Morphometrical, Histopathological, and Cytogenetical Ameliorating Effects of Green Tea Extract on Nicotine Toxicity of the Testis of Rats

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Abstract

This current study aimed at evaluating the toxic effect of nicotine and the possible protective role of green tea extract on some organs of Swiss albino mice by using histological, histomorphometrical and cytogenetical studies. Male Swiss albino mice were divided into four groups. The first group served as control and was injected intraperitoneally (i.p.) with distilled water (1 ml DW/daily). The second group was injected with green tea extract (40 mg/kg b.w./i.p./daily), animals of the third group were injected with nicotine (2.5 mg/kg b.w./i.p. daily) and those of the fourth group were injected with nicotine (2.5 mg/kg b.w./i.p./daily) and green tea extract (40 mg/kg b.w./i.p./daily). The experimental period was four successive weeks. Nicotine treatment induced histological changes in both the lung and testicular tissue as revealed by light microscopy. It also induced histomorphological changes in the lung represent by significant decrease in elastic fibers and in the testicular tissue, a significant decrease in the number of Leydig cells. These changes were obtained by the computer image analyser. Combine administration of nicotine with green tea extract resulted in marked ameliorations of the testicular changes using histological and histomorphological observations. The combine administration of nicotine with green tea extract caused also an improvement in the ratio of PCEs/NCEs and a significant decrease in the increase of the MnPCEs numbers induced by nicotine treatment alone. In conclusions, the administration of green tea extract might suppress the cytotoxicity and mutagenic activity of nicotine. We suggest that green tea extract may be useful in combating tissue injury and genotoxicity caused by nicotine toxicity.

Keywords: Smoking; Nicotine; Antioxidant; Green tea extract; Histological; Histomorphometrical; Cytogenetical

Introduction

Cigarette smoking has been the most popular of taking nicotine since the beginning of the 20th century. In which it has been identified injurious to human health. Each cigarette contains about 10 milligrams of nicotine [1]. Nicotine and its metabolites are also being investigated and researched for the treatment of a number of disorders, including Alzheimer’s disease, attention deficit disorder and Parkinson’s disease [2]. It induces oxidative stress both in vitro and in vivo and contributes with a major proportion to the net oxidative stress imposed by tobacco use, and at the same time, depletes antioxidant defense mechanisms [3]. The addictiveness of nicotine is being mediated by neuronal nicotinic acetylcholine receptors in the central nervous system [4] and is the cause of the continuing use of tobacco products. Gossian et al. [5] stated that cigarette smoking can affect the fertility of rats. The adverse effects of cigarette smoke on Leydig cell function in animals have been reported [6], Kapawa et al. [7], showed that cigarette smoke exposure results in secretor deficiency of Leydig cells and Sertoli cells leading to impaired epidymal sperm maturation process and diminished capacity of spermatozoa to penetrate oocytes. In addition, paternal cigarette smoke exposure affected the embryonic ability for implantation. Polyzos et al. [8], recorded also an increased risk for spontaneous abortions, and birth defects association with cigarette smoking. Genetic mutations in sperm were recorded by Yauk et al. [9] on exposed male mice to tobacco smoke. Yamamoto et al. [10] and Kim et al. [11] on their studies on cigarette smoking and nicotine (respectively) suggested a secretory dysfunction of the Leydig cells, and also a deficiency in sperm maturation and spermatogenesis. Reddy et al. [12] studied ascending concentrations of nicotine on testis and recorded a reduction in testis weight. There is substantial evidence from human studies that cigarette smoke contains harmful mutagens and carcinogens; that may induce defective semen quality, nuclear DNA damage of spermatozoa, and compromise the chances of pregnancy [13]. However, Sergerie et al. [14] have found no association between smoking and sperm function or sperm nuclear DNA damage. Cigarette smokers have increased DNA strand breaks [15], DNA adducts [16], and chromosomal abnormalities [17,18] in their sperm. Doolittle et al. [19] concluded that neither nicotine nor its major metabolites cotinine, nicotine-N-oxide, and trans-3,4-dioxycotinine caused genotoxic effects with or without metabolic activation. In contrast, other groups of researchers found a modest increase in sister chromatid exchange in Chinese hamster ovary cells [20], and in bone-marrow cells of mice with chromosomal aberration [21]. Other studies reported negative results in sister chromatid exchanges, chromosomal aberrations and micronucleated bone marrow cells of Sprague-Dawley rats nose exposed to the mainstream smoke generated by either traditional cigarettes or cigarettes which heat but do not burn tobacco [22]. Many references reported that nicotine has deleterious toxic effects through increased production of free radicals and reactive oxygen species (ROS) [23,24].

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Natural antioxidants with free radical-scavenging activity such as polyphenols from green tea extracts have received much attention as potential, non-toxic, treatments for oxidative-stress-related pathological conditions. Green tea has also many biological functions including antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial properties, antimutagenic, lowering plasma cholesterol and triglyceride levels, reducing blood pressure and platelet aggregation in human, animal, and in vitro studies [25].

The antioxidant properties, reactive oxygen species scavenging, and cell function modulation of flavonoids could account for the large part of pharmacological activity of green tea [26].

Material and Methods

Experimental animals

Eighty Male Swiss albino mice aged 9 – 12 weeks and weighing 25–30 gm were used in this study. Nicotine was purchased in the form of colorless liquid in glass bottle at 100% concentration from faculty of Pharmacy, Cairo University, Egypt. The mean LD₅₀ for intraperitoneal administration of nicotine to 8-week-old mice (29.6 g) was reported as 12.5 mg/kg.

Green tea extract was purchased in form of tablets, each contained 200mg of green tea extract; it obtained from Techno-mad Groups Company, Egypt. Each tablet was dissolved in distal water and solution was injected intraperitoneal to experimental animals at dose level of (40 mg/kg.b.w.) according to [27].

Polychromatic erythrocytes and normochromatic erythrocytes were two types of erythrocytes could be distinguished in mouse bone marrow on the basis of their staining characteristics with May-Grünnwald-Giemsa stain [30].

The animals were divided randomly into 4-groups of each group have 20 animals:

**Control group:** The animals were injected intraperitoneal with distilled water (0.1 ml/ 10 gm.b.w.) daily for four weeks.
Green tea extract treated group: The animals were daily intraperitoneal injected with green tea extract (40 mg / kg body weight / day) for four weeks.

Nicotine treated group: The animals were daily intraperitoneal injected with nicotine (2.5mg/ kg / day) for four weeks.

Nicotine green tea extract treated group: The animals were daily intraperitoneal injected with nicotine (2.5mg/kg/day) concomitantly with green tea extract (40 mg / kg body weight/day) for four weeks.

Statistical analysis: Statistical analyses were carried out using analysis of variance (One Way ANOVA) and Duncan’s Test to determined difference between group means and stander errors. The values are mean ± S.E. for five samples for each week. P-values ≥0.05 were considered as significant, while P-values ≥0.001 were considered as highly significant.

Results

The testis is the primary male sex organ and is also, an important endocrine gland. Sections in testis of control group revealed that seminiferous tubule; each has a definite basement membrane and a lumen which may contain a few spermatozoa (Figure 1 and Figure 2). Macroscopical examination of testis sections in male Swiss albino mice treated with green tea extract alone for one, two, three and four weeks respectively revealed normal testicular architecture with complete normal spermatogenic layers and well developed sperm. Interstitial Leydig cells showed no structural abnormalities (Figure 3).

Histological examination of testis sections in male Swiss albino mice treated with nicotine for one, two, three and four weeks respectively, revealed variable degrees of alteration according to the time of treatment and compared to control.

Marked reduction of sperm in some seminiferous tubules, in addition to, the appearance of few scattered pyknotic nuclei in the basal cell layers. Interstitial Leydig cells showed also a mild reduction (Figure 4). Other seminiferous tubules showed areas of cystic degenerative changes accompanied with exfoliated spermatogenic cells in the lumen. (Figure 5) and thickened, hyalinized wall of arterioles were occasionally observed in the widened intertubular regions with eosinophilic deposition (Figure 6) at the third week of nicotine treatment.

At fourth week, sections of the testis from male Swiss albino mice treated with nicotine for four weeks showed scattered apoptotic cells at the lumen portion of many seminiferous tubules (Figure 7). Many seminiferous tubules showed nuclear vacuolation (Figure 8) together with large apoptotic cells were seen between layers (Figure 9). Widened interstitial areas with marked reduction in interstitial Leydig cells (Figure 10a & 10b).

Histological examination of sections in the testis of male Swiss albino mice treated with nicotine and green tea extract for four successive weeks revealed different degrees of amelioration in response to the time of treatment and compared to the intervals of nicotine group. Mild reduction in germ layers and sperm appeared in focal seminiferous tubules. Scattered seminiferous tubules had exfoliated germ cells in the lumen (Figure 11) and Interstitial Leydig cells showed mild increased compared to the nicotine group at the same interval (Figure 12). At the last week of treatment, mild reduction in sperm occasional seen in few seminiferous tubules (Figure 13). Interstitial spaces were within normal limit and Leydig cells approached normal appearance and distribution compared to the nicotine at the same interval (Figure 13).

Histomorphometrical studies

The histomorphometrical changes induced by intraperitoneal injection of nicotine at (1/5 LD50 = 2.5mg/kg to mice) as well as the protective effects of green tea at (40 mg /kg.b.w.) for one, two, three
and four weeks with or without nicotine were investigated in the testis of male mice using image analysis. Numbers of Leydig cells in testis were shown in (Table 2).

It could be concluded that the number of Leydig cells in testis section throughout all intervals of green tea extract administered was within the accepted spontaneous range for control.

The nicotine treatment for one week induced insignificant decrease in the numbers of Leydig cells (18.10 ± 2.28) compared to the control group (23.40 ± 1.78). A significant decrease in the number of Leydig cells was induced when nicotine treatment took place for two weeks compared to the control group (23.70 ± 1.72) (P < 0.05). Animals received nicotine for three and four weeks, exhibited also a highly significant decrease in the number of Leydig cells (12.4 ± 1.97 and 9.5 ± 1.46, respectively) when compared control group (23.90 ± 1.94 and 24.60 ± 2.24), (P < 0.01).

Treatment with green tea extract prevented the decrease in the numbers of Leydig cells observed in the nicotine treated male Swiss albino mice, with different degrees. It showed also a general increase in these cells with increase periods of administration. When treatment with nicotine and green tea extracts took place for one and two weeks (21.30 ± 1.88 and 19.70 ± 2.29) an insignificant increase in the number of Leydig cells was detected in comparison with the nicotine treatment at same intervals (18.10 ± 2.28 and 15.30 ± 1.78, respectively) or control group (23.40 ± 1.78). While nicotine and green tea extract treatment for three weeks (19.50 ± 2.59) caused a significant increase in the number of Leydig cells and a highly significant increase for four weeks (21.60 ± 2.39) of treatment in comparison to the three and four weeks of the nicotine treatment (12.4 ± 1.97), (P < 0.05) and (9.5 ± 1.46); (P < 0.01). These increases (21.60 ± 2.39) come up to the same range of control value, but still insignificantly lower to the control (24.60 ± 2.24).

As depicted in (Figure 13), the numbers of Leydig cells were significantly reduced in the nicotine treated groups, while the treatment with nicotine and green tea extract significantly increase their numbers compared to the control groups.

**Cytogenetical studies**

The cytogenetic damage induced by intraperitoneal injection of nicotine at (1/5 LD50 = 2.5mg /kg to mice) as well as the anti-...
The treatment of nicotine and green tea extract to the animals was effective in reducing the mean number of MnPCEs compared to the control group (4.6 ± 0.74). For two and three weeks of treatment a significant increase mean number of MnPCEs (9.60 ± 1.166 and 15.00 ± 1.224 respectively), (P < 0.01) were obtained when compared to control values (4.20 ± 0.58) at same treatment, while a highly significant increase was observed following control group (4.6 ± 0.74). For two and three weeks of treatment a significant increase mean number of MnPCEs (9.60 ± 1.166 and 15.00 ± 1.224 respectively), (P < 0.01) is highly significant.

In each group, twenty animals were used, five animal for each studied week. 1000 cells / animals were examined. Data are represented as sum of micronucleated polychromatic erythrocytes (MnPCE) showing in (Figure 13).

### Table 1: The effect of nicotine, green tea extract and nicotine with green tea extract treatment on the mean number of micronucleated polychromatic erythrocytes in male Swiss albino mice.

<table>
<thead>
<tr>
<th>Time Of Treatment</th>
<th>Parameter</th>
<th>%PCE</th>
<th>%NCE</th>
<th>%PCE / NCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>%PCE</td>
<td>64.60 ± 0.05</td>
<td>65.68 ± 1.17</td>
<td>Ba</td>
</tr>
<tr>
<td>Week 2</td>
<td>%NCE</td>
<td>54.90 ± 0.05</td>
<td>61.02 ± 0.76</td>
<td>Ba</td>
</tr>
<tr>
<td>Week 3</td>
<td>%NCE</td>
<td>52.30 ± 0.75</td>
<td>62.16 ± 0.76</td>
<td>Ba</td>
</tr>
<tr>
<td>Week 4</td>
<td>%PCE / NCE%</td>
<td>70.62 ± 3.92</td>
<td>54.00 ± 1.02</td>
<td>Ba</td>
</tr>
</tbody>
</table>

In each group, twenty animals were used, five animal for each studied week. 1000 cells / animals were examined. Data are represented as mean ± standard errors of 5 counting values in each studied week of treatment for each group. Capital letters represent comparison between different weeks within each group. (P < 0.05) is significant and (P < 0.01) is highly significant.

### Table 2: The effect of nicotine, green tea extract and nicotine with green tea extract treatment on the ratio of mean percentage of polychromatic erythrocytes to NCEs.

In each group, twenty animals were used, five animal for each studied week. 1000 cells / animals were examined. Data are represented as mean ± standard errors of 5 counting values in each studied week of treatment for each group. Capital letters represent comparison between different weeks within each group. (P < 0.05) is significant and (P < 0.01) is highly significant.

### Discussion

The treatment of nicotine and green tea extract to the animals was effective in reducing the mean number of MnPCEs with the time of treatment. As depicted in (Figure 13), the mean number of MnPCEs was significantly increased in nicotine treated groups and treatment with nicotine and green tea extract significantly reduced the mean number of MnPCEs compared to control group.

(Table 2) summarizes the results of bone marrow cytotoxicity related to the treatment with nicotine and / or green tea extract. The statistical analysis of the data is also provided. The treatment with green tea extract for one, two, and three and even for four weeks did not induce evident bone marrow cytotoxicity in comparison to the control group.

Nicotine treated male Swiss albino mice, for one, two, three and four weeks resulted in highly significant alterations of all monitored parameters (%PCEs, %NCEs & %PCEs / %NCEs), but with different intensity and distinctive time trends. When compared to the control group (45.40 ± 0.953).

Animals that treated with nicotine and green tea extract, for three and four weeks induced a significant increase of the mean percentage and four weeks of nicotine treated group. (P < 0.05) is significant and (P < 0.01) is highly significant.

### Histological studies

In the present study, the histological examination of testis sections taken in the male Swiss albino mice treated with nicotine for one and two weeks showed mild to moderate reduction in spermatogenic...
series and reduction in sperm count in some seminiferous tubules. This was accompanied with scattered nuclear pyknotic change in the basal cell layers observed in first week of treatment. Prominent degenerative changes were also noticed in few seminiferous tubules after the second week.

This was in agreement with the work done by [12] stated that administration of different doses of nicotine reduces the weight of the testis, and the number of spermatocytes and spermatids and Sertoli cells accompanied with thickening of the tunica propria with a daily treatment with nicotine. Elshal et al. [13], reported that cigarette smoking was associated with immature spermatocytes, sperm head defect and disturbances in spermatooza chromatin and DNA integrities in idiopathic infertile subject. Kapawa et al. [7], also revealed that cigarette smoke-exposure impaired epididymal sperm maturation process and diminished capacity of spermatooza to penetrate oocytes. The structural changes observed in the seminiferous tubules of the current study could be explained by the work done by Ahmadnia et al. [31] who attributed these changes to the vascular insufficiency resulting from nicotine toxicity. Aydos attributed the damage in testicular tissue to the direct cytotoxic effects of nicotine on spermatogenic cells or via inhibition of prostaglandin’s synthesis, that play a functional role in the reproduction system of the male mice and mainly initiating and completing spermatogenesis and steroidogenesis in the testis. Reddy et al. [12] revealed that nicotine is central nervous system depressors that can inhibit the neural stimulus essential for the release of pituitary gonadotrophins.

In the present study the combined treatment of nicotine and green tea extract ameliorated the histological changes in testicular tissue induced by nicotine alone. This was evidenced by normal appearance of spermatogenic layers and sperm in the seminiferous tubules. The interstitial spaces and Leydig cells appeared also in normal shape. Hafez et al. [25], found that oral pretreatment of rats; with green tea at a dose 1.7% to the exposure to radiation exerted a noticeable amelioration in the structure of kidney, testes, spleen, and heart.

Sriram et al. [26] revealed also that EGCG at a dose of 20 mg/kg body weight significantly improved the body weight, enzymic and non enzymic antioxidants and considerably decreased the lipid peroxidation marker levels. The ameliorating effect of green tea extract in the present study may be attributed to its antioxidant properties [32]. In conclusion, the present studies indicating the efficiency of green tea extract to restore the original appearance of testicular tissue.

Histomorphometrical studies

In the present study, the histomorphometrical examination of testis tissue of animals treated with nicotine revealed gradually significant decrease in mean number of interstitial Leydig cells in testicular tissue throughout the experimental weeks compared to the control level.

The observed reduction in Leydig cells in testis section of male Swiss albino mice in the present study may be attributed to several features of apoptosis exhibited by nicotine [11], Cohen et al. [33] suggested that nicotine activated specific intracellular death-related pathways. In addition, nicotine enhanced the expression of the activated form of caspase-3 and caspase-3 enzyme activity (one of the key executors of apoptosis, which is responsible either partially or wholly for the proteolytic cleavage of many proteins) [34].

In the present work, histomorphometrical measuring of Leydig cells in animals treated with green tea extract treatment and nicotine recorded that mean number of interstitial Leydig cells returned gradually to normal. In the present study, the ameliorated effect of green tea extract on mean number of Leydig cells due to its antioxidant activity [35], free radical scavenging properties [36,37]. It suppresses ROS formation through its polyphenolic contents such as catechins imparting astrign antioxidant activity.

In conclusion, green tea extract could reduce the nicotine toxicity and caused amelioration in the number of Leydig cells in testis of male Swiss albino mice.

Cytogenetically studies: The Present study evaluated that, nicotine is clastogenic in the mean number of micronucleated polychromatic erythrocytes (MnPCEs) and ratio of mean percentage of polychromatic to normochromatic erythrocytes (PCEs/ NCEs). It caused gradually and highly significant increase in the mean number of MnPCEs; however caused significant decrease in ratio of mean percentage of PCEs/ NCEs compared to control. The micronucleus technique has been proposed as a useful tool for measurement of genotoxicity [29].

Our findings are consistent with Bandyopadhyaya et al. [38]; Argentin and Cicchetti [39]; Arabi [40] who stated that nicotine, the well known addictive chemical of tobacco and active medication for several diseases and is a potential genotoxic compound. In the present study, genotoxicity properties of nicotine may be attributed to excess production of two highly mutagenic nitrosamine, N'-nitrosonorotine (NNN) and 4-(methylamino)-1-(3-pyridyl)-1-butanone (NNK) which product from nicotine during tobacco curing or burning [41,42,43]. Also, in the present study, genotoxicity of nicotine also may be attributed to excess production of malondialdehyde (MDA), is end product of lipid peroxidation as recorded by Sheng et al. [44]; Sudheer et al. [45].

In the present study, the treatment with nicotine and green tea extract caused also an improvement in the ratio of mean percentage of PCEs/NCEs and a significant decrease in the increase of the mean number of MnPCEs induced by nicotine toxicity alone. This result is in agreement with Fujii et al. [46,47] that, demonstrating that green tea did not pose toxic nor adverse events. Ogura et al. [48], also demonstrated no significant increase in micronucleated polychromatic erythrocytes (MN-PCEs) in the bone marrow.

Another reason for the beneficial effect of green tea extract on genotoxicity of nicotine can be attributed to the antioxidant properties and ROS scavenging properties [49,37,32] and antimutagenicity activity [50,51] of green tea extract [42]. In conclusion, the results of the present study showed that green tea extract act as protective against genotoxicity of nicotine due its antimutagenicity activity and antioxidant properties.

References


