The effects of Hormonal Manipulation on Azoxymethane-Induced Colorectal Carcinogenesis Carcinogenesis in Rats

Ratlarda Azoksimetan ile Oluşturulan Kolorektal Karsinogenezde Hormonal Maniplopolyonun Etkileri

Purpose: Epidemiological evidence suggests that estrogen may affect the natural history of colon cancer. We investigated the role of estrogens in Azoxymethane induced carcinogenesis in rats by examining the effect of oopherectomy and orchiectomy with and without estrogen replacement on tumor burden and distribution throughout the colon, type and invasiveness.

Material and Methods: Total 120 inbred Wistar rats were used. Two gender groups divided into six subgroups in each arm. Group F3, F5 and F6 oopherectomized, group M3, M5 and M6 orchiectomized. Group F2, F4, F5, F6 and Group M2, M4, M5, M6 received Azoxymethane (7.5 mg/kg), but F1, F3 and M1; M3 did not and served as control. Group F4, F6 and M4, M6 received Estrogen which was given 0.015mg/kg daily for 25 weeks. All animals sacrificed 25 weeks after first AOM injection.
Introduction

In last decade, several lines of epidemiologic, clinical and experimental evidences have been reported showing that estrogen hormones may be involved in malignant colorectal cancer (CRC).1-9 In a review of the literature; studies including case-controls or cohorts with 3 meta-analyses, 23 studies reported a protective effect, whereas one study reported an adverse effect of ERT.10 Risk reductions were generally similar among recent ERT versus those who were on ERT for 5 years.11-15 The sex differences in site-specific incidence, the protective effect of increasing parity and the reduced risk among women taking postmenopausal hormones, are all elements suggesting that sex hormones may play a role. Estrogen use confers overall protection, with a reduction in the incidence of colon adenoma and carcinoma of about 25-50%.5 At all ages, women are less likely than men to develop colon cancer and epidemiological studies have demonstrated that colorectal cancer incidence and mortality rates are lower in women than men.6-8 The protective effects of female hormones are also evident in families with HNPCC, because the lifetime risk of developing colon cancer is significantly lower in females than in males (30% versus 74%, respectively).16 These findings have led many investigators to search for the biological mechanisms by which estrogen may affect the pathogenesis of colorectal cancer. In the presented study, we have attempted to evaluate the influence of hormonal manipulation on colorectal carcinogenesis in rats of both sexes. The precise biological mechanisms employed by estrogen to stimulate protective factors have not yet been identified. We investigated the role of estrogens in intestinal carcinogenesis in rats by examining the effect of oopherectomy and orchiectomy with and without estrogen replacement on tumor number and distribution throughout the colon, type and invasiveness in this experimental study.

Materials and Methods

Experiment: This study was approved by local ethical committee and was carried out in accordance with the Helsinki Declaration. Male (n=60) and female (n=60) inbred Wistar rats weighing 160-180 gr were used. Rats were housed in groups of three in rooms artificially illuminated at a temperature of 21-24 °C. All animals were fed on standard laboratory died and water ad libitum at the Pamukkale University Faculty of Medicine, Animal Laboratory. The animals were checked daily for signs of distress or

Results: Gonadectomy with carcinogen treatment resulted in 4-6 fold increase in mitotic indices in both females and males compared to control groups. Castrated animals exhibited significantly higher frequency of tumor formation as compared to controls. Although gonadectomy did not significantly alter the distribution of colon cancer in both sexes, a significantly higher percentage of these tumors were invasive in castrated males.

Conclusion: Although this is an experimental study, it is clear that colonic cancer appears to be an estrogen-dependent tumor with a wide therapeutic window and the longer colon epithelium is under the influence of these hormones the more pronounced is the effect of an alteration in hormonal state upon carcinogenesis of the colon.

Key words: Experimental carcinogenesis, Azoxymethane, Estrogen, Chemoprevention
anemia, animals and their food were weighed weekly. During the course of the experiment, there were no statistically significant differences in body weight or food consumption among the various study groups. Specification of the groups displayed in Table 1. Both oopherectomy and orchiectomy carried out in six weeks old animals to simulate the effect of postmenopausal statues before any effect of hormonal changes takes place on to the colonic mucosa of the rats. Under ketamine and xylazine anesthesia, the skin prepared and the dorsal midline incision made for oopherectomy. The ovary pulled out, a single ligature is placed around the oviduct, blood vessels, fat and is severed with single cut. Same procedure is repeated on the opposite side. The muscle and skin layers closed with polyglactin suture material. Estrogen treatment started postoperative first day in group F4, M4, F6 and M6. Successful oopherectomy was documented by histological comparison of uterine mucosa from ovariectomized animals, animals treated with estrogen replacement, and control group. Under ketamine and xylazine anesthesia the scrotum was prepared and an incision is made in each scrotal sac at the tip for orchiectomy. A single ligature is placed around the vas deferens and spermatic blood and vessels; and is cut distal to the ligature. The scrotum is then closed with absorbable sutures. Successful orchietomy was documented by histological examination of testicles of orchietomized male animals. Estrogen (200mg, Estradiol benzoate, Sigma Chemical Co., St. Louis, MO) were given 0.015mg/kg body weight daily throughout the rest of experiment.

Rat model of colon cancer: Azoxymethane (100 mg, Sigma Chemical Co., St. Louis, MO) was diluted in 120ml sterile water for injection, and refrigerated at 5°C until use. A single batch of azoxymethane was used throughout the experiment. AOM started 14 days after the operation and were given subcutaneously (7.5mg/kg body weight) once a week in related groups (F2-M2, F4-M4, F5-M5, F6-M6).

Pathological examination: All animals sacrificed 25 weeks after first AOM injection. At the end of the experiment, animals were exanguinated under ketamine and xylazine anesthesia, their intestinal tracts were removed from caecum to distal rectum, opened, flushed with saline, and examined under 33 magnifications to obtain tumor counts. The colon and terminal ileum were opened on the antimesenterial aspect, any elevation of the mucosa suspicious for tumor was counted, measured, and its anatomic site recorded. The colon and rectum dissected into four parts, i.e., ascending, transverse, descending, rectosigmoid, and rectum according to their anatomical locations. All susceptive areas removed and fixed in 10% buffered formalin solution. 4μm paraffin sections were stained with Hematoxylin&Eosin (H&E). Histological type (dysplasia: histologic abnormality of an adenoma according to the degree of atypical cells, adenocarcinoma: carcinoma originating from colonic cell, mixed carcinoma: adenocarcinoma that contains signet-ring or mucinous cells, invasive adenocarcinoma: cancer cells penetrate through the muscularis mucosa into the submucosa) and number of lesion, dysplastic crypt ratio (dysplastic crypt count/crypt count X 100) and mitotic ratio (mitotic cell count/cell count X 100) were examined. At least 20 crypts examined for the demonstration of epithelial proliferation. The tumors were counted and an individual blinded to the animal’s genetic status and treatment group performed analyses of the different animal tissues.

**Statistical analysis:** Statistical evaluation was done using SPSS 12.0. Differences among mean values were assessed using Mann-Whitney U test. Comparisons between groups made with $\chi^2$ test. ANOVA with Bonferroni corrections was used within each female and male group. In all cases, $p<0.05$ was considered as the statistical criterion to determine significant differences.

### Table 1. Experimental groups.

<table>
<thead>
<tr>
<th>FEMALE</th>
<th>MALE</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (intact control)</td>
<td>M1 (intact control)</td>
</tr>
<tr>
<td>F2 (intact+AOM)</td>
<td>M2 (intact+AOM)</td>
</tr>
<tr>
<td>F3 (Oopherectomized)</td>
<td>M3 (Ophericetomized)</td>
</tr>
<tr>
<td>F4 (intact+E+AOM)</td>
<td>M4 (intact+E+AOM)</td>
</tr>
<tr>
<td>F5 (Oopherectomized+AOM)</td>
<td>M5 (Ophericetomized +AOM)</td>
</tr>
<tr>
<td>F6 (Oopherectomized+E+AOM)</td>
<td>M6 (Ophericetomized +E+AOM)</td>
</tr>
</tbody>
</table>

**Abbreviations:**

- E: Estrogen given
- AOM: Azoxymethane

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**Results**

**Effects on mitotic indices**

Gonadectomy increased the mitotic indices throughout the colon significantly in both male (M3) and female (F3) groups when compared control male (M1) and female (F1) groups in all colonic regions (p<0.05). But the increase was higher in males (M1) when compared females (p<0.05). This means that changes in hormonal homeostasis are more prominent in males. Carcinogen treatment with AOM increased the mitotic indices in groups M2-F2 as expected (p<0.05). Both estrogen and AOM treatment in intact rats has also resulted in increase the mitotic indices in all regions of the colon both males (M4) and females (F4) when compared control groups (M1, F1) (p<0.05). This increase is statistically lower when M4-F4 groups compared with M2-F2 (p<0.05). This means that estrogen treatment is decrease mitotic indices both in intact males and females after AOM treatment. AOM treatment after both oopherectomy and orchiectomy in M5 and F5 had the highest increase in mitotic indices in all groups and throughout the colon.

<table>
<thead>
<tr>
<th>Table 2. Mitotic index changes in different colonic regions.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>M1</td>
</tr>
<tr>
<td>1 (intact control)</td>
</tr>
<tr>
<td>2 (intact+AOM)</td>
</tr>
<tr>
<td>3 (Gondx)</td>
</tr>
<tr>
<td>4(intact+E+AOM)</td>
</tr>
<tr>
<td>5 (Gondx+AOM)</td>
</tr>
<tr>
<td>6(Gondx+E+AOM)</td>
</tr>
</tbody>
</table>

**Effects of azoxymethane**

Frequency of colonic tumors and site distribution in the various groups were given in Table 3. Colonic tumor distribution was in favor of ascending, descending and sigmoid colon in both sexes (p<0.05).

**Pathological evaluation**

Male and female rats receiving azoxymethane showed two types of macroscopic lesions. First type of gross lesions mostly appeared as poly-p-type lesions, which proved to be invasive adenocarcinoma or mixed tumors, that these were mostly in groups (M5-F5, M6-F6) (Figure 1). Second type was the elevated areas of colonic mucosa, which proved to be moderately differentiated non-invasive carcinoma or dysplasia (Figure 2). Although gonadectomy did not significantly alter the distribution of colon cancer in both sexes, a higher percentage of these tumors were invasive in castrated females (p<0.05). Opposite was also true for well-differentiated tumors. When comparing group 5 and group 6, estrogen treatment in both sex groups resulted in decrease in the invasiveness (p<0.05) (Table 4).

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Table 3. Site distribution and the number of colorectal tumors (Mean±SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>M1</th>
<th>F1</th>
<th>M2</th>
<th>F2</th>
<th>M3</th>
<th>F3</th>
<th>M4</th>
<th>F4</th>
<th>M5</th>
<th>F5</th>
<th>M6</th>
<th>F6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascending</td>
<td>0</td>
<td>0</td>
<td>25±1.8*</td>
<td>23±0.6*</td>
<td>0</td>
<td>0</td>
<td>22±1.6*</td>
<td>26±1.3*</td>
<td>45±1.8*</td>
<td>50±1.4*</td>
<td>37±1.1</td>
<td>33±0.7*</td>
</tr>
<tr>
<td>Transverse</td>
<td>0</td>
<td>0</td>
<td>13±1.2</td>
<td>6±2.2</td>
<td>0</td>
<td>0</td>
<td>20±1.8*</td>
<td>7±0.7</td>
<td>14±0.9</td>
<td>17±1.3</td>
<td>15±2.3</td>
<td>13±1.9</td>
</tr>
<tr>
<td>Descending</td>
<td>0</td>
<td>0</td>
<td>36±0.8</td>
<td>33±1.9*</td>
<td>0</td>
<td>0</td>
<td>42±2.2*</td>
<td>34±2.3*</td>
<td>74±1.6*</td>
<td>79±1.4*</td>
<td>53±0.9*</td>
<td>43±1.6*</td>
</tr>
<tr>
<td>Sigmoid</td>
<td>0</td>
<td>0</td>
<td>14±2.1</td>
<td>10±1.1</td>
<td>0</td>
<td>0</td>
<td>16±1.5</td>
<td>12±0.9</td>
<td>28±2.1*</td>
<td>36±1.1*</td>
<td>25±0.4*</td>
<td>25±0.4*</td>
</tr>
<tr>
<td>Rectum</td>
<td>0</td>
<td>0</td>
<td>13±0.6</td>
<td>13±0.8</td>
<td>0</td>
<td>0</td>
<td>13±1.8</td>
<td>10±1.3</td>
<td>9±0.9</td>
<td>11±0.8</td>
<td>8±2.4</td>
<td>8±0.7</td>
</tr>
</tbody>
</table>

* vs p<0.05 the other sites within group

Table 4. Histopathological types of azoxymethane induced colorectal tumors.

<table>
<thead>
<tr>
<th>Group</th>
<th>Well-differentiated adenocarcinoma</th>
<th>Moderately differentiated adenocarcinoma</th>
<th>Mixed tumors</th>
<th>Invasive tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>1(intact control)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2(intact+AOM)</td>
<td>35±0.8</td>
<td>39±1.1</td>
<td>60±1.6</td>
<td>50±1.4</td>
</tr>
<tr>
<td>3(Gondx)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4(intact+E+AOM)</td>
<td>33±0.4</td>
<td>36±0.8</td>
<td>49±1.3</td>
<td>44±1.7</td>
</tr>
<tr>
<td>5(Gondx+AOM)</td>
<td>57±2.1</td>
<td>51±1.7</td>
<td>108±1.6</td>
<td>115±0.2</td>
</tr>
<tr>
<td>6(Gondx+E+AOM)</td>
<td>39±0.2</td>
<td>44±0.4</td>
<td>74±1.8</td>
<td>59±1.8</td>
</tr>
</tbody>
</table>

Abbreviations:
Gondx: Gonadectomy (Oopherectomized/Orchiectomized), E: Estrogen AOM: Azoxymethane
* p<0.05 vs group 3 and group 4 in same sex in mix tumors, # p<0.05 vs group 3 and group 4 in same sex in invasive tumor, α p<0.05 vs group 5 in same sex

Figure 1. Invasive type carcinoma

Figure 2. Flat type carcinoma
Discussion

The peak incidence of CRC for both men and women comes approximately 10 years after the onset of age-related decline in hormone production, an appropriate time span for completion of carcinogenesis once a protective effect has been removed. Studies that support a hormonal basis for the etiology of colon cancer indicate that age-adjusted colon cancer incidence rates are somewhat higher for men than for women. Women have more proximal tumors and more Micro Satellite Instability (MSI) than men as independent of tumor site. Women were less likely than men to have MSI+ tumors at a young age and more likely to have unstable tumors at an older age. Furthermore, parity has been inversely associated with the development of colon cancer; therefore, subgroups of the population with low parity, such as nuns, have higher rates of colon cancer than populations where parity is high. Hormone Replacement Treatment (HRT) has been shown to decrease the risk of developing both incidence and size of polyps or colon cancer. For estrogens and androgens a number of intestinal functions seem to be affected by alterations in hormone concentration. The activity of estrogens and antiestrogens in both males and females is mediated through binding of these compounds to Estrogen Receptor (ER)s, which are ligand-activated transcription factors. There are at least two major ERs, known as ER α and ER β. These two ER isoforms appear to have a differential tissue distribution: ER β is the predominant isoform in breast and uterine tissue; and ER α is expressed in significant quantities in the urogenital tract, the central nervous system, and endothelial cells. In the colonic tissue, expression of ER β protein is significantly higher than that of ER α. The two ER isoforms also exhibit differences in binding affinity, potency, and efficacy after interaction with various estrogenic compounds. Beside this, multiple isoforms namely ER β 1 and ER β have been identified. ER α is present at very low levels in the colon, with no difference in mRNA or protein expression between normal colon, adenomas, and colon cancers and no differences between males and females. ER β protein is the predominant isoform present in normal colon, and expression of ER β protein may be decreased in colon cancers. The ratio of ER α: ER β protein observed in normal colon is the reverse of that found in human endometrium. Tumor prone tissues responsive to hormones, such as breast and endometrium, have overall higher expression levels of ER α than ER β. These observations led to the theory that binding of estrogens to ER α induces a cancer-promoting response, whereas ER β binding is potent inhibitor of cellular proliferation and/or transformation. Cancers of the distal and proximal colon displayed a similar degree of loss of ER β expression. The expression of ER α in normal and malignant colon is minimal, available data regarding the progressive loss of ER β in tumors of the proximal colon suggest that the decline of ER β expression may also underlie the pathogenesis and progression of these tumors. Different mechanisms have been suggested in the literature for the estrogenic effect on colonic mucosa and colonic carcinogenesis sequence. 1-Exogenous estrogens may reduce the risk of large bowel cancer by decreasing bile acid concentration or by direct effects on colonic mucosa, e.g., prevention of cell proliferation. 2-Estrogen-induced carcinogenesis is induction of peroxisome proliferation; Estrogen-induced peroxisome proliferation is associated with carcinogenesis in the liver and may be important in the development of APC-associated colorectal tumors. 3-Diet is known to influence the development of colonic cancers, with high consumption of fruits and vegetables conferring a protective effect. These food categories contain a variety of phytoestrogens that might be capable of modulating estrogen receptor activity. Phytoestrogens appear to have a greater affinity for ER β than they do for ER α. 4-Issa has shown that hypermethylation, and, thus, reduced expression, of the ER in the colon is a concomitant of aging. Furthermore, they have shown that colon tumors almost universally arise from cells that have lost ER expression. On the basis of this finding, one could hypothesize that HRT may reduce the risk of colon cancer in women by reducing the likelihood of ER methylation and, thus, the pool of cells that give rise to colon tumors. 5- As pointed out by Breivik, heredity is an important factor in non polyposis colon cancer, an inherited colon cancer syndrome in which MSI is seen in nearly all colon cancers. Women with hereditary non polyposis colon cancer have half the risk of developing colorectal adenomas as their male relatives. Estrogens also play in important role in the growth of colon carcinoma cells lines. 1,2-Dimethylhydrazine and AOM are two well known
agents in experimental carcinogenesis.\textsuperscript{45-48} Using AOM as a carcinogenic agent in 7.5 mg/kg dose preferred for our study. Since, it was found that AOM has a dose dependent action on colonic mucosa. When the dosage doubled (15mg/kg), left colonic tumor development incidence is significantly increases. Nevertheless, 7.5 mg/kg dose evenly distributed mitoses and tumors throughout the colon due to the advised dose of AOM.\textsuperscript{49}

In presented study, we found that following bilateral oopherectomies the colonic crypt appeared to reach a new steady state, which is characterized by small crypt size, a decrease in the number of differentiated cells, an increase in the number of proliferative cells (mitotic activity index), this is not the case for bilateral orchiectomy group. This outcome is consistent with the results in the literature.\textsuperscript{50} Male rodents have higher aberrant crypt or tumor formation rates compared with females in several colon cancer models.\textsuperscript{46-49,52} Mitotic index have increased in all colonic regions of the female rats after gonadectomy (F3), it is also true for counterparts in group M3, but the difference for this group was not significant. Tumor distribution was in favor of ascending, descending and sigmoid colon in both sexes in our study. Previous reported results on tumor locations after experimental carcinogenesis were confusing. There might be a stronger inverse association of female hormone use with proximal colon cancer\textsuperscript{63,54} or a stronger association with distal colon cancer\textsuperscript{55} or no difference\textsuperscript{56} according to the literature. Tumor locations were mostly in distal parts of the colon for our study. The present study has also shown that estrogen treatment causes decreased tumor development rate in carcinogen treated rats in both sexes. This finding is consistent with the results in literature. Tumor locations were mostly in distal parts of the colon for our study. Another data have been reported as well in the mouse colon cancer model for familial adenomatous polyposis that show reduced tumor numbers in intact females compared with ovariectomized females.\textsuperscript{21} Taken together with that finding and non-significant differences in invasiveness between the sexes and significant inter group analyses, in our study led us to think that estrogen prevents tumor formation, but after tumor development there was also inhibitory effect on tumor cells and they become less invasive. Although, we haven’t studied estrogen subgroup receptors in the group of Azoxymethane induced tumors, Odagiri proved that the relationship between steroid receptors and morphologic features in dimethylhydrazine-induced colon carcinogenesis.\textsuperscript{61} Although it is dangerous to extrapolate results obtained from rats in this study, susceptibility of rats to chemical carcinogens raises the question that whether data obtained from animal studies applicable to human carcinogenesis. However, the successes observed in the use of hormonal manipulation as a treatment for cancer in man may not be due entirely to a change in the in vivo hormonal status.
Conclusion
In conclusion, hormone-associated carcinogenesis is a complex process with species and tissue-specific differences in receptor expression, receptor isoform distribution, and ligand metabolism as well as significant cross talk between the different signaling pathways that govern cell fate. The gastrointestinal tract was regarded until last two decade as a non-target organ. This study, although it is experimental, appear to indicate that in colonic cancer appears to be an estrogen-dependent tumor with a wide therapeutic window and the longer colon epithelium is under the influence of these steroids the more pronounced is the effect of an alteration in hormonal state upon carcinogenesis of the colon. In both sex of rats, estrogen demonstrated protective effects in colonic epithelium. This compound should therefore be given further consideration as a chemo preventive or therapeutic agent in colon cancer.

References


