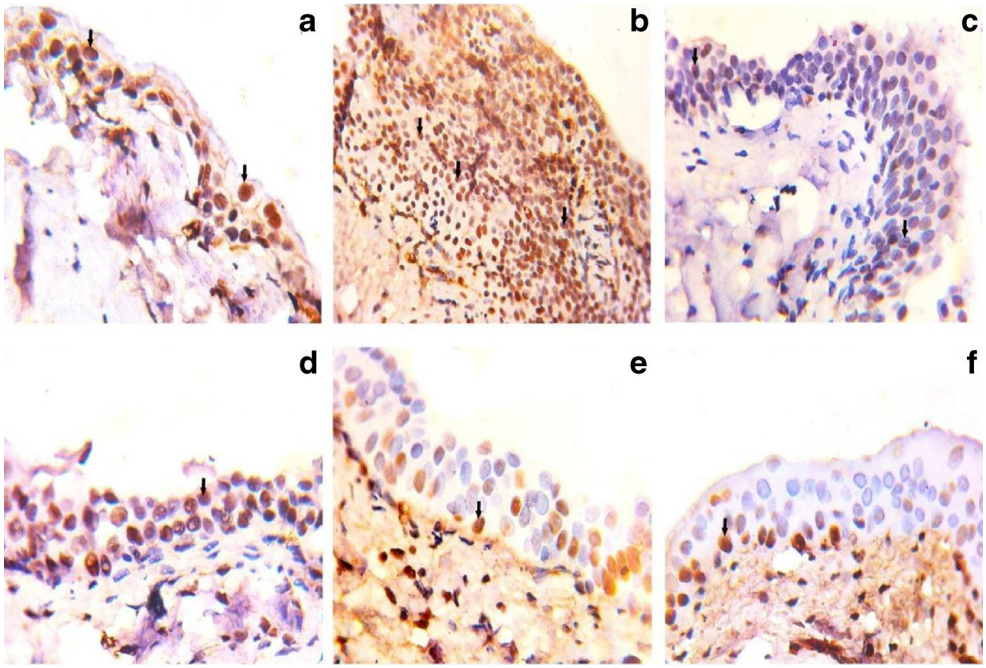

Fig. 1 Immunohistochemistry analysis of PCNA in control and experimental animals bladder

score of positively stained cells in control and experimental animals in each group are shown in Table 1.

Expression of Apoptotic Caspase 3 Expression in Control and Experimental Groups

The strong expression of caspase-3 in the urothelium from control, diindolylmethane and lupeol alone treated groups was observed (Fig. 4a, e and f). Weak or no expression of caspase 3 was observed in the bladder urothelium of BBN + DMA treated tumor rats (Fig. 4b). However, immunohistochemical analysis showed moderate expression of caspase 3 in the diindolylmethane and lupeol with BBN + DMA treated rats (Fig. 4c and d). Diindolylmethane and lupeol treatment induced the apoptotic caspase 3 protein expression in the urinary bladder carcinoma. The immunoexpression pattern of Caspase-3 and the score of positively stained cells in control and experimental animals in each group are shown in Table 1.

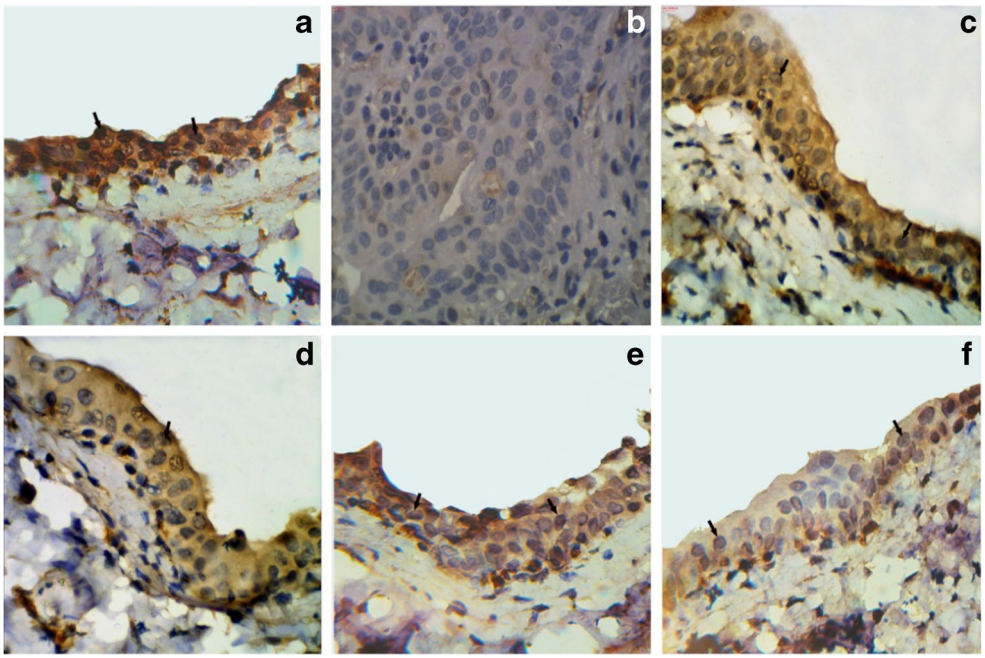

Fig. 2 Immunohistochemistry analysis of BAX in control and experimental rat bladder

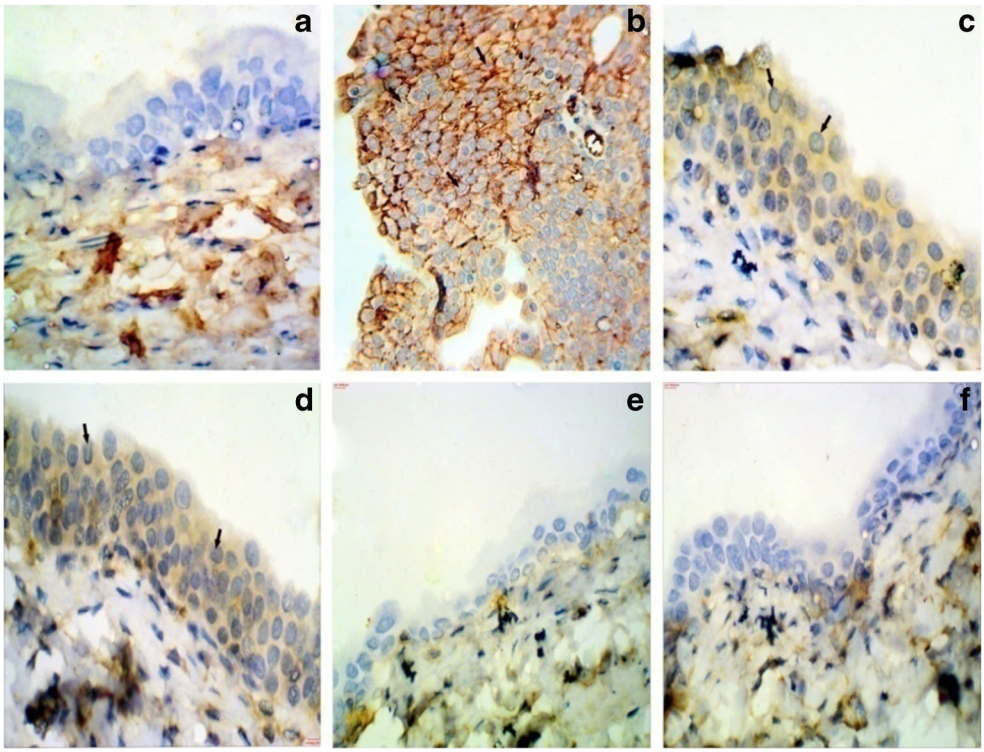

Fig. 3 Immunohistochemistry analysis of Bcl-2 in control and experimental animals bladder

Expression of Apoptotic Caspase 9 Expression in Control and Experimental Groups

The strong expression of caspase-9 in the urothelium from control, diindolylmethane and lupeol alone treated groups was observed (Fig. 5a, e and f). Weak or no expression of caspase 9 was observed in the bladder urothelium of BBN + DMA treated tumor rats (Fig. 5b). However, immunohistochemical analysis showed moderate expression of caspase 9 in the diindolylmethane and lupeol with BBN + DMA treated rats (Fig. 5c and d). Diindolylmethane and lupeol treatment induced the apoptotic caspase 9 protein expression in the

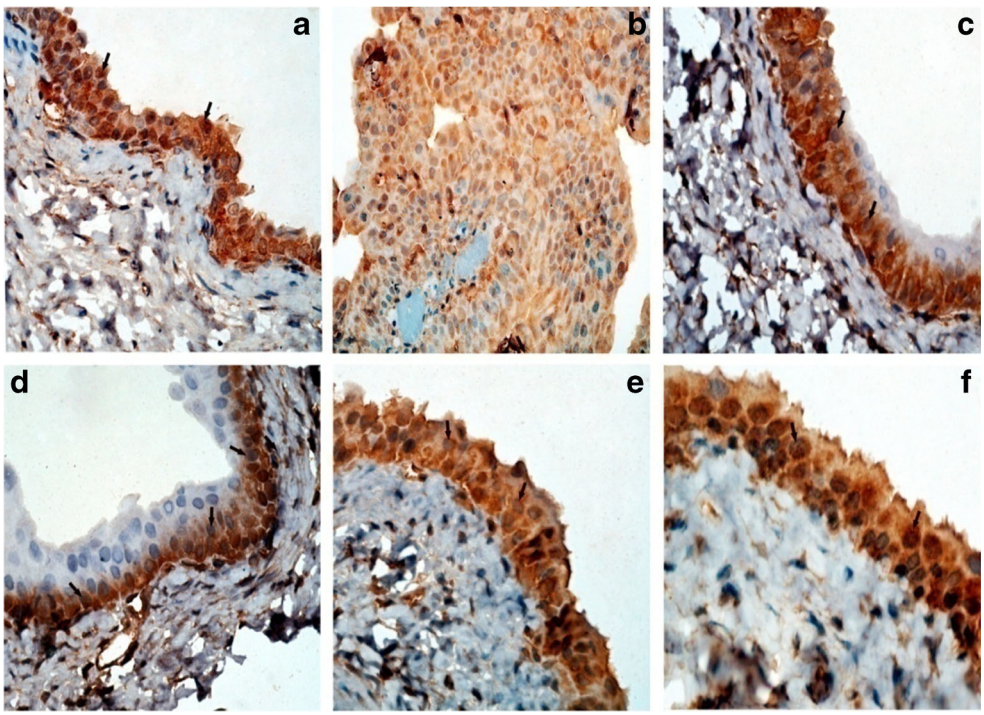

Fig. 4 Immunohistochemistry analysis of caspases 3 in control and experimental animals bladder

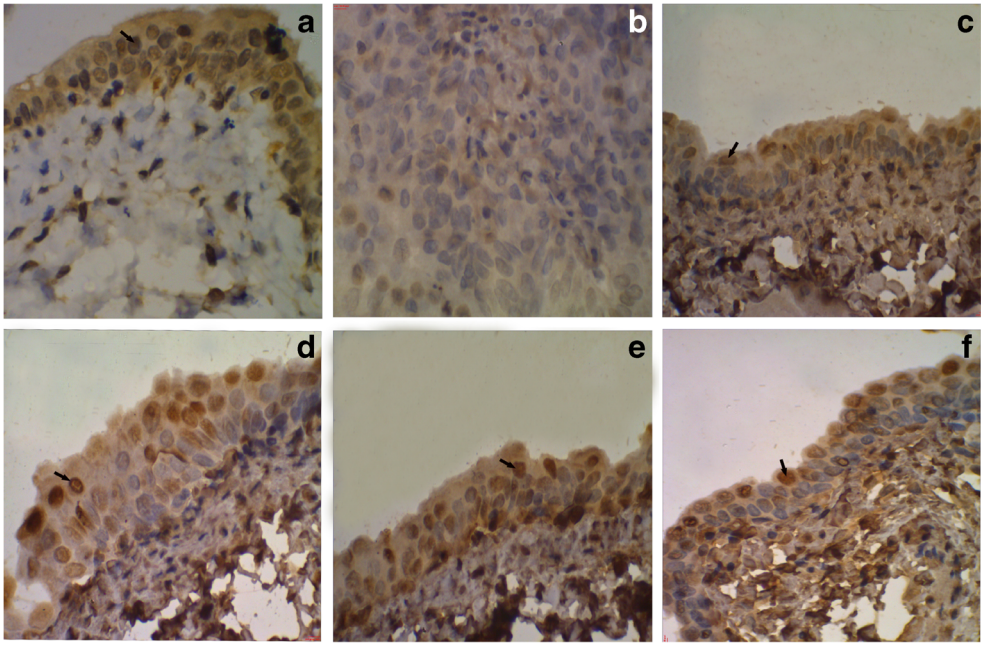

Fig. 5 Immunohistochemistry analysis of caspases 9 in control and experimental animals bladder

urinary bladder carcinoma. The immunoexpression pattern of Caspase-9 and the score of positively stained cells in control and experimental animals in each group are shown in Table 1.

Discussion

The process of carcinogenesis selects against apoptosis to initiate, promote, and perpetuate the malignant phenotype and apoptosis-inducing ability is considered to be a primary factor in evaluating the chemopreventive efficacy of a candidate agent. Research imparts that interplay of several proteins results in either inhibition or activation of the apoptotic cascade in carcinogenesis and chemoprevention [21]. Bladder cancer has been shown to resist programmed cell death with altered expression of both pro-apoptotic and anti-apoptotic proteins [22]. Loss of caspase-3 expression observed in our study was associated with bladder carcinogenesis. Immunohistochemical staining for PCNA provides significant clinical information which may be useful in the treatment of bladder cancer [23]. Overexpression of PCNA, Bax, caspases and Bcl-2 with downregulation of cytokeratins observed in carcinomas may confer a selective growth advantage on tumor cells [24]. Agents that inhibit cell proliferation and induce apoptosis are known to have immense potential in chemoprevention and chemotherapy. DIM is cytotoxic to cancer cells and inhibits growth of multiple tumor types and these responses are accompanied by activation of growth inhibitory and proapoptotic pathways [25]. Diindolylmethane increased the expression of bax and caspase 3 inducers of apoptosis in breast and prostate cancer cells [26, 27]. Kim et al. reported that diindolylmethane increased the activation of Bax, caspase and decreased the Bcl-2 levels in the colon cancer cells [28]. It has shown that DIM activates the pro-apoptotic proteins Fas, FasL and death receptor 5, leading to caspase-dependent apoptosis [29].

Lupeol-induced apoptosis was associated with caspase dependent mitochondrial cell death pathway through activation of Bax, caspases and decrease in Bcl-2 expression [30]. Saleem et al. reported that Lupeol can adopt multi-prong strategy to target multiple signaling pathways leading to induction of apoptosis and inhibition of growth of pancreatic cancer cells [31]. Lupeol treatment resulted in significant inhibition of cell viability in a dose-dependent manner and caused apoptotic death of this cell line with activation of caspase-3 expression [32]. It completely in hibit the oral buccal cancer in golden syrian hamster by its antioxidant properties [33]. Lupeol inhibited cell proliferation and induce apoptosis as well as cell cycle arrest of PCNA-1 cells and might offer a therapeutic potential advantage for human pancreatic cancer chemoprevention or chemotherapy [34]. Lupeol suppressed tumor growth in melanoma-bearing mice by attenuating proliferating cell nuclear factor (PCNA) which is highly expressed in melanoma or other tumors [35]. Our previous study proved that Diindolylmethane and Lupeol is a potent Cox-2 inhibitor, which also possess the anti-inflammatory properties by suppressing the NFκβ and TNFα by activating the tumor suppressor PTEN [36]. Based on our immunohistochemical analysis, we have observed limited expression of Bax and elevated expression of Bcl-2 in bladder tumor tissues harvested from BBN treated animals which may explain evasion of apoptosis in tumors. It is noteworthy that overexpression of Bcl-2 has been associated with downregulation of Bax. Hence these reports are in agreement with the results of the

present report and we speculate that DIM and lupeol would be inhibiting the bladder carcinogenesis in rats. The administration of DIM and lupeol had shown the inhibitory actions on chemical induced rat bladder carcinogenesis. Owing to the nature of biological potency and apoptotic inducing property of indole and triterpene compound might be the one of the mechanism to support the anticarcinogenic actions of them. The study had revealed the effects of DIM and lupeol on promotion stage of carcino- genesis in rat bladder. We were already shown the inhibitory actions in initiation stage of carcinogenesis in rats. Our laboratory is studying the effects of DIM and lupeol in metastasis formation of secondary tumors in animal models.

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Compliance with Ethical Standards

Conflict of Interest The author(s) declared no potential conflicts of interest.

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