Enhancement of Urinary Bladder Carcinogenesis by the Role of Chronic Bacterial Infection-induced Inflammation (Imunnohistochemical and Biochemical studies)

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Abstract

Background: Bacterial infections traditionally have not been considered major causes of cancer. Recently, however, bacteria have been linked to cancer by two mechanisms: induction of chronic inflammation and production of carcinogenic bacterial metabolites. The most specific example of the inflammatory mechanism of carcinogenesis is *Escherichia coli* infection. *E. coli* has been epidemiologically linked to urothelial carcinoma of the urinary bladder by its propensity to cause lifelong inflammation. This inflammation is in turn thought to cause cancer by inducing cell proliferation and production of mutagenic free radicals and N-nitroso compounds.

Material and methods: After each 3, 6 and 9 months of daily oral administration of dibutyl amine (DBA) plus sodium nitrate (nitrosamine precursors) in drinking water, curcuma in grinding diet and bladder injection with *E. coli*, rats were sacrificed. The excited bladder were dissected, processed and stained with H&E and anti-Ki67 immunohistochemical stains. This was followed by Elisa for caspse-3 and statistical analysis.

Results: The current results indicated that *E. coli* infection in the bladder tissues increases the carcinogenic ability of nitrosamine precursors through caused marked alteration in the form hyperplastic, dysplastic and metaplastic urothelium. Also, there was a statistically significant increase in ki67 immunoreactivity in urothelium. However, a statistically significant decrease in the concentration of caspase-3 in bladder tissue consequently caused the process of carcinogenesis. All these changes were less marked after curcuma treatment when compared with the group that not treated with curcuma.

Conclusion: Bacterial infection of the urinary bladder may play a major additive and possible role in bladder carcinogenesis. Rhizome of curcuma may have a protective action during induction of urinary bladder tumors.

Keywords: Bladder carcinogenesis - *E. coli* - curcuma – DBA - Ki67-Immunohistochemistry - Caspase-3.

Introduction

Bladder carcinoma is the most common malignancy of the urinary tract (Ploeg *et al*., 2009), where the cells lining the urinary bladder lose the ability to regulate their growth and start dividing uncontrollably. This abnormal growth results in a mass of cells that form a tumor (Good, 2003). Urothelial carcinomas account for more than 90% of urinary bladder cancer cases

(Hwa-Chain et al., 2011), and about 4% of all malignant tumors and approximately 7% of all urinary tract malignancies (WHO, 1997). Bladder cancer affecting the urinary tract worldwide and it accounted for 386,000 cases and 150,000 deaths in 2008 (Jemal *et al*., 2011). In Egypt, carcinoma of the bladder is the most prevalent cancer and account 30.3% of all cancers at the National Cancer Institute (NCI), Cairo (Khaled *et al*., 2004). The incidence of the bladder cancer in Egypt was 26 / million / year in males and 7 / million / year for females **(**Mokhtar, 1991**).**

Uropathogenic *Escherichia coli* (UPEC) are responsible for approximately 90% of urinary tract infections (UTI) (Todar, 2007). Caspases, free radicals and other proteins have been included in the pathogenesis of many diseases (Choudhary and Wang, 2009). Caspases are a group of cysteine proteases that cleave after an aspartic acid residue of a specific recognition site. This extrinsic activation then triggers the hallmark caspase cascade characteristic of the apoptotic pathway (Alnemri *et al*., 1996). Caspase-3 is a major effectors caspase that plays a critical role in the apoptotic cascade (Porter, 1999).

On the other hand, the Ki67 is a molecule that can be easily detected in growing cells in order to gain an understanding of the rate at which the cells within a tumor are growing, where Ki67 has been suggested as having prognostic value in renal cell carcinoma and urothelial neoplasms of the urinary bladder (Yurakh *et al*., 2006). This 345 kDa protein is present in proliferating cells in all cell-cycle phases (G1, S, G2 and M) and absent in quiescent cells (G0); thus, it can be used as a marker of growth fraction (Gerdes *et al*., 1984).

Aim of the study:

The present work was designed to study the possible role of chronic inflammation induced by *E. coli* in the urinary bladder of rats, the protective role of curcuma, in addition to the early detection for ki67 (as proliferative index) and caspase-3 (apoptotic modulator) during bladder carcinogenesis.

Material and Methods

Material

1- Chemicals

Dibutylamine $[CH_3.\ (CH_2)_3]_2NH$], is a colorless liquid with ammonia odor and molecular weight of 129.244 gm/mol. and Sodium nitrate [$NaNo_3$], is a white powder or colorless crystals with sweet smell were purchased from BDH ltd pool (England) and Arabic Laboratory Equipment Co. (Egypt) respectively. All other chemicals were of the highest purity commercially available.

2- The natural product (curcuma)

Curcuma was purchased from shop perfumery and was identified by Medicinal and Aromatic Plants Department, National Research Centre, Giza, Egypt.

3- Microorganism *E. coli*

E. coli originally isolated from the urine of patients with urinary tract infection (Nasser Institute, Cairo, Egypt). The *E. coli* were grown overnight in brain-heart infusion broth (BHI broth) medium then heat-killed in boiling water

for 30 min. The suspension was centrifuged and the sediment was suspended in phosphate buffered saline at a density 2 x 10^6 cells/ml and stored in 30-ml portions at -20°C until used. Once the suspension was thawed, any portion remaining after use was discarded (Yamamoto *et al*., 1992).

4- Experimental Animals and Dosing

The experiment was conducted in accordance with the provisions of the guide for care and use of laboratory animals (NIH, 1985). One hundred and fifty adult male albino rats, each weighing 60-80 gm. were kept in the laboratory under constant conditions of temperature 25ºC, and were maintained on a standard diet and water *ad libitum* for at least one week before the experimental study.

The experimental rats were divided into five groups as follow:

Group 1: Fed on the basal diet and normal water and left in normal condition, this group was served as control group.

Group 2: Bladder injected with 2x10^6 organisms suspended in 0.1 ml of phosphate-buffered in 2.1% NaCl solution (Yamamoto *et al*., 1992). This group was served as *E. coli* group.

Group 3: Administered drinking tap water containing 1.32 ml/L DBA and 2 gm/L sodium nitrate (El Gendy *et al*., 2007). This group was served as nitrosamine precursors group.

Group 4: Administered drinking tap water containing 1.32 ml/L DBA and 2 gm/L sodium nitrate and the bladder was injected with 2x10^6 *E. coli* bacteria in 0.1 ml salin. This group was served as *E. coli* and nitrosamine precursors group.

Group 5: Fed normal grinding diet supplemented with 1% of curcuma, drink tap water containing 1.32 ml/L DBA and 2 gm/L sodium nitrate and bladder was injected with 2 x 10^6 *E. Coli* bacteria in 0.1 ml saline. This group was served as *E. coli* plus nitrosamine precursors and curcuma group.

Diet was prepared three times a week just before the feeding time. DBA and sodium nitrate were mixed with the drinking water using magnetic stirrer for a period of 20 min.

Methods:

At the end of the experimental period, animals were sacrificed and the whole bladder was removed, washed with saline, dried, cut into weighted pieces and frozen -80ºC until used. Bladders were obtained to carry out histopathological and immunohistochemical studies. Body weight of rats in each group was recorded at the beginning of the experiment and at the end of each stage of scarification.

1- Histopathlogical findings:

For histopathological studies, bladder tissue pieces were fixed in 10 % formalin, blocked in paraffin, sectioned (4-6 µm) and stained with hematoxyline and eosin according to Banchroft *et al.* (1996).

2- immunohistochemical observations:

Sections of bladder fiwere immunostained using study anti Ki67 primary antibody for 60 minutes, followed by the secondary antibody application using labeled streptavidine –biotin. Sections were counterstained with hematoxyline

(Bancroft and Gamble, 2002) and analyzed by image analyzer for calculation of Ki67 labeling index (Ki67 LI). The results were expressed as mean ± standard error (Ki67 LI = mean ± SE %) using SPSS 11 for windows.

2.1 Immunoreactivity scoring

The sections were screened for positive cells, defined as cells with nuclear staining. The amount of Ki67 staining was scored in percentages. The areas with maximal immunohistochemical staining were used for scoring. A total, 300-500 cells were scored. In the visual estimation, only definitely brown nuclei were recorded as positive. The results were expressed as percentage of immunoreactive infected cell nuclei (Antoine *et al*., 2007).

3- Biochemical measurements

Total protein was determined according to Gornall *et al*. (1949). Caspase-3 concentration was determined using the CaspACE™ Assay System, Colorimetric kit according to the manufacturer's instructions.

4- Statistical analysis

All data were expressed as the mean ± standard error (SEM). The statistical significance of differences among means was assessed by the one way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons. Survival analysis was utilized to analyze the survivability of all animal groups. Differences between two groups were assessed by student's t test. Statistical differences were considered significant at $p \leq 0.05$. The least significant difference (LSD) test was used to separate the mean values according to Steel and Torrie (1981).

Results

A- Histopathological findings

Bladder histophathological changes due to bacterial infection, nitrosamine precursors and therapeutics effect of curcuma are shown in (fig.1). Examination of H and E sections of the urinary bladder of the control rats showed normal histological structure that consisted of the lining mucosal epithelium (transitional epithelium) since intermediate between non keratinizing squamous and pseudostratified columnar epithelium that about 5-7 cell layers, covered by superficial urothelium that is single layer of umbrella cells with eosinophilic cytoplasm and prominent nucleoli, while Intermediate urothelial cells are cuboidal to low columnar with well defined borders with ovoid nuclei, basal urothelial cells are more cylindrical and longitudinal nuclear grooves. The urothelium with underlying lamina propria is thin, contains loose to dense connective tissue, blood vessels, lymphatic, adipose tissue and muscularis that consists of longitudinal and circular muscle bundles. In this group there was no histopathological alteration during all treatment periods (Fig. A -1). In *E coli* group there were focal stratification and hyperplasia in the lining mucosal epithelium, maintains morphologic evidence of maturation from basal cells to superficial cells, absence of cytologic atypical, while the underlying lamina propria had sever congestion in the blood capillaries

with inflammatory cells infiltration (Fig. B -1). In nitrosamine precursors group, dysplasia appeared in the mucosal lining epithelium that characterized by lack of maturation in basal and intermediate cell layers (not full thickness). Uurothelial thickness (may be increased or decreased) and superficial umbrella cells were present. Atypical cytological changes were restricted to intermediate and basal cells, loss of cytoplasmic clearing and nuclear polarity, nuclear enlargement, nuclear membrane irregularities and nuclear hyperchromasia, while the underlying lamina propria showed congestion in newly formed capillaries (Fig. C -1). In nitrosamine precursors plus *E coli* group, the serosal layer showed granuloma like formation is a tiny collection of macrophages in which immune system attempts to wall off substances that it perceives as foreign but is unable to eliminate. Such substances include infectious organisms such as bacteria with inflammatory cells infiltration (Fig. D -1). In nitrosamine precursors plus *E. coli* group and protected with curcuma, showed normal bladder layers except congestion in the blood capillaries in the lamina propria (Fig. E -1).

B- Immunohistochemical observations

Urothelium of control and curcuma treated rats stained with immunostained for Ki67 showed few weak positive stained nuclei indicating the cell division of some urothelial cells (Figs. 2A and 2E). However, most of the urothelium showed strongly positive stained nuclei in rats treated with *E. coli* and nitrosamine precursors plus *E. coli* (Figs. 2B and 2D). Also, the urothelium of rats treated with nitrosamine precursors plus *E. coli* and curcuma illustrated that, the positively stained nuclei were markedly decreased approximated control section from that of nitrosamine precursors plus *E. coli* treated rats (Fig. 2E).

As shown in Table 2 and fig. 3, the effect of nitrosamine precursors, *E. coli* and curcuma on urinary bladder ki67 proliferating index were presented, and the analysis of variance revealed that, there was a significant difference between different treatment groups. Nitrosamine precursors plus *E. coli* treated rats and *E. coli* treated rats after 9 months illustrated a significant increase in urinary bladder Ki67 labeling index. However, rats protected with curcuma and treated with nitrosamine precursors plus *E. coli* showed a high significant decrease and depletion in the number of Ki 67 positive reaction in all layers of the epithelium with respect to control group and very high significant decrease when compared with nitrosamine precursors plus *E. coli* treated animals.

C- Detection of caspase-3

As shown in table (2), the present results indicated that, a comparison between mean level of caspase-3 concentrations in groups showed that, all treated groups were recorded lower values than control during the first 3 months, lowest values in nitrosamine precursors plus *E. coli* group and followed by the group that protected by curcuma. During 6 and 9 months, nitrosamine precursors plus *E. coli* group was recorded also the lowest values, while the group

that protected with curcuma started in increasing of caspase-3 concentrations when compared to nitrosamine precursors plus *E. coli* group.

Table (1): Urinary bladder Ki67 labeling index as a result of carcinogenicity of *E. coli*, nitrosamine precursor and curcuma administration during different intervals.

Intervals / Groups	3 months (%)	6 months (%)	9 months (%)
Control	**2.22 ± 0.13^{a}**	**2.22 ± 0.13^{a}**	**2.22 ± 0.13^{a}**
E.coli	**4.44 ± 0.67^{b}**	**4.53 ± 0.97^{b}**	**14.02 ± 2.73^{b}**
Nitrosamine precursors	**5.02 ± 0.53^{b}**	**5.61 ± 0.23^{b}**	**6.25 ± 0.32^{a}**
Nitrosamine precursors plus *E. coli*	**13.91±1.04^{c}**	**18.63 ± 0.94^{c}**	**20.75 ± 2.2^{a}**
Nitrosamine precursors plus *E. coli* plus curcuma	**5.6 ± 0.99^{b}**	**3.60 ± 0.43^{d}**	**3.07 ± 1.00^{c}**
F value	**35.48531**	**108.7714**	**25.61225**
F crit	**2.578739**	**2.578739**	**2.578739**

Table (2): Effect of *E. coli*, nitrosamine precursor and curcuma administration on caspase-3 concentrations of bladder tissues during different intervals.

Intervals / Groups	3 months (µM)	6 months (µM)	9 months (µM)
Control	**95.1 ± 2.40^{a}**	**95.17 ± 2.40^{a}**	**95.17 ± 2.40^{a}**
E. coli	**61.5 ± 6.52^{b}**	**71.67 ± 18.97^{b}**	**93.67 ± 8.24^{a}**
Nitrosamine precursors	**34.83± 3.83^{c}**	**31 ± 6.04^{c}**	**29.5 ± 0.85^{b}**
Nitrosamine precursors plus *E. coli*	**20.5 ± 2.29^{d}**	**16.67 ± 0.72^{d}**	**14.83 ± 1.01^{c}**
Nitrosamine precursors plus *E. coli* plus curcum	**28 ± 1.51^{e}**	**29.3 ± 4.05^{c}**	**34 ± 3.47^{c}**
F value	**74.77341**	**13.95873**	**89.09777**
F crit	**2.75871**	**2.75871**	**2.75871**

Within each column, means superscript with different letters are significantly different ($P \leq 0.05$).

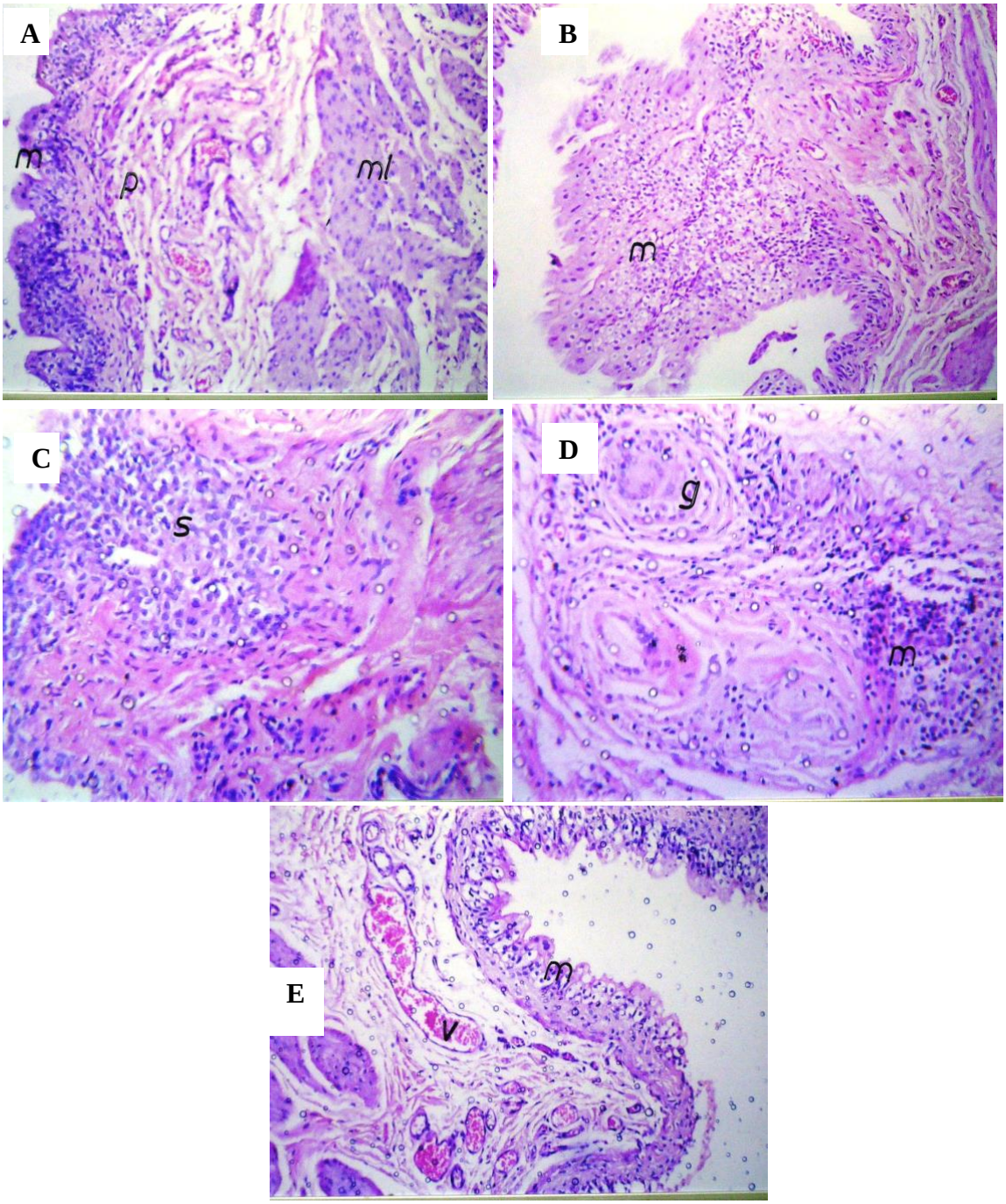

Figure (1): Light micrograph showing **(A)** normal bladder tissues in control gp. **(B)** hyperplasia, there is an increase in the number of urothelial layers (more than 7 layers) and stratified urothelium in *E coli* gp. **(C)** showing dysplasia, consists of an expansion of immature cells, with a corresponding decrease in the number and location of mature cells in the mucosal lining epithelium (s) in nitrosamine precursors gp**.(D)** granuloma formation (g), is a tiny collection of macrophages in which immune system attempts to wall off substances that it perceives as foreign but is unable to eliminate. Such substances include infectious organisms such as bacteria with inflammatory cells infiltration (m) in serosal layer showed in nitrosamine precursors plus *E. coli* gp**.(E)** normal bladder layers except congestion in blood capillaries of lamina propria (v) in nitrosamine precursors plus *E. coli* plus curcuma gp. (H and E, X40

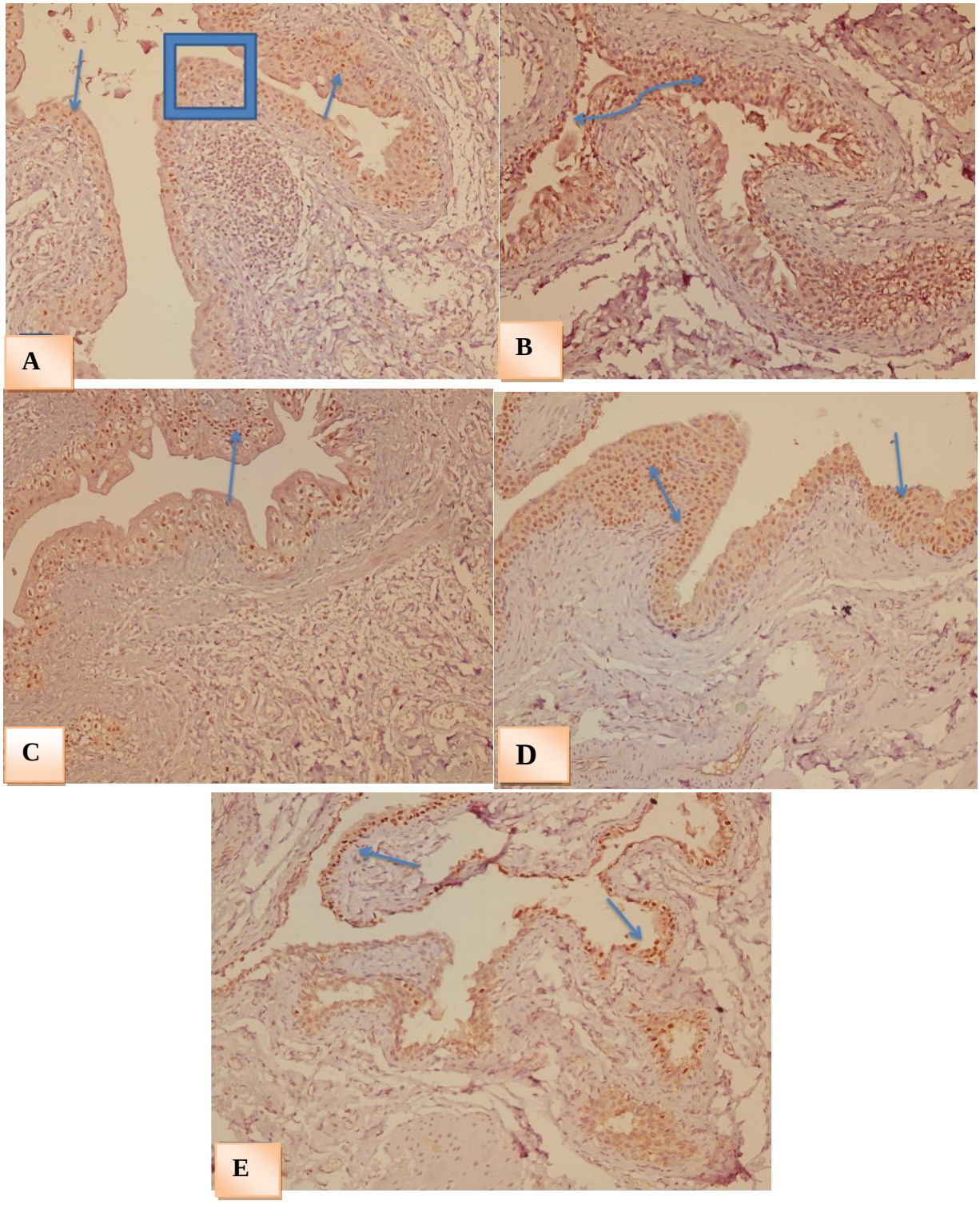

Figure 2: Immunostaining micrograph of Ki67 expression in different groups (the positivity is brown nuclear staining), Control **(A),** *E. coli* gp **(B)**, nitrosamine precursors gp **(C),** nitrosamine precursors plus *E. coli* gp **(D)** and nitrosamine precursors plus *E. coli* gp treated with roots of curcuma **(E).** (Labeled Streptavidin Biotin, X 200).

Discussion

An experimental study in rats had shown associations between chronic infections with Escherichia coli and early bladder neoplasia (Davis et al., 1991). Several studies concluded that, urinary tract infectionpromotes urinary tract carcinogenesis in rat and infection with *Escherichia coli* produced permanent infection, acute and chronic inflammation and urothelial hyperplasia (Schetter *et al*., 2010).

The results in the current study indicated that, *E. coli* infection in the bladder tissues increased the carcinogenity of nitrosamine precursors which is in agreement with Ashmawey *et al*. (2011). This may be due to the increase of production of nitrite and nitrosamine by *E. coli*. These results are also in agreement with Rex *et al*. (2008), who found that, N-butyl-N-(4-hydroxybutyl) nitrosamine has a strong ability to induce rat bladder cancer. The present study showed that, bacterial infection of the urinary bladder may play a major role in bladder carcinogenesis, both by helping in-situ nitrosamine synthesis and by augmenting carcinogenesis by nitrosamines. This result may agree with Jhamb *et al*. (2007) who stated that, when animals receiving amine and nitrate, indicating re-absorption of the carcinogen synthesized in situ to induce distant organ transformation. In another case, *E. coli* infection augmented bladder carcinogenesis by N-nitrosobutyl (4-hydroxybutyl) amine (NBHBA) as indicated by earlier appearance of bladder tumours.

Some mechanisms were suggested for the tumor enhancing effect of *E. coli* induced inflammation in the present experiment. Firstly, the formation of the intracellular bacterial communities (IBC), secondly, Uropathogenic (UPEC) colonization is counteracted by the innate host responses to infection, which include soluble defensive molecules, cytokine secretion and neutrophil influx which increased bladder inflammation and polymorphonuclear cell (PMN) influx in response to the proinflammatory cytokine, interleukin 6 (IL-6) and the PMN chemo tactic cytokine, IL-8, respectively. This host inflammatory response is augmented by *E. coli* invasion of urothelial cells (Schilling *et al*., 2001). Tissue damage caused by the influx of neutrophils, as well as the generation of secreted toxins by UPEC, also likely facilitate bacterial dissemination throughout the multiple layers of bladder epithelium (Higgs and Pollard, 2000). Thirdly, the initiation of bladder carcinogenesis could be due to the nitrosation of secondary amines with ingested or metabolically derived nitrite that leads to *N*-nitrosamine formation (Hill, 1988).

In the present study, the Ki67 index is a useful marker of cell proliferation and was significantly greater in rats that received nitrosamine precursors and *E. coli*. These results agree with El-kott (2007) who reported that, urinary bladder Ki67 labeling index was very high in N-methyl-N-nitrosourea (MNU) precursors group when compared with control rats. However, rats which were protected with

garlic extract and treated with N- MNU precursors showed a high significant increase with respect to control group and very high significant decrease when compared with MNU precursors treated animals. the present results were also in agreement with Tsuji *et al*. (1997) who concluded that, immunohistochemical analysis for Ki67 are useful prognostic indicators in patients with urinary bladder cancer who undergo radical cystectomy.

The present findings have shown that *E. coli* infection was associated with a significant decrease in the concentration of caspase-3 protein, one form of mediators of apoptosis pathways. However, it is significant that tumors did not develop in the groups treated with *E. coli* plus carcinogen. This suggests that, inflammatory reaction by *E. coli* only is insufficient to induce tumors but may be sufficient to augment neoplastic changes induced by carcinogen.

The current results have also demonstrated the presence of caspases-3 protein in bladder tissues but it was down regulated than that found in control group and this may be due to functional deletion or mutation in the caspases-3 gene (Yang *et al*., 2001).This results is in agreement with Burton *et al*.(1999) who suggested that, the measurement of caspase 3 levels may be useful in monitoring patients with bladder carcinoma in situ, to identify both those at increased risk for invasive bladder cancer and those who are more likely to have a response to intravesicular immunotherapy. The current results were also in agreement with another reports by Karamitopoulou *et al*. (2010). The decreased detection of active caspase-3 carcinomas suggested that, alterations in interrelated apoptosis markers may play an important role in the progression of urothelial carcinoma from a superficially infiltrating to a muscle invading tumour and would help to better characterize a subpopulation of T1 carcinomas that could profit from early cystectomy or more aggressive adjuvant chemotherapy. Active caspase-3 might be an important prognostic factor in bladder cancer.

In the current study, curcuma treated group showed significant decrease in the reduced caspase-3 level. This result indicated the ability of curcuma with its rich anti carcinogens substance to protect bladder from sever carcinogenic action of nitrosamine and minimize its effect. The present findings were in agreement with Katia et al. (2009) who reported that, Curcuma could be an option for treatment of bladder cancer and with Kawamori et al. (1999) who concoluded that, chemopreventive activity of curcuma has been indicated when administered before, during and after carcinogenic treatment as well as when administered during the promotion and progression phase of colon carcinogenesis in rats. Also the present results were in concomitant with Tian *et al*. (2008) who stated that, curcuma could prove an effective chemopreventive and chemotherapy agent for bladder cancer, i*n vivo* study revealed curcuma did induce apoptosis in situ, inhibit and slow the development of bladder cancer, where exposure

of human bladder cancer cells to curcuma resulted in the induction of apoptotic cell death and caused cells to arrest in the G2/M phase. At the same time, curcuma treatment did not cause any side-effect.

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دور الإلتهاب الناجم عن الإصابة البكتيرية المزمنة المسببة لسرطنة المثانة

(دراسة هستوكيميائية وبيوكيميائية)

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تعتبر الإصابة البكتيرية من الأسباب الشائعة المسببة للسرطان . فى الأونة الأخيرة إرتبطت البكتيريا بحدوث السرطان عن طريق آليتين هما: حدوث الإلتهاب المزمن وإنتاج الأيضات البكتيرية المسببة للسرطان.

ويعد الإلتهاب الناجم عن الإصابة البكتيرية بالإيشيريشيا كولاى من أحد العوامل المسببة لسرطان المثانة عن طريق ميلها الى حدوث إلتهاب مدى الحياه والذى يساعد على تكاثر الخلايا وإنتاج الشقائق الحرة المطفرة ومركبات النيتروزو.

وقد هدفت هذه الدراسة على دور الإصابة البكتيرية المزمنة المسببة لسرطان المثانة وذلك عن طريق حقن مثانة ذكور الفئران البالغة ببكتيريا الإيشيريشيا كولاى وإعطائهم جرعة يومية من ثنائى بيوتيل أمين و نترات الصوديوم (سلائف النيتروسامين) مذابة فى ماء الشرب وإستخدم الكركم كواقى كيميائى لتقييم قدرته العلاجيه أثناء حدوث عملية سرطنة المثانة وذلك لمدة تسعة شهور حيث أخذت العينات كل ثلاث شهور وذلك عن طريق تشريح الفئران وأخذ المثانة وتحضيرها لإجراء الدراسات النسيجية بإستخدام صبغة الهيماتوكسيلين والإيوسين والصبغة الهستوكيميائية المناعية ضد الكى أى 67 ثم تحليل الصور بإستخدام محلل الصورة لحساب المؤشر التكاثرى وتلا ذلك إجراء دراسة الإليزا لتعيين بروتين الكاسبس-3 فى أنسجة المثانة والتحاليل الإحصائية.

وقد أظهرت هذه الدراسة أن العدوى البكتيرية لأنسجة المثانة زادت من قدرة سرطنة النيتروسامين وتسببت فى تغيرات ملحوظة فى طبقة الظهارة البولية (الطبقة المبطنة لجدار المثانة) عن طريق تكاثر خلاياهاعن الطبيعى وتغير شكل الأنوية وظهور خلايا جديدة وغيرها. كما لوحظت زيادة ذات دلالة إحصائية فى الصبغة الهستوكيميائية المناعية ضد أنتيجين الكى أى 67 داخل أنوية خلايا تلك الطبقة.

وكذلك أظهرت الدراسة وجود إنخفاض ذات دلالة إحصائية فى تركيز الكاسبس-3 فى أنسجة المثانة . بينما كانت هذه التغيرات أقل وضوحا عند إستخدام الكركم مقارنة بنفس المجموعة التى لم تعالج به .

ومما سبق نستخلص أن العدوى البكتيرية للمثانة البولية تلعب دورا رئيسيا خلال عملية سرطنة المثانة وتلعب جذور الكركم دورا وقائيا خلال عملية السرطنة. ويعتبر كلا من الكى أى 67 والكاسبس-3 أحد دلائل الأورام التى تساعد على الإكتشاف المبكر لهذا المرض.