Protective Effect of Green Tea (*Camellia snensi*) Extract on P53 Gene Mutation and Reproductive Toxicity in Male Rat


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Abstract: The present study aimed to detect the protective effect of green tea extract on the mutant frequency of p53 gene and reproductive toxicity in rat testis. 60 male rats were divided into 3 groups, group (1) were given a 2% green tea extract for 7 weeks pre and post benzo(a)pyrene treatment, group (2) were given the same does of green for 7 weeks post b(a)p treatment, while group (3) used as b(a)p control. After 1st 7 weeks of experimental period, all groups were injected (i/p) with 50mg./kg b(a)p for three consecutive days. Blood, liver testis samples and epididymal sperms were collected for evaluation of testosterone, P53 gene mutation, histopathological and sperm analysis respectively. The results indicated that, application of PCR-SSCP for exons 5-9 revealed antimutagenic effects of green tea with various percentages in exons 7, 8 and 9. Green tea relieved the toxic effects of b(a)p significantly (p < 0.05) on testicular weight (1.20 ± 0.04 vs. 1.15± 0.02 gm.) and health, sperm motility (80.00 ± 3.54 vs. 60.00± 3.54 %), sperm count (80.00 ± 4.47 vs. 70.00 ± 3.54 million /ml) and maintained testosterone concentration (1.90 ± 0.07 vs. 1.50± 0.17ng/ml). It could be concluded that, green tea contains antagonistic substances to some carcinogenic and mutagenic chemicals and suppress testicular degeneration.

Key words: Green Tea • Mutation • P53 Gene • Testis • Testosterone • Benzo (A) Pyrene • Rats

INTRODUCTION

P53 is one of the most frequently mutated gene in human cancers, located on the short arm of chromosome 17p13 in most mammals, encodes a 53 kDa phosphoprotein capable of binding to DNA and acting as a transcription factor. Upon physical or chemical DNA damage, functional P53 can either arrest the cell cycle or induce apoptosis and may be involved in the recognition of DNA damage and DNA repair [1, 2].

Tea is the most widely consumed beverage in the world. There are three basic types of regular tea; green (20%), black (78%) and oolong (2%) according to processing methods [3]. Green tea contains many nutrients, but the primary is the polyphenols. The chief polyphenols in green tea are flavonoids (catechin and proanthocyanidins), with the four major polyphenols being epicatechin, epicatechingallate, epigallocatechin and epigallocatechingallate. Of the four, epigallocatechingallate is regarded as the most significant active constituent. Other compounds found in green tea include an unusual amino acid, theanine and others as, tannins, lignin, organic acids, proteins, sugars and chlorophyll [4].

A number of reports have documented the chemoprotective effect of green tea consumption on various types of cancers such as those of bladder, prostate, esophagus and stomach [4]. This property was attributed to the presence of polyphenols in green tea [5]. Common use of antimutagens and anticarcinogens in everyday of life is an effective measure for preventing cancer and genetic diseases. Antioxidant properties of tea have vast potential as protective agents against toxic effects of some mutagens [6]. Moreover, Gupta *et al.* [7] demonstrated significant antimutagenic and anticlastogenic effects of both green tea polyphenols in multiple mutational assays. DeBoer [8] found that green tea and linoleic acid can modulate the mutagenic potency
of different chemical carcinogens including the dietary heterocyclic amine, the environmentally important aromatic hydrocarbon b(a)P and the food contaminant aflatoxin B1. In mice, Zhang et al. [9] observed that, green tea displayed an average of 50% inhibition of lung tumors induced by b(a)P, regardless of p53 gene status. Benzo(a)pyrene is a contaminant that occurs in the environment together with other polycyclic aromatic hydrocarbons (PAHs) as a product of incomplete combustion or hydrolysis of organic material containing carbon and hydrogen.

Many studies suggested that green tea may be protective towards cancers of the squamous cell carcinoma induced by 7,12 dimethyl benzo-anthracene in Hamsters [10], gastrointestinal tract in mice [11], breast cancer growth and endothelial cells in vitro assays and in animal models [12], lung, oral cavity, esophagus, stomach, small intestine, colon, skin, prostate, mammary glands, liver, pancreas and bladder [13]. Kusum et al. [14] found that, green tea catechins (GTCs) may intervene with breast cancer development (induced by long-term exposure to b(a)p) by blocking carcinogen-induced ROS elevation, Raf-independent extracellular signal-regulated kinase (ERK) activation, cell proliferation and DNA damage. Tarek et al. [15] noticed that Co-administration of green tea extract to insecticide treated-animals alleviates the reproductive toxicity and testicular oxidative damage.

The increasing interest in the health proprieties of green tea and its catechin polyphenols level led to a significant rise in scientific investigations for prevention and therapeutics in several diseases. Therefore, the present study aimed to detect the protective effect of green tea extract on the mutant frequency of p53 gene and reproductive toxicity in rat testis induced by benzo (a) pyrene.

**MATERIALS AND METHODS**

The present study was conducted at the Department of Biochemistry and Chemistry of Nutrition, Faculty of Veterinary Medicine, Cairo University in collaboration with the Department of Animal Reproduction and Artificial Insemination, National Research Center, Cairo, Egypt.

Materials used included, green tea was purchased from local store, Benzo(a)pyrene (Sigma Aldrich - B10102), corn oil, DNA extraction kit (Fermentas Life Science Co.) Oligonucleotide primer for exons 5-9 of rat P53 gene, Taq polymerase and molecular weight marker 100 bp ladder (Promega). Other chemicals were molecular biology grade.

**Experimental Animals:** Total number of 60 male albino rats (3 weeks old of 80-100 gm. BW) was obtained from laboratory animals' colony, Ministry of Public Health, Helwan, Egypt. Animals were kept in clean metal cages under strict hygienic measures, maintained on balanced diet; food and water were offered ad-libitum.

**Experimental Design:** After a period of three weeks acclimatization, animals were classified into three equal groups 20 rats for each and subjected to the following treatments according to table (a). Water and green tea were offered ad-libitum.

**Blood and Tissue Sampling:** At the end of the first 7th week of the experimental period and before the administration of benzo(a)pyrene, 10 rats from groups 1 and 3 were sacrificed, the liver was stored at -80°C for DNA isolation. At the end of experiment period, all rats from all groups were sacrificed; liver was removed, flushed and stored at -80°C for DNA isolation.

Blood samples were collected from rats just before scarifying and centrifuged at 3000 rpm for 15 min for serum collection and were stored at -20°C for testosterone assay. Epididymal sperms were collected by flushing method for morphological studies. Testis from sacrificed rats were collected and preserved in 10% formol saline for histopathological examination.

Preparation of green tea extract Aqueous tea extract (2% w/v) was prepared every other day by adding100ml of boiling water to 2gm of green tea leaves followed by filtration after standing at room temperature for 30 minutes [16].

**DNA Extraction:** High quality genomic DNA was extracted from preserved liver samples of all treated and control groups, the total DNA was measured at 260nm optical density according to Sambrook and Russell [17]. The extracted DNA of each sample was kept frozen at -20°C until used.

**Synthesis of Oligonucleotides Primers:** Oligonucleotide primers flanking exons 5, 6, 7, 8 and 9 of the rat p53 gene were designed to amplify exons 5-7 [18] and exons 8, 9 [19]. The sequence of these oligonucleotide primers was described in table (b).
Table (A): Experimental design

<table>
<thead>
<tr>
<th>Experimental period</th>
<th>1st 7 weeks</th>
<th>3 consecutive days</th>
<th>2nd 7 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (1)</td>
<td>Green tea 2gm% (w/v)</td>
<td>b(a)p 50mg/kg b.w.</td>
<td>Green tea 2gm% (w/v)</td>
</tr>
<tr>
<td>Group (2)</td>
<td>Water</td>
<td>b(a)p 50mg/kg b.w.</td>
<td>Green tea 2gm% (w/v)</td>
</tr>
<tr>
<td>Group (3)</td>
<td>Water</td>
<td>b(a)p 50mg/kg b.w.</td>
<td>water</td>
</tr>
</tbody>
</table>

Table (B). Sequence of oligonucleotide primers of exon 5 to 9.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Target exon</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5-GAT TCT TTC TCC TCT CCT AC-3</td>
<td>Portion of Exon 5 (3’ end)</td>
<td>158</td>
</tr>
<tr>
<td>R1</td>
<td>5-TGT AGA TGG CCA TGG CAC GG-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>5-GTC ACC TCC ACA CCT CCA CC-3</td>
<td>Portion of Exon 5 (3’ end)</td>
<td>165</td>
</tr>
<tr>
<td>R2</td>
<td>5-CCT GGA CAA CCA GTT GTA AC-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>5-GGC TCT GAC TTA TTC TTG C-3</td>
<td>Exon 6</td>
<td>158</td>
</tr>
<tr>
<td>R3</td>
<td>5-GTG GTA TAG TCG GAG CCG AC-3</td>
<td>Exon 6</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>5-GTG GTA CGG TAT GAG CCA CC-3</td>
<td>Exon 7</td>
<td>175</td>
</tr>
<tr>
<td>R4</td>
<td>5-CAA CCT GCC ACA CAG CCT CC-3</td>
<td>Exon 7</td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>5-CTT ACT GCC TTG TGC TGT GC-3</td>
<td>Exon 8, 9</td>
<td>382</td>
</tr>
<tr>
<td>R5</td>
<td>5-CTT AAG GGT GAA ATA TTC TCC-3</td>
<td>Exon 8, 9</td>
<td></td>
</tr>
</tbody>
</table>

Polymerase Chain Reaction (PCR): The primer-directed enzymatic amplification of specific rat P53 gene (exon 5, 6, 7, 8 and 9) fragments was done by PCR. The amplification was performed in a final volume of 50µl containing 100ng of DNA template, 50pm of each primer, 5 μl of 10x PCR buffer, dNTPs 1μl, 0.25 μl Taq polymerase (Fermentas, Germany). The PCR conditions were performed through initial denaturation at 95°C for 5min., followed by 35 cycles of denaturation at 95°C for 1min, annealing at 53°C for exon 5, 59°C for exon 7, 56°C for exon 8 and 9 for 1min to all and extension at 72°C for 1min, followed by final extension at 72°C for 5min. The amplified fragments of different exons were verified in 2% agarose gel, positive PCR product detected using UV. The positive PCR reaction was subjected to SSCP analysis for detection of point mutation using 12% polyacrylamide gel [20].

Reproductive Parameters: Testosterone concentrations in the collected serum samples were determined by ELISA [21], testis and epididymal weights from scarified rats were determined and subjected to histopathological examination [22]. Spermatozoa were obtained from cauda epididymides, the motility, count, vitality and morphological characters of sperms were determined [23]. Nuclear maturation was evaluated by aniline blue stain [24].

Statistical Analysis: Data were analyzed using simple one way ANOVA according to Brunning and Kintz [25] and the statistical significance between means was compared using Duncan multiple range test; (p<0.05) was considered significant. All tests were performed using computer package of the statistical analysis system (SPSS, version 16).

RESULTS AND DISCUSSION

The analysis for mutations in exon 5 and 6 of p53 gene by conventional SSCP-PCR revealed that, b(a)p did not induced any mutation in these exons (Fig. A and B -tab. 1). On the other hand, mutation induced by b(a)p can be detected only in exon 7 (Fig. D and E, tab.1) and the amplified portion of exon 8 and 9 (Tab. 1, Fig. F) with a ratio of 80% for both. The same results of Hassouna [26] who found that b(a)p did not induced point mutation in exon 5 and 6 of rat p53 gene. Noordin et al. [27] found no mutation in exon 5 of p53 gene. Treatment of rats with green tea alone did not induced any mutation in any of the examined exons (exon 5, 6, 7 and 8-9) as migration patterns for these treated samples were as those of the normal control ones (Fig. A, B, C and F. Tab. 1).
The present study revealed that rats treated with green tea for 7 weeks post i/p of b(a)p induced 25% antimutagenic effect in exon 7 (Tab. 1, Fig. D) and 0% in exon 8 & 9 (Tab. 1, Fig. G). In line, treatment with green tea for 7 weeks pre and post i/p of benzo(a)pyrene induced 25% of antimutagenic effect for exon 7 (Tab. 1, Fig. D) and 75% for exon 8&9 (Tab. 1, Fig. H). Our results coincide with Jiang et al. [28] who noted 63% decrease in mutation frequency induced by b(a)p in green tea treated mice, as well as 53% decrease in GC- ->TA transversions mutation. Indicating that green tea extract significantly suppressed b(a)p-induced mutations in the Lac I transgene in vivo. Many mechanisms for cancer prevention by green tea have been proposed based on studies in cell lines, which demonstrated the modulation of signal transduction and metabolic pathways by (-)-epigallocatechin-3-gallate (EGCG ). These molecular events may result in cellular changes, such as enhancement of apoptosis, suppression of cell proliferation and inhibition of angiogenesis. Nevertheless, it is not known whether these are the molecular mechanisms of inhibition of carcinogenesis in animals and humans [29,30]. In this respect, it can be explained that green tea phenols react with reactive oxygen species, such as superoxide radical, singlet oxygen, hydroxyl radical, peroxy radical, nitric oxide, nitrogen dioxide and peroxynitrite [16]. These radical species may contribute to the inactivation of epidermal growth factor receptor and telomerase, while hydrogen peroxide may contribute to cell apoptosis [31]. Green tea can inhibit the oxidation of lipoproteins induced by Cu2+ in vitro [13]. Stearns and Wang [32] demonstrated that EGCG in combination with taxane may provide a novel therapeutic treatment of advanced prostate cancer and had an additive effect to increase the expression of apoptotic genes (p53, p73, p21 and caspase 3) and the apoptosis percent observed in vivo and in vitro [33]. Green tea polyphenols was shown to inhibit the expression of carcinogen activating enzymes such as cytochromes P450 (CYP) and increase the levels of enzymes that detoxify carcinogens [34]. It blunted the increase in expression of CYP1A1 and 1A2 induced in mice liver and lung by b(a)p [35]. In addition, it can induce Phase II drug metabolizing enzymes glutathione-S-transferase (GST) [36], induction of apoptosis, inhibition of cell proliferation or cell cycle arrest, through the Nrf-2 pathway and alteration in cell signaling through nuclear factor-jB [37]. Moreover, EGCG up-regulate gene expression of â-glutam-ytransferase, glutamate cysteine ligase and hemeoxygenase 1in mice [38].

The present study cleared that, green tea stopped the harmful effect of b(a)p on testis and maintain its weight from reduction when administered for 7 weeks post or pre and post b(a)p exposure (Fig. 5). Similar findings to Maima et al. [39] and Marina et al. [40] who explained that green tea content of polyphenol and other antioxidant substances antagonized the adverse effect of b(a)p. These results confirmed by histopathological findings in b(a)p samples in the form of vacuolar degenerative changes in the seminiferous epithelium of some seminiferous tubules. There was congestion, oedema in the interstitial tissues (Fig. 9). Some seminiferous tubules showing arrest of spermatogenesis and detachment of the spermatocytes in the lumen of the tubules (Fig.7). Meanwhile, some seminiferous tubules revealed spermatogenesis was still evident degenerative changes in the sertoli cells emphasized by the appearance of polymeric vacuoles of variable sizes (Fig.8). In addition to mild degree of degeneration and necrosis in the spermatogenic germ cells of seminiferous tubules associated with moderate thickening and hyalinization of their basement membranes (Fig.10). Lumen of tubules was free from spermatids. These histopathological findings were coincidence with that recorded by EL-Shahat et al. [41] and Gawish et al. [42]. The epididymal weight was decreased significantly in group 2 comparing to group 1 and 3 as green tea supplementation for 7 weeks post b(a)p injection has the most reducing effect (Fig.5). In this respect, Rachid et al. [43] found that green tea extract could improve the adverse effect of nicotine that diminished epididymal weight and can adjust it to normal values in rats.

Concerning the effect of green tea on sperm motility, data in figure(6) brought to mind that, green tea extract for 7 weeks post or pre and post b(a)p injection significantly (P< 0.05) increased sperm motility % comparing with b(a)p control rats. In line, Yousef et al. [44] in rats and Kenji et al. [45] in mice. The improvement of sperm motility by green tea could be attributed to the presence of isoflavones and other polyphenol [46]. These polyphenols are very efficient antioxidant, reduced the production of hydrogen peroxide, scavenger of oxygen free radicals and protected sperm DNA against oxidative damage [47]. Although green tea supplementation for 7 weeks pre and post i/p injection of b(a)p caused numerical
increased sperm count, it was insignificant comparing to other two groups and significant (p < 0.05) decrease in abnormal sperms (Fig.6), similar to Jassem et al. [48] in rats and Kenji et al. [45] in mice. Green tea administration for 7 weeks pre and post b(a)p injection significantly (p<0.05) improved serum testosterone concentration, while administration for 7 weeks post b(a)p treatment did not induce this effect (Fig.5), similar findings to Yu et al. [49] who said that green tea in the diet increase plasma testosterone of rats. This may be attributed to the presence of polyphenols constituent that stimulate testosterone production by leydig cells [50], through activity of intracellular calcium by catechins and/or via the enzyme activities of 17α-hydroxysteroid dehydrogenase by epicatchins [49].

In summary, it can be concluded that oral administration of green tea, to rats for 7 weeks after or before and after I/p injection of the potent carcinogen b(a)P, provided antimutagenic effect and therefore cancer chemoprotection and prevent testicular degeneration, may be throw antagonistic action or supportive treatment.

Table 1: Mutagenic and antimutagenic ratio of treated substances.

<table>
<thead>
<tr>
<th>P53 gene exons</th>
<th>Mutagenic ratio of b(a)P</th>
<th>Pre &amp; post</th>
<th>post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 5</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Exon 6</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Exon 7</td>
<td>80%</td>
<td>25%</td>
<td>25%</td>
</tr>
<tr>
<td>Exon 8 and 9</td>
<td>80%</td>
<td>75%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Fig. 1,2,3,4: Agarose gel electrophoresis (2%) of PCR amplitons of green tea and b(a) p treated rats p53 gene exon 5, 6, 7, 8-9 respectively. Lane M, 100 bp ladder size marker, all the products are at the expected size.
Fig. A: SSCP analysis of PCR amplicons of rat P53 gene (exons 5) shows no mutation, lanes 1-6 represent green tea, lanes 7-12 represent b(a)p. Fig. B: SSCP analysis of PCR amplicons of rat P53 gene (exons 6) shows no mutation, green tea lanes (1-6) and b(a)p lanes (7-11). Fig. C: SSCP analysis of PCR amplicons of P53 exon 7 of green tea pre b(a)p treatment showed no mutation. Lane 1 represent b(a)p control, lane 2 represent non treated control, other lanes of green tea. Fig. D: show SSCP of PCR amplicons of exons 7 pre and post b(a)p treatment. Lanes 1-4 green tea pre and post b(a)p treatment, lanes 5-8 green tea post b(a)p treated samples, 9-10 represent b(a)p control samples. Fig. E: SSCP for PCR amplicons of exons 7 of post b(a)p treated groups, where lanes 1-5 represent green tea post treated group 6-10 b(a)p treated group. Fig. F: show SSCP of PCR amplicons of exons 8-9, lanes 1-8 represent green tea pre b(a)p treated samples, 9-10 represent b(a)p control samples. Fig. G: SSCP for PCR amplicon with exons 8-9 of post b(a)p treated groups, where lanes 1-4 represent green tea post treated group, lanes 5-8 represent b(a)p control group. Fig. H: show SSCP of PCR amplicons with exons 8-9 of pre and post b(a)p treated groups in which lanes 1-4 represent green tea pre and post b(a)p treated samples.
Plat (3): reproductive parameters and histopathological finding of examined testis

Fig. 5: Effect of green tea supplementation pre & post and post b(a)p treatment on testicular weight, epididymal weight and testosterone concentration in comparing to b(a)p control group.

Fig. 6: Effect of green tea supplementation on sperm motility, count and abnormal forms in comparing to b(a)p control group.

Fig. 7: Testis of a rat intoxicated with b(a)p (group 3) showing degeneration and necrosis in the spermatogenic cells with focal germ cell depletion (H&E. X 200).

Fig. 8: Testis of group (1) revealing vacuolation of Sertoli cells with inhibited release of mature spermatids. (H&E. X400).

Fig. 9: Testis of group 2 showing arrest of spermatogenesis and detachment of the spermatocytes in the lumen of the tubules (H&E X 100).

Fig. 10: Showing mild degeneration and necrosis in the spermatogenic germ cells associated with moderate thickening and hyalinization of their basement membranes.
REFERENCES


