Abstract

Objective: This work is designed to study the possible role of chronic inflammation induced by E. coli in the urinary bladder of rats, the protective role of soybean flour, in addition to the role of oxidative and nitrosative stresses during bladder carcinogenesis.

Material & Methods: This study was done on one hundred and fifty adult male albino rats (50 - 60 gm) that divided into five groups; a) Normal control group, b) Dibutyl amine and sodium nitrate treated group, c)E.Coli treated group, d) Dibutyl amine and sodium nitrate treated group plus E. coli, and e) Dibutyl amine and sodium nitrate treated group plus soy bean flour. Survival rate and histopathological changes during the period of treatment were recorded. Level of malondialdehyde, glutathione, catalase, hydrogen peroxide, Total antioxidant capacity and nitric oxide were measured. RNA extracted from bladder tissues was determined in addition to P16 level and caspase-3 expression.

Results: Survival analysis showed a significant decrease (p< 0.001) in E.Coli and dibutyl amine plus sodium nitrate treated groups in comparing with the normal and other treated groups. Also, E. coli infection in the bladder tissues increases the carcinogenic ability of nitrosamine precursors, and enhances oxidative and nitrosative stresses via increasing levels of nitric acid, hydrogen peroxide and malondialdehyde. Regarding the molecular changes, extra bands have been found in E. coli and E. coli + carcinogen treated groups approximately at 16-18KD which are not present in the other groups.

Conclusion: Bacterial infection of the urinary bladder may play a major additive and synergistic role in bladder carcinogenesis. Our results have also shown that soy bean flour may have a protective action during induction of urinary tumors.

Key Words: Bladder carcinogenesis; E. Coli; Soybean; DBA; P16

1. Introduction

Urothelial carcinoma, or transitional cell carcinoma is the most common cancer type of the lower urinary tract. Like other malignant neoplasms, urothelial carcinoma develops through multiple genetic and epigenetic changes that lead to alterations in growth, differentiation, and apoptotic control. In the United States, approximately 71,000 individuals (53,000 males and 18,000 females) develop bladder cancer each year, and 14,000 die from the disease. In North America and Europe, urothelial transitional cell carcinomas (TCCs) comprise over 90 percent of bladder cancers. In Egypt, carcinoma of the bladder is the most prevalent cancer, accounting for 31% of all cancer cases. Currently, it ranks first in males representing 16.2% of male cancer. Escherichia coli (E. coli) infection is by far the most common cause of UTI, accounting for 80-85% of community-acquired infections and 50% of hospital-acquired infections. Free radicals, caspases, and p16 protein have been implicated in the pathogenesis of many diseases. There is a critical balance between free radical generation and antioxidant defenses. It has been suggested that oxidative and nitrosative stresses may be associated with malignancy. Caspases are a group of cysteine proteases that cleave after an aspartic acid residue of a specific recognition site. Activation of these enzymes is a biochemical hallmark of apoptosis. Caspase-3 is a major effector caspase that plays a critical role in the apoptotic cascade. P16 gene encodes p16 protein that competes with cyclin D for binding to CDK4. So, the present study aimed to;(a) evaluate the possible role of chronic inflammation induced by E. coli in the urinary bladder of rats, (b) study the protective action of soybean flour against the carcinogenesis of
DBA and sodium nitrate, (c) determine the role of oxidative and anti-oxidative stress during the carcinogenesis, and (d) examine the expression of p16 and caspase-3 genes during bladder carcinogenesis.

2. Material and Methods

2.1. Experimental Animals and Dosing

One hundred and fifty adult male albino rats, weighing 50 - 60 gm were included and divided into five groups, as follows: (Gr1): Normal control feed the standard diet. (Gr2): Received dibutyl amine (DBA) 1000 ppm and sodium nitrate 2000 ppm in drinking. (Gr3): E. Coli treated; the rats infected by 0.5 ml PBS containing suspension of E. coli in the bladder, as previously described by11 (1 × 10⁸ cells suspended in 0.5 ml of phosphate-buffered 2.1% NaCl solution) (Gr4): Received dibutyl amine (DBA) and sodium nitrate in drinking water and infected by E. coli in the bladder. (Gr5): DBA and sodium nitrate treated and received the standard diet containing 20% of defatted soy bean flour (protein content 48%, fat content 6%) mixed in the diet all over the experimental period and infected with E.Coli. At the end of the experiment, animals were decapitated and 5 ml of blood was collected. The present experiment was continued 40 weeks.

2.2. Sampling and Processing

At the end of the experiment, animals were scarified and blood was collected into heparinized tube. The clear plasma samples were separated by centrifugation at 3000 rpm for 10 min. and then plasma was kept at -80 °C and used later in bioassays. For molecular studies, tissues of bladder were removed immediately from sacrificed animals, washed with saline, dried, cut into weighted pieces immediately frozen in liquid nitrogen until assayed. For histopathological studies other bladder tissue pieces were fixed in 10% formalin, blocked in paraffin, sectioned, and stained with hematoxyline and eosin. Finally, the samples were send to Pathology department - National Cancer Institute Cairo, Egypt, for histopathological examination.

2.3. Biochemical measurements

Total protein and Malondialdehyde (MDA) were determined according to12,13 respectively. Thiobarbituric acid (TBE) react with malondialdehyde (MDA) in acidic medium at temperature of 95 °C for 30 minute to form thiobarbituric acid reactive product. The absorbance of the resulting pink product can be measured at 534 nm wavelength.

Glutathione content was determined following the method reported by14. Kit were purchased from Biodiagnostic chemical company- Dokki, Giza, Egypt. Catalase activity and hydrogen peroxide were measured biochemically according to15,16 respectively. Total antioxidant capacity was assayed according to previously done. On the other hand, Nitric oxide was determinate by colorimetric method according to the paper published earlier.18

2.4. Molecular studies

Polyacrylamide gel for protein electrophoresis (SDS-PAGE) was done according to19. RNA was extracted from tissues and cells by using total RNA isolation kits (QIAGEN cat # 74004)

2.4.1. Determination of P16 levels and caspase-3 expression

Reverse transcriptase PCR was performed using QIAGEN OneStep RT-PCR Kit (cat # 210212), each RT-PCR was performed in 25 µl containing 1 µg of purified RNA, 5 µl of 5X buffer, 1 µl dNTPs (25 mM), 2 µl of 25 ng from sense and anti-sense (As) for each gene (sigma co.), 2µl enzyme mix and completed to 25 µl by nuclease free water. Caspase 3 and p16 were amplified using specific primers (Sigma Co) described by20,21 respectively. β-actin (sigma co.) was used as an internal control to yielded a PCR product of 146 bp on electrophoresis.22 β-actin sequence was as follows: Sense: 5'-TTG CTG ATC CAC ATC TGC TG-3' Antisense: 5'-GAC AGG ATG CAG AAG GAG AT-3'. The samples were incubated at 50 °C for 60 minute, then the samples were denaturated at 95 °C for 5 minute and subjected to 35 round of thermal cycling (T- gradient, Biometra- Germany). Each cycle consist of denaturation for 1 min at 95 °C, annealing for 1 minute at different annealing temperatures as shown in Table 1, and extension for 1.5 min at 72 °C. All samples were analyzed twice for gene profile by RT-PCR on different days to ensure reproducibility of the results. PCR products were analyzed via 2 % agarose gel electrophoresis.

Table-1: Showing caspase-3 and p 16 primer sequences, annealing temperature and PCR product size

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
<th>Annealing temp</th>
<th>Size bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase–3</td>
<td>5'-ACG GTA CGC GAA GAA AAG TGA C-3' 5'-TCC TGA CTT CGT ATT TCA GGG C-3'</td>
<td>58 °C</td>
<td>282</td>
</tr>
<tr>
<td>P16</td>
<td>5'-CAT CTC CGA GAG GAA GGC GGA CT-3' 5'-CGC AGT TCG AAT CTG CAC CAT AG-3'</td>
<td>57 °C</td>
<td>239</td>
</tr>
</tbody>
</table>

2.4.2. Quantitation of PCR product

Gel were video-photographed and the bands of the photagraved gel were scanned as digital peaks and the
2.5. Statistical Methods and Data analysis
Data analysis was done using SPSS version 14.0 for windows (Statistical Package for the Social Sciences Inc, Chicago, Illinois). Means were compared by independent-samples t-test. Percentage change was also calculated. All data are expressed as the mean ± the standard error (SEM). The correlation coefficient (R) was determined by linear regression analysis. The statistical significance of differences among means was assessed by the one way analysis of variance followed by Duncan’s test for multiple comparisons. Kaplan-Meier 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.

3. Results
3.1. Survival rate
Survival analysis of all groups showed that best survival was recorded to the control group (96.66 %) followed by Gr2 (86.66%). Animals of Gr3 (46.66%) and Gr4 (33.33%) showed the least survival of all, respectively. On the other hand, Gr5 recorded a good survival (70 %). In addition group of animals that treated with Carcinogen plus E. coli (G4) died earlier as compared with control and other groups (p<0.001).

3.2. Histopathological findings
Bladder histopathological changes due to bacterial infection are shown in fig-1.

3.3. Biochemical studies (oxidative and anti-oxidative stress markers)
Table 2 shows the effect of E. coli, nitrosamine precursor and soybean administration on the hydrogen peroxide, nitric oxide, malondialdehyde, total antioxidant capacity, catalase and glutathione level.

3.4. Molecular Alterations Analysis: SDS-polyacrylamide gel for protein electrophoresis
Fig-2 show the molecular weight of different bands for control and other treated groups. The electrophoretic mobility showed extra bands in E. coli and E. coli + carcinogen treated groups approximately at 16-18KD which
are not present in the other groups.

**3.5. Expression of Caspase 3**

Expression of caspase-3 was considered by dividing the area under the curve of the normal control by the corresponding value of β actin (caspase-3/β actin) which was considerate as a base line for the expression equal 100%. The percent of changes from normal control of caspase-3 expression showed a decreased level in *E. coli*, carcinogen, *E. coli* + carcinogen and *E. coli* + carcinogen + soybean treated groups, -5.4, -45.6, -53.1, -19.6% respectively, as shown in Figure 3. Treatment with soybean significantly decreased the expression of Caspase-3 (-19.6%) in compared by control group.

**3.6. Expression of p16**

The percent of changes from normal control of p16 expression showed a decreased level in *E. coli*, carcinogen and *E. coli* + carcinogen treated groups, -5.3, -26.6 and -37.3% respectively, whereas it showed slightly increased level of p16 expression (+1.4%) in *E. coli* + carcinogen + soybean treated group, as demonstrated in Figure 4. The highest down regulation was found in G4 (-37.3%).

**Figure-2: Electrophoretic pattern of protein in control (lane 1) and different treated groups lane 2,3,4 and 5) after staining by Coomassie Brilliant Blue G-250**

**Figure 3: RT-PCR analysis of mRNA expression of caspase3 in control (Lane 1) and different treated groups (Lanes 2-5). The first lane (M) is a 1000 base pair (bp) ladder, lan 6; negative control (sample without RNA). β actin (146 pb) was used as internal control. Expression of caspase-3 m RNA was compared between different treated groups by calculating p16 / β actin % ratio for each group**

**Figure 4. RT-PCR analysis of mRNA expression of p16 in control (lane 1) and different treated groups Lanes 2-5. The first lane (M) is a 1000 base pair (bp) ladder; Lane 6, represent negative control (sample without RNA). p16 / β actin% ratio in control group was considerate as a base line of expression equal 100%, then the levels of expression of the rest groups calculated and compared to the level of the base line for each case.**

**4. Discussion**

Several reports indicate that urinary tract infection promotes carcinogenesis in the urinary tract of the rat, and infection with live *Escherichia coli* resulted in persistent infection and diffuse urothelial hyperplasia in addition to acute and chronic inflammation.23,24 Survival analysis of the current results showed a significant decrease (p< 0.001) in E.Coli and DBA plus sodium nitrate treated groups in comparing with the normal and other treated groups. Also, the results indicated that *E. coli* infection in the bladder tissues increase the carcinogenic ability of nitrosamine precursors which is in agreement with25 whom indicated that the N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) has a strong ability to induced rat bladder cancer. In addition the results showed that *E. coli* infection to bladder tissues increase the carcinogenic ability of nitrosamine precursors and this may be due to increase of nitrite production by the bacteria and continuous production of nitrosoamine.26 Our results indicate that bacterial infection of the urinary bladder may play a major additive and synergistic role in bladder carcinogenesis, by helping in-situ nitrosamine synthesis and by augmenting carcinogenesis by nitrosamine in agreement with reports showing that chronic inflammation and irritation in the urinary bladder are associated with increased cancer initiation at the site of inflammation.27
Three mechanisms were suggested to account for the tumor enhancing effect of *E. coli* induced inflammation in the present experiment. First, it is well documented that the synthesis of nitric acid and nitrosamine(s) can be stimulated greatly by the cytosol of macrophages activated by lipopolysaccharide (LPS) and γ-interferon. Second, *E. coli* infection accelerated urothelial proliferation. This may have augmented the mutagenic effect of the carcinogen. Third, increase of lipopolysaccharide may induce a prolonged oxidative and nitrosative stresses which results in DNA damage and mutation. Our findings have shown that *E. coli* infection was associated with a significant increase in the concentration of H2O2, one form of active oxygen species. However, it is significant that tumors did not develop in the groups treated with (*E. coli* + carcinogen). This suggests that oxidative stress by itself is insufficient to induce tumors but may be sufficient to augment neoplastic changes induced by carcinogen. The present study demonstrated an increase in the hydrogen peroxide (H2O2) which considered to be an important molecule contributing to oxidative damage in animals treated by carcinogen precursors and *E. coli* than other treated group in agreement with a previous study indicated that reactive oxygen species are involved in the process of cancer initiation and promotion, as evidenced by the role of antioxidants in preventing or delaying the onset of certain cancers. Our results indicated a significant increase in the level of nitric oxide especially in *E. coli* + carcinogen, in agreement with other report postulated that nitric oxide has been shown to contribute to cell and tissue injury when it is formed at high rates. So, it can be concluded that free radicals are potent DNA mutagenic species, which likely exert their carcinogen effects by providing transformed cells with greater plasticity for malignant progression through enhanced DNA mutational rates. In agreement with this finding, free radicals have been shown to predispose to the onset of cancer in vivo.

Regarding the role of antioxidants in the present study, several trials have found that supplementation with antioxidants can reduce the risk of cancer or inhibit the further development of cancer precursors and has produced neither benefit nor harm. Multiple epidemiological studies have also found an association between low plasma concentrations of antioxidants, and developing cancer. Our findings have also shown marked decrease in reduced glutathione level in both carcinogen and carcinogen + *E. coli* treated groups, in agreement with whom stated that reduced glutathione levels (GSH) were significantly lower in bladder carcinoma patients as compared with the normal persons. This may be as part of the glutathione reacts with NO to form nitrosoglutathione. Also, our results indicated low level of the total antioxidants capacity in all treated groups especially in carcinogen + *E. coli* treated group, this a combined with high histological grade. Hydrogen peroxide levels may also be influenced and regulated by the action of antioxidants, including the enzyme catalase, which converts hydrogen peroxide to water, as well as by the scavenger effect of glutathione. The results showed that bladder cancer tissues expressed catalase less frequently than normal tissues. Catalase is expressed predominantly within peroxisomes, where it dismutates the ROS and H2O2 formed as a result of oxidation of long chain fatty acids. The possible mechanism of the decrease in catalase activity, levels may be strongly related to the overproduction of ROS similar to what has been emphasized in previous studies. Also, we found a decrease in catalase activity in accordance with increase of nitric oxide, hydrogen peroxide MDA levels suggesting that imbalance in the antioxidant enzyme system occurs in parallel with oxidative injury rather than a causative event. Accordingly reported that excessive oxidative stress suppresses the activity and expression of catalase. Increased ROS attacks need to be scavenged by antioxidant enzyme activity such as with catalase. Under some conditions, nitrite can be reduced to nitric oxide an effective inhibitor of catalase. Also, soybean treated group show no significant decrease in the reduced glutathione level. The results indicated the ability of soy flour with its rich anti carcinogens substance to protect bladder from sever carcinogenic action of nitrosamine and minimize its effect. This may be supported by evidence from animal studies indicating that; increased intake of soy products has been linked to reduce risk of breast, colon and prostate cancer. Soy products contain high concentrations of several isoflavones, saponins, phytic acid and dietary fibers. In a recent meta-analysis done by, they reported an approximately 30% reduction of prostate cancer risk associated with increased soy products consumption. The protective action may derive in part from induction of G2-M cell cycle arrest, apoptosis and inhibition of angiogenesis. Soy protein intake has been reported to inhibit oxidative modification of LDL in vitro which
was attributed to radical scavenging activity of genistein. Furthermore, antioxidative and antiatherosclerotic effects of soy isoflavones have been reported. Therefore, soybean bioactive components may be particularly effective in the prevention of bladder cancer progression by providing higher levels of bioactive components to bladder tumor via both blood circulation and urinary exposure. The epidemiologic association between soy consumption and bladder cancer development or progression has not been established. In a case-control study, soy juice intake was associated with a non-significant reduction of bladder cancer risk. In addition, experimental studies have shown that soy bioactive components, such as genistein, may have antitumor cancer activity. Genistein inhibited the growth of a bladder tumor xenograft. Zhou et al. evaluated the effects of several soy components such as genistein, soy phytochemicals, and soy protein on bladder tumor growth and found that genistein and genistein-rich soy phytochemicals had more potent effects in inhibiting the growth of a murine bladder cancer xenograft.

Regarding the molecular changes in the all studied groups, our results have shown significant differences in protein electrophoretic pattern between the different treated groups and normal control group. In some instances, there was a differential up-regulation or down-regulation. In many cases, a protein was not limited exclusively to either normal or cancerous state. We believe that our experimental approach facilitated these findings and this may be due to treatment with E. coli or carcinogen precursors which may cause an induction or inhibition in the synthesis of some polypeptides in the rat bladder tissues. As cancer progresses, the protein profile will gradually change, and each individual cell can display a different pattern of change. One possible explanation for completely disappearance of some proteins in different groups is that the gene (S) responsible for certain proteins had been completely suppressed which is generally in accordance with the other findings obtained by. Our results have also demonstrated presence of caspases-3 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. Additionally, caspase-3 activation has been detected in response to a soy treatment. Mechanistically, chemoprevention with dietary soybean could be achieved by stimulating metabolic inactivation or detoxification of potential carcinogens, inhibition of abnormal cell proliferation, suppression of persistent inflammation, induction of programmed cell death and halting/delaying the angiogenic process. In the current era of molecular target-based chemoprevention, many food factors, especially soybean present in our regular diet, have been explored as promising cancer chemopreventive agents, which modulate the function of one or more of redox-regulated transcription factors and apoptotic pathway and this with agreement with several report indicated ability of variety of apoptotic stimuli, including chemotherapeutic agents, irradiation, and cytokines to activate caspases. Regarding p16 level, a marked decrease in p16 gene expressed RNA in different groups especially in carcinogen + E. coli treated group. P16 expression has been studied extensively in several tumor types, and is typically down regulated as expected for a tumor suppressor gene. Our data indicate that soy flour have the ability to overcome the decrease in p16 and caspase-3 expression by unknown mechanism may be by decrease oxidative stress and lipid per oxidation and this open a new promising goal in gene therapy with tumor suppressor genes, such as p16, for treatment of bladder tumor patients, and would be easy to apply. These results were also confirmed by other studies which analyzed p16 mRNA. Losses of tumor suppressor genes are involved in many types of cancer, including bladder cancer. Loss of p16 activity by deletion, point mutation, or hypermethylation has been detected frequently in bladder cancer, resulting in a G1 regulation defect. Chromosome 9p deletion appears to be an early event in bladder carcinogenesis and has been found in >50% of superficial bladder cancer. P16 binds specifically to CDK4 and CDK6 and inhibits these two kinases. Interestingly, cyclin D1 activates CDK4 and CDK6, so that p16INK4 acts as a specific regulator of cyclin D1-dependent kinases. It is therefore likely that p16INK4 alterations can also be involved in cancer development. In another study, deletions in chromosome 9p, where the CDKN2 gene is located (and without changes in 9q), were found in 92% of SCC (10 of 11) of Egyptian and Swedish origin, compared with only 10% of TCC (11 of 110) from a literature-based sample. Sharpless et al. reported that the cyclin-dependent kinase inhibitor p16INK4a can induce senescence of human cells, and its loss by deletion, mutation, hypermethylation, LOH or epigenetic silencing is among
Asian Journal of Medical Sciences 2 (2011) 31-40

5. Conclusion

In conclusion, E. coli infection to the bladder of rats increases the carcinogenic ability of nitrosamine precursors; this may be due to the increase of production of nitrite and nitrosamine by E. coli. Bacterial infection enhanced oxidative and nitrosative stresses by increase levels of nitric acid, hydrogen peroxide and malondialdehyde and these combined by low levels of antioxidant enzymes specially catalase and and reduced glutathione, so bacterial infection of the urinary bladder may play a major additive and synergistic role in bladder carcinogenesis. The results indicated that soy flour has the ability to modulate the decrease in p16 and caspase3 expression by unknown mechanism, may be by decrease oxidative stress and lipid per oxidation.

Acknowledgement: We would like to acknowledge Prof. Dr. Soheir Abdellatif, Professor of Clinical pathology, NCI, Cairo University, for providing E.coli strain. Also, many thanks for Dr. Samir Ahmed, Assistant Lecturer of Pathology, NCI, Cairo University, for his role in pathological examination.

6. References


