Expression of Nuclear Factor Kappa P65 ;Ki-67 and Caspase-3 in Chemically -Induced Bladder Carcinogenesis

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Abstract - The increased expression of NF-kB associated with carcinogenesis resulted in cell proliferation and stop apoptosis, in bladder carcinogenesis induced by dibutylamine / sodium nitrate mediated by e-coli infection. This study was designed to evaluate the potential protective effect of curcum in rats administered nitrosamine precursors; dibutylamine (DBA) and sodium nitrate (NaNO3); and infected with Escherichia coli (E. coli) and also to monitor changes in nuclear factor the Kappa B p65 (NF-kB p56) pathway and its downstream products, Bcl-2 and interleukin-6 (IL-6), in parallel with nitrosamine precursors, E. Coli and curcum treatment. Rats were divided into three groups (n = 25 each): Group I a normal control group, group II administered DBA/NaNO3 in drinking water and group III was administered DBA/NaNO3 in drinking water, infected with E. coli and receiving standard diet containing 1% curcum powder; and group IV received DBA/NaNO3 +E.coli+ curcum. Dibutylamine was given as 1000 ppm, NaNO3 was given as 2000 ppm in drinking water, infected by 0.1 ml saline containing suspension of E. coli in the bladder (approximately 2 X 10^6 organisms) and received standard diet containing 1% curcum powder mixed in the diet. Histopathological examination and immunohistochemical studies indicated that the curcum treated group featured a lower incidence of urinary bladder lesions, and lower levels of NF-kB, Bcl-2 and IL-6 and Ki67 protein than the group receiving nitrosamine precursor and infected with E. coli. These findings suggested that curcum may have a protective role during the process of bladder carcinogenesis by inhibiting the NF-kB pathway and its downstream products.

keywords: Bladder carcinogenesis; Curcum; E.coli; DBA; NF-κBp65, Ki67; Immunohistochemistry; Caspase-3.

Introduction

Bladder carcinoma is one of the most common malignancy of the urinary tract (Gornall et al., 1949; LoTempio et al., 2005; Pradeep et al., 2007). Urothelial carcinomas account for more than 90% of urinary bladder cancer cases. Bladder cancer affecting the urinary tract worldwide and it accounted for 386,000 cases and 150,000 deaths in 2008 (Pradeep et al., 2007). In Egypt, carcinoma of the bladder is the most prevalent cancer and account for 30.3% of all cancers at the national cancer institute (NCI), Cairo. The incidence of the bladder cancer in Egypt was 26 / million / year in males and 7 / million / year for females. Uropathogenic E. coli (UPEC) is responsible for approximately 90% of urinary tract infections (UTI) seen in individuals with ordinary anatomy(Schetter et al., 2010).

Nitrosamines are considered one of the most important environmental carcinogens (Hong et al., 2012). It was found that N-nitrosamines can be formed in bladder in the presence of nitrate reducing bacteria. It is estimated that 20–25% of all human cancers are caused by chronic infection and inflammation (Steel and Torrie, 1981) (Thangapazham et al., 2006). One of the key molecules that link chronic inflammation and cancer is represented by NF-kB family of transcription factors (Steel and Torrie, 1981) (Thangapazham et al., 2006). NF-kB regulates the transcription of genes for proinflammatory cytokines (e.g. IL-6 and tissue necrosis factor alpha TNFα), adhesion molecules (Barnes and Karin, 1997) and the expression of several pro-survival genes (e.g. Bcl-2) (Calzado et al., 2007).

On the other hand, Ki67 is a molecule that detected in growing cells, it has been a prognostic value in renal cell carcinoma and urothelial neoplasms of the urinary bladder (Viartour et al., 2005). 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).

Herbal medicine and spices can be used as preventive measurement against cancer due to their antimicrobial, antioxidant, and antitumorigenic properties, as well as their direct suppressive effect on carcinogen bioactivation (Jankovic and Radosavljevic, 2007). More natural and dietary compounds including curcumin have been recognized as cancer chemopreventive agents due to its non-toxic and anti-carcinogenic properties (Ploeg et al., 2009).

Curcumin (diferuloylmethane) is a major constituent of the yellow spice turmeric derived from the rhizomes of Curcuma longa. It is safe and nontoxic and has demonstrable antitumor, anti-inflammatory, anti-apoptotic, and antioxidant properties. Curcumin also inhibits tumor metastasis, invasion, and angiogenesis (Karin et al., 2002; Leitte et al., 2009). NF-kB regulates the transcription of genes for proinflammatory cytokines (e.g. IL-6 and tissue necrosis factor alpha TNFα), adhesion molecules (Barnes and Karin, 1997) and the expression of several pro-survival genes (e.g. Bcl-2) (Calzado et al., 2007).

Materials and Methods:

Experimental Animals and Dosing:

One hundred male albino rats, weighing 50 - 60 gm were divided into four groups (n = 25 each): group(1): normal control group, group (2): received nitrosamine precursor; 1000 ppm DBA and 2000 ppm NaNO3; in drinking water as previously described by (El-Gendy et al., 2010), group (3) received nitrosamine precursor; 1000 ppm DBA and 2000 ppm NaNO3; in drinking water as previously described by (El-Gendy et al., 2010) and infected by 0.1 ml saline containing suspension of E. coli in the bladder (approximately 2 X 10⁶ organisms), as previously described by (Goel et al., 2008) and group (4): received DBA/NaNO3 in drinking water, infected with E. coli and received standard diet containing 1% curcum powder (obtained from commercial market) mixed in the diet, 2 weeks prior E.coli infection and all over the experimental period (Sadik et al., 2008).

Laboratory Procedures:

Six rats from each group were sacrificed at the 3rd, sixth, nineth month of the experiment. The bladder of the rats were excised immediately after scarifying rats and divided into two pieces; the first stored in 10% formalin for histopathological and studies according to (Bancroft and Stevens, 1996), for immunohistochemical studies of nuclear factor kappa NF-kB,ki67 protein expression according to (Bancroft and Stevens, 1996), the second piece was washed three times with saline and stored for total protein extraction to be used for determination of caspase-3 protein expression and rats in different groups were scarified; bladder was removed and an autopsy samples were taken from the urinary bladder of rats in different groups, to carry out an Immunohistochemical studies, histopathological samples fixed in 10% formalin saline for twenty four hours. The results were expressed as mean ± standard deviation (Ki67 LI = mean ± SD %) using SPSS 11for windows.

Other specimens of bladder were removed immediately from sacrificed animals, washed with saline, dried, cut into weighed pieces and kept frozen at -80°C then tissue homogenate was prepared according to (Hong et al., 2012).

Biochemical measurements: Total protein was determined according to (Gerdes et al., 1984).

NF-kB p65:

NF-kB p65 determination by ELISA kit (Glory Science Co., Ltd, USA) following the manufacturer instructions according to (Hong et al., 2012).

Immunohistochemical Study.

The immunohistochemical study was carried out by the treatment of the paraffin sections with xylene and applying Ki67primary antibody according to (Antoine et al., 2007).

Caspase-3:

Caspase-3 protein expression was determined using the CaspACE™ Assay System, Colorimetric kit according to the manufacturer's instructions (Promega Corporation, USA).

Histopathological Study

1. For histopathological studies the formalin stored bladder tissue was processed and stained with hematoxyline and eosin according to (Bancroft and Stevens, 1996).

Statistical Analysis

All data were expressed as the mean ± standard error (SEM). The statistical significance of differences among means was assessed by 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 ≤ 0.05. The least significant difference (LSD) test was used to separate the mean values according to (Munday et al., 2008).

Results:

The Histopathological Results:

Histopathological changes of bladder are presented in figure 1. The highest incidence of lessions was in the Group received nitrosamine precursor and infected with E. coli (group III) and was represented by hyperplasia and dysplasia, while the curcum treated group (group IV) showed only minor changes represented by congestion in the blood capillaries of lamina propra, focal desquamation and focal hemorrhage. Curcum treated group (group IV) also showed lower level of NF-κB, than the group received nitrosamine precursor and infected with E. Coli.

Biochemical Results

The mean ± SD of NF-kB p65 level was significantly lower in curcum treated (group IV) compared with nitrosamine precursor plus E. coli (group III) all over the experiment duration. At three months interval, NF-kB p65 level was significantly higher in group III (1.19 ± 0.19 ng/ml) compared with control group (0.57 ± 0.07 ng/ml),whereas NF-kB p65 level in curcum treated (group IV) (0.66 ± 0.05 ng/ml) was significantly lower, compared with group II( nitrosamine precursor plus E. coli). At six months interval, NF-kB p65 level was significantly higher in group II (1.52 ± 0.21 ng/ml) compared with control group (0.61 ± 0.08 ng/ml) and NF-kB p65 level in curcum treated group (group III) (0.68 ± 0.03 ng/ml) was significantly lower than group II. At nine months interval, NF-kB p65 level was significantly higher in group II (1.72 ± 0.14 ng/ml) compared with control group (0.70 ± 0.11 ng/ml) and NF-kB p65 level in curcum treated group (group III) (0.73 ± 0.08 ng/ml) was significantly lower compared with
group II. On the other hand curcuma treated group (group III) (325.3 ± 8.92 ng/ml) was significantly lower compared with group II (table 1).

Immunohistochemical Observations

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

As shown in Table 2 and fig. 2, 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 treated 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

Detection of Caspase-3:

The mean ± SD of caspase-3 level in nitrosamine precursor plus E.coli-treated group (20.5) as shown in table (3) was significantly lower compared to the nitrosamine precursor-treated (34.83) group, while co-administration with curcuma resulted in more decrease in the level of caspase-3 compared to nitrosamine precursor-treated group.

Discussion

Nitrosamines are known carcinogenic agents. However, the mechanism of action is not well understood. Generation of reactive oxygen species could be an important cause of toxicity of nitrosamines. These cellular pro-oxidant states can initiate oxidation of lipids and other biomolecules (Ozbek et al., 2011; Ploeg et al., 2009; Porter and Janicke, 1999).

E. coli enhancing effect on the carcinogenicity of nitrosamine precursor can be explained by the ability of the bacteria to increase nitrite level which through subsequent nitrosation can give rise to highly carcinogenic N-nitroso compounds (Higgy et al., 1987). The ability of E. coli infection to increase nitrite level can be explained by several mechanisms. First E. coli is capable of reducing nitrate to nitrite by a membrane-bound nitrate reductase enzyme (Kunnumakkara et al., 2007). Second, it was proven that E. coli lipopolysaccharide (LPS); a major cell wall component of E. coli; when instilled intravesically or intraperitoneally is capable of production of inducible nitric oxide synthase (iNOS) (Tripathi and Jena, 2010; Chen et al., 2006) which is an endogenous source of nitrite. In our study curcuma could have led to decrease in nitrosamines synthesis and decreasing the carcinogenic ability of nitrosamines precursor by the reduction in iNOS level as (Viatour et al., 2005) reported that curcumin treatment showed anti tumorigenic potential by significantly reducing the levels of iNOS.

The results in the current study indicated that E. coli infection in the bladder tissues increased the carcinogenicity of nitrosamine precursors which is in agreement with Higgy et al., 1987), (Palmeira et al., 2010) and (Ashmawey et al., 2011)This may be due to the increased production of nitrite and nitrosamine by E. coli.

Activation of NF-kappaB occurs mainly via I-kappaB kinase (IKK)–mediated phosphorylation of inhibitory molecules (Thangapazham et al., 2006).

In the present study curcumin treated group showed lower incidences of urinary bladder lesions, lower level of NF-κB, than group receiving nitrosamine precursor and infected with E. coli. These results indicate that curcumin has a strong protective effect during the process of bladder carcinogenesis. Curcumin blocks the NF-kappa B signaling and inhibits IKK activation, thereby suppressing proliferation of head and neck squamous cell carcinoma (Aggarwal et al., 2006).

The following result is also in agreement with ((flankovic and Radosavljevic, 2007; Kawamori et al., 1999; Todar, 2007)) who reported that blockage of PI3K/ AKT signaling pathway led to altered balance between pro-apoptotic (increase in Bax level) and anti-apoptotic members (decrease in Bcl-2 level) of Bcl-2 family.

In the present study the Ki67 labeling index is very high, These results are in line with those of (El-Kott, 2007; Nishimura et al., 2008; Steel and Torrie, 1981) suggesting that, immunohistochemical analysis for Ki67 are useful prognostic indicators in patients with urinary bladder cancer who undergo radical cystectomy.

Results of the current study 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 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 (Viatour et al., 2005).

Results of the following study are in agreement with (9; 24; 40, and 53), the decreased detection of active caspase-3 carciomas suggest that alterations in interrelated apoptosis markers may play an important role in the progression of urothelial carcinoma. Active caspase-3 might be an important prognostic factor in bladder cancer.
In the current study, curcumin treated group show significant decrease in the reduced caspase-3 level.

These results agree with (Kamat et al., 2009; Karamitopoulou et al., 2010; Sarkar et al., 2009) concluded that chemopreventive activity of curcumin was clear when administered before, during and after carcinogenic treatment, during the promotion and progression phase of colon carcinogenesis in rats.

In conclusion, E. coli infection might play a role in the development of bladder cancer, and it may be mediated by activation of NF-κB pathway resulting in inhibition of apoptosis and increased inflammation while curcumin treatment was able to reduce the incidence of bladder lesions and this was accompanied by reduced level of NF-κB, caspase-3 and Ki67, incidence of bladder lesions and this was accompanied by reduced level of NF-κB, Bcl-2 and IL-6 suggesting that curcumin can prevent the deleterious effect of nitrosamine precursor plus E. coli by inhibiting NF-κB pathway and its products.

Acknowledgement

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References


Figures

Figure (1): Light micrograph showing (A) control gp. (B) nitrosamine precursors gp. (C) nitrosamine precursors plus *E. coli* gp. (D) 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), Nitrosamine precursor’s gp (B), Nitrosamine precursors plus E. coli gp(C) and Nitrosamine precursors plus E. coli gp group treated with roots of curcum (D). Labeled Streptavidin Biotin (LSAB) Method (original magnification X 200).
### Table (1): Tissue homogenate level of NF-κBp65 (ng/ml) in different treated groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>3 months (ng/ml)</th>
<th>6 months (ng/ml)</th>
<th>9 months (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean ± S.D</td>
<td>Range</td>
</tr>
<tr>
<td><strong>Group I</strong></td>
<td>0.51 - 0.67</td>
<td>0.57 ± 0.07</td>
<td>0.53 - 0.67</td>
</tr>
<tr>
<td></td>
<td>0.51 ± 0.07</td>
<td>0.61 ± 0.08</td>
<td>0.70 ± 0.11</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td>0.57 - 1.05</td>
<td>1.02 - 1.50</td>
<td>1.39 - 2.09</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td>0.84 ± 0.17a,da,d</td>
<td>1.27 ± 0.20a,da,d</td>
<td>1.66 ± 0.27a,b,da,d</td>
</tr>
<tr>
<td></td>
<td>0.94 - 1.40</td>
<td>1.30 - 1.87</td>
<td>1.58 - 1.95</td>
</tr>
<tr>
<td><strong>Group IV</strong></td>
<td>1.19 ± 0.19ab,c</td>
<td>1.52 ± 0.21ab,c</td>
<td>1.72 ± 0.14ab,c</td>
</tr>
<tr>
<td></td>
<td>0.60 - 0.74</td>
<td>0.63 - 0.71</td>
<td>0.63 - 0.86</td>
</tr>
<tr>
<td></td>
<td>0.66 ± 0.05b</td>
<td>0.68 ± 0.03b</td>
<td>0.73 ± 0.08b</td>
</tr>
</tbody>
</table>

- a: Significantly different from control group (group I) at P < 0.05.
- b: Significantly different from DBA and *E. coli* group (group II) at P < 0.05.
- c: Significantly different from *E. coli* + nitrosamine precursors + group (group III) at P < 0.05.

### Table (2): Urinary bladder Ki67 labeling index as a result of carcinogenicity of *E. coli*, nitrosamine precursor and curcuma administration during different intervals.

<table>
<thead>
<tr>
<th>Groups</th>
<th>3 months (%)</th>
<th>6 months (%)</th>
<th>9 months (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>2.22 ± 0.13a*</td>
<td>2.22 ± 0.13a*</td>
<td>2.22 ± 0.13a*</td>
</tr>
<tr>
<td><strong>Nitrosamine precursors</strong></td>
<td>5.02 ± 0.53b*</td>
<td>5.61 ± 0.23b*</td>
<td>6.25 ± 0.32b*</td>
</tr>
<tr>
<td><strong>Nitrosamine precursors plus</strong> E. coli</td>
<td>13.91 ± 1.04c</td>
<td>18.63 ± 0.94c</td>
<td>20.75 ± 2.2c*</td>
</tr>
<tr>
<td><strong>Nitrosamine precursors plus</strong> E. coli plus curcum</td>
<td>5.6 ± 0.99b</td>
<td>3.60 ± 0.43d</td>
<td>3.07 ± 1.00c</td>
</tr>
</tbody>
</table>

- a: Significantly different from control group (group I) at P < 0.05.
- b: Significantly different from DBA and *E. coli* group (group II) at P < 0.05.
- c: Significantly different from *E. coli* + nitrosamine precursors + group (group III) at P < 0.05.

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>3 months (μM)</th>
<th>6 months (μM)</th>
<th>9 months (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>95.1 ± 2.40a*</td>
<td>95.17 ± 2.40a*</td>
<td>95.17 ± 2.40a*</td>
</tr>
<tr>
<td><strong>Nitrosamine precursors</strong></td>
<td>34.83 ± 3.83c</td>
<td>31 ± 6.04c</td>
<td>29.5 ± 0.85c</td>
</tr>
<tr>
<td><strong>Nitrosamine precursors plus</strong> E. coli</td>
<td>20.5 ± 2.29d</td>
<td>16.67 ± 0.72d</td>
<td>14.83 ± 1.01d</td>
</tr>
</tbody>
</table>

- a: Significantly different from control group (group I) at P < 0.05.
- b: Significantly different from DBA and *E. coli* group (group II) at P < 0.05.
- c: Significantly different from *E. coli* + nitrosamine precursors + group (group III) at P < 0.05.