Possible protective effect of applying vitamin E to the rats having been exposed to cigarette smoke: an animal study

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To show the possible healing effects in ovarian apoptosis that deteriorates due to smoking by giving vitamin supplement to the rats having empirically been exposed to cigarette smoke. Swiss albino rats that are 12–14 weeks old and 25.19 ± 4.34 gr were chosen for the experiment. The experimental groups were formed by dividing female rates into groups of six in different cages. Experimental groups: Group A: Control female rats (n=6), Group B: Female rats that were exposed to cigarette smoke (n=6), Group C: Female rats that were given antioxidant along with cigarette smoke (n=6). Midluteal phase was determined by taking a smear from female rats. The rats were euthanized by applying overdose alphamine- alphasine. Ovarian tissue taken from the rats was evaluated under a light microscope by means of immunohistochemical method. Not any significant difference was observed in statistical terms when ovarian follicle numbers were compared (p< 0.05). While the number of the cells stained by caspase -3 (immunoreactive cells) were on the increase, though not important in statistical terms, in the group subjected to cigarette smoke (Group B), there wasn’t any crucial difference between the group applied vitamin E (Group C) and control group in statistical terms. (p< 0.05). An increase, though not important, was detected in ovarian apoptosis with smoking. Similar findings were identified between the control group and the group given vitamin E. Therefore, it was concluded that vitamin E supplement has a curative effect on the negative effects of the cigarette.

Keywords: ovarian apoptosis, cigarette, vitamin E

INTRODUCTION

Apoptosis is generally a case in which self- destruction of cells take place, regulated by genes, systematical, needing RNA, protein synthesis and energy, and which protects homeostasis in organisms. In normal tissues, apoptosis (programmed cell death) occurs in a small number of cells as a part of the physical process. However, if the tissue has been exposed to any toxic substance, an increase in the number of the apoptosized cells is observed. There are at least 4700 known chemical substance in cigarette smoke. Most of these compounds are pharmacologically active, toxic,
mutagentic and carcinogenic. There is much evidence about the toxic effects of the cigarette on reproductive system (Golding et al., 1990; Janoff et al., 1987; Tümerkülöz, 1988).

While smoking, a number of free radicals and reactive oxygen metabolites are generated. Free radicals cause much oxidative damage on DNA, carbohydrates, proteins and membrane lipids within cells with a great number of different molecules. It makes one think that protective effect might be possible by means of antioxidants.

Vitamin E or alpha tocopherol is the most important antioxidant that is present in biological membranes, dissolves in fat and has the ability to break chains (Horwitt, 1986). In body tissues, it plays a major role in binding free radicals in early stages and protecting cell membranes from their damage. However, no scientific studies have yet confirmed whether oral antioxidants can reach the targeted area like ovary (Taylor, 2001). Further studies are necessary to enlighten these matters and to reveal the possible protective effects of antioxidants. In this study, we aim to show the potential positive effects of vitamin E, which has antioxidant capacity, on ovary in the cases of cigarette smoke exposition.

MATERIALS AND METHODS

The study was carried out by taking approval from Gazi University Animal Ethics Committee with the support of Turgut Ozal University (No: G.U. ET–10.042). 12-14 week old and 25.19±4.34 gr Swiss albino rats were chosen for the study. The rats were supplied from GUDAM, Gazi University Medical Faculty. 18 female rats were used in the study. Test period was 10-week-long. Three experimental groups were formed by dividing female rats into groups of six in different cages.

Experimental Groups

Group A: Control female rats (n=6)
Group B: Female rats that were exposed to cigarette smoke (n=6)
Group C: Female rats that were given antioxidants along with cigarette smoke (n=6)

In order to place experimental animals with their cages and expose them to cigarette smoke, 2 adjacent cages (Cage A-B) were built, each of which is 150*120*80 cm in size, made from transparent plastic, has a window that is 15*15 cm and two covers on the front side, has a 20*10 cm compartment inside the window, has 15 holes on its floor that are 3 cm in diameter. Excessive smoke were headed out through the holes on the floor.

During the experiment, the animals were fed with tap water and feeds supplied by Oğuzlar Animal Feed Company.

Cage A was exposed to tobacco smoke for 10 weeks. An ash-tray and cigarette machine were placed in the compartment which was behind the window. The rats in this cage were exposed to 20 cigarettes per day for 10 weeks. The experimental animals in Cage A which were planned to take antioxidant (Vitamin E) were injected 50 mg / kg intraperitoneal (i.p.) vitamin E (Evigen, Aksu Farma; 300 mg dl-Alfa Tokoferol Asetat) at the same time every day. The other groups were injected the same dosage of 0.9% NaCl intraperitoneal (i.p). At the end of the ten-week period, midluteal phase was determined by taking smear samples from the rats. The rats were euthanized by applying overdose alphamine-alphasine. Ovarian tissues taken from the rats were prepared with immunohistochemical methods and studied under a light microscope in Histology-Embryology Department of Gazi University. In immunohistochemical staining performed with Caspase-3 primary antibody on the ovarian sections of all experimental and control groups involvements were evaluated in terms of density and percentage of involvement amount by choosing five sections randomly in 400 magnification on each glass. Involvement density was semiquantitatively scored as 0 (0, no involvement), 1 (+, weak immunoreactivity), 2 (+++, intermediate level of immunoreactivity), 3 (+++, high level of immunoreactivity). The amount of involvement was scored as 1 (between 0% and 10%, local) 2 (11%-50%, zonal), 3 (51%-100, generalized) by proportioning the cells / structures in which immunoreactivity took place and the total cells / structures. The scores of concentration and quantity having been found for each glass were multiplied by each other, and a single value for that glass was reached by collecting these results.

Cigarette machine: It was made from two partitioned, transparent colored plastic. A fan was placed in the middle of the machine.

A rectangular apparatus was assembled to place cigarettes. Thus, it became possible to give the cigarette smoke inside. Samsun Cigarette (Tar: 15 mg, Nicotine: 1 mg, Carbonmonoxide: 14 mg) was used.

Immunohistochemical Method

Tissue samples that were taken were identified for 72 hours in a 10% formalin solution in room temperature. The identified tissues were passed through the light microscope tracking method, and paraffin blocks were obtained. Incisions that were 4-5 µm in thickness were taken to poly- l-lysine coated microscope slides with a microtome (Leica SM 2000, Germany). For deparaffinization the incisions were held in incubator for a night at 37° C and the following day at 60° C for an hour and they were exposed to 15°2 xylol. To dehydrate, the tissues were passed through 100%, 96% and 80% alcohol series respectively, each of which lasted 10 minutes. After dehydrated tissues were rinsed with
Table 1. Follicle Counts

<table>
<thead>
<tr>
<th></th>
<th>Tobacco</th>
<th>Tobacco+Evita</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial follicle</td>
<td>12 (2)</td>
<td>13.5(2)</td>
<td>13.5(2.25)</td>
<td>0.101</td>
</tr>
<tr>
<td>Developing follicle</td>
<td>23 (1.25)</td>
<td>23 (1.25)</td>
<td>22.5 (2.25)</td>
<td>0.756</td>
</tr>
<tr>
<td>Atretic follicle</td>
<td>3 (0.5)</td>
<td>3 (1.5)</td>
<td>3 (1.5)</td>
<td>0.825</td>
</tr>
<tr>
<td>Corpus luteum</td>
<td>13.5(1.25)</td>
<td>13.5(1.50)</td>
<td>13.5 (1,5)</td>
<td>0.957</td>
</tr>
</tbody>
</table>

Statistical Method

Data analysis was carried out by a packaged software called SPSS for Windows 11.5. With Shapiro Wilk test, it was identified if the distribution of continuous variables was normal. Descriptive statistics were shown as median (IQR: Interquartile range). The importance of difference in terms of Caspase–3 median values in the groups was alternately assessed by Kruskal-Wallis test. Mann Whitney test with Bonferroni correction was used to detect the group or groups that caused significant difference in the cases where Kruskal-Wallis test result was considered important. With the help of Spearman's correlation test, it was analyzed whether there was significant association in statistical terms between continuous variables. The results for p<0.05 were regarded as statistically significant.

RESULTS

When the ovarian follicle numbers of the rats were compared, no statistically important difference was observed (p<0.05). Follicle counts according to groups are shown in Table 1. The involvement in germinal epithelium in the ovarian tissue samples of the control group was viewed as too low in the immunohistochemical evaluation which was carried out with Caspase–3 antibody. In all the developing follicles, atretic follicles and stroma, Caspase-3 immunoreactivity was detected from mild to severe. In addition to the observation of Caspase–3 involvement in granulosa cells, oocyte and secondary follicles whose antrum was examined, it was also pointed out that immunoreactivity was weaker in theca interna cells. It was also recognized that there was a more intense and medium weak involvement in corpus luteum in contrast to stroma. In the ovarian tissue samples of the group that was exposed to cigarette smoke, caspase–3 immunoreactivity in the developing follicles was similar to that of the control group. Involvement in both stroma and corpus luteum was weak medium just like the previous group. In caspase–3
Photograph 1. Primary follicles (▲), germinal epithelium (→) and atretic follicles (←) are detected (x400) in the ovarian tissue sections of the control group in Caspase-3 immunostaining.

Photograph 2. Primary follicle (▲) and corpus luteum (●) are detected (x400) in the ovarian tissue sections of the control group in Caspase-3 immunostaining.

immunohistochemical staining in the ovarian tissue samples of the group that was exposed to vitamin E as well as cigarette smoke, there wasn’t any significant difference in all the structures of the tissues compared to the group exposed to cigarette smoke.
Photograph 3. Secondary follicle (.kafka) and theca cells (★) are detected (x400) in the ovarian tissue sections of the control group in Caspase–3 immunostaining.

Photograph 4. Primary follicles (.kafka) and corpus luteum (■) are detected (x400) in the ovarian tissue sections of the group exposed to tobacco in Caspase–3 immunostaining.

**COMMENT**

One of the significant conclusions we have drawn is that there is an increase in ovarian apoptosis with smoking. Prevalence of tobacco use has highly increased in recent years and it substantially associated with endocrine changes. Not only does it have serious peripheral effects, but also it may cause changes in hypothalamic
gonadotropine excretion in central nervous system (Nusbaum et al., 2000). Having some effects on hormonal parameters, smoking also effects reproductive system. Bodis et al. (Bodis et al., 1996; Evers et al., 1998) have studied the effects of nicotine on human follicle cells in vitro. They have suggested that nicodine causes an increase in E2 secretion depending on dosage and that high levels of E2 in early periods may cause impairment in ovarian response and lower levels of oocyte.

In some studies, it has been revealed that cigarette smoke extracts inhibits aromatase activity in granulosa cells. As the inhibition of granulosa-luteal cell functions may cause corpus luteum deficiency, it is regarded as one of the mechanisms underlying the increased early pregnancy loss among women who are exposed to cigarette smoke (Neal et al., 2005; Barbieri et al., 2000).

In some other studies, it has been reported that smoking causes early menopause and a decrease in ovarian reserve (Shara et al., 1994).

The effects of cadmium cotinine and benzopyran, all of which are present in cigarette smoke, on human gamete cells have been demonstrated. Cadmium, about 1.0-2.0 mg in each cigarette, accumulates in ovary, testicles, epididymis and vesicula seminalis depending on the number of the cigarettes smoked. Cotinine, which is a nicotine metabolite, disrupts meiotic formation of oosite and follicle maturation by binding to proteins in nucleus and cytoplasm (Mumcu, 2002; Racowsky et al., 1989). Zenses et al. have reported a clear and significant cadmium increase in follicular fluid of those who smoke (Zenses et al., 1995). Trapp et al. proved that rhodonite, which is one of the ingredients of tobacco, is also found in follicular fluid (Trapp et al., 1986). Benzopyrene from polycyclic aromatic hydrocarbon group, of which 6-40 ng is present in each cigarette, has been shown to cause DNA damage in ovarian cells and inhibit the aromatase activity in granulosa cells in vitro (Mumcu, 2002; Barbieri et al., 2000).

Another important result obtained in our study is that there is a decrease in apoptosis rates with vitamin E supplements. While smoking, a number of free radicals and reactive oxygen metabolites are generated. Free radicals cause much oxidative damage on DNA, carbohydrates, proteins and membrane lipids within cells with a great number of different molecules (Elenbogen et al., 1991; Park et al., 1998). However, vitamin E, by bonding free radicals in early stages, shows its effect in the protection of cell membranes. Vitamin E, which plays an important role in the functions and structure of membranes, has the property to protect unsaturated fatty acids in cell membranes. Vitamin E intake strengthens the immune response. By reducing the cyclooxygenase activity of trombosit, it regulates trombosit aggregation. It also has been revealed that it has a role in mitochondrial function and hormonal production in nuclear acid and protein metabolism.

In the group that was given vitamin E in our study, the number of the immune positive cells was detected similar to the control group. Just like the group that was only exposed to cigarette smoke, not an increase in the number of the immune positive cells was detected. When the group given vitamin E and control group were compared, the fact that the number of immune positive cells does not increase supports our hypothesis. Therefore, vitamin E supplementation has an antioxidant curative effect. Similar to our study, in an animal study conducted by Asadi et al. (Asadi et al., 2012), cigarette has been shown to increase apoptosis and smoking-induced ovarian apoptosis is reduced by vitamin E supplement.

Rahmawati et al. have also carried out a similar study (Rahmawati et al., 2014). It has been demonstrated in this study that cigarette causes oxidative damage, which results in reduced number of overian follicles and this can be treated with various doses of α tocopherol.

As a result, it is known that exposure to cigarette smoke leads to an increase in follicle loss. Depending on the circumstances such cases as menopause and subfertility can be seen. In order to increase the response to treatment, antioxidant therapy support may be beneficial for the patients with low ovarian reserve, who smoke and for whom treatment is planned to ensure fertility. We are in the opinion that vitamin E supplementation may be a part of the treatment in this respect, particularly for the patients who smoke; however, further randomized controlled studies are required.
REFERENCES


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