Effects of green tea and vitamin E in the testicular tissue of streptozotocin-induced diabetic rats

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ABSTRACT

الأهداف: التحقق من الأثر العلاجي أو الوقائي لآثار الشاي الأخضر لأنسجة خصى الفئران المصابة بالسكري أما بعامل وحيد أو مرتبطة بفيتامين E.

الطريقة: أجريت هذه الدراسة في كلية الطب، جامعة غازي، خلال الفترة من مايو حتى أغسطس 2011م لمدة 10 أسابيع. اشتملت الدراسة على 84 فأر روستار يزن 300–250 غرام. تم تقسيمهم إلى 8 مجموعات (شاهد، غير مصابة بالسكري واستخدمت فيتامين Eجرعة 0.4 ملغ/كلغ/نانوغرام، مجموعة غير مصابة بالسكري واستخدمت الشاي الاخضر 300 ملغ/كلغ/نانوغرام، غير مصابة بالسكري واستخدمت الشاي الأخضر 300 ملغ/كلغ/نانوغرام، غير مصابة بالسكري واستخدمت فيتامين E مع الشاي الاخضر، مجموعة السكري (60 ملغ/كلغ بالوريد) فيتامين E، مجموعة السكري مع الشاي الأخضر، مجموعة السكري مع فيتامين Eمع الشاي الأخضر. تم قياس مؤشر الاستجابة والتكاثر باستخدام مضاد PCNA الهيستولوجية المناعية ومقايسة تنل. كما قمنا بقياس الانتكاسة باستخدام مقياس جونسون ومقياس جونسون ومقياس أبعاد الأنبوب و السماكة الظاهرة.

النتائج: أظهر الفحص الهيستولوجي المناعي في مجموعة السكري تغيرات انتكاسية في الأنبوب الظاهر مع نقص إحصائي في خلايا PCNA و السماكة الظاهرة، وزيادة في استماتة الخلايا. وهذه النتائج جميعها تعتبر آثار وقائية للسكري تم الحصول عليها في مجموعة الأكسدة.

خاتمة: أن العلاج بفيتامين E والشاي الأخضر في مجموعة السكري يعد فعال بشكل أكثر من العلاج لهذا تعتبر عوامل الأكسدة علاج مساعد لعجز الأنجاب.

Objectives: To investigate the possible therapeutic or protective effects of green tea in diabetic rat's testicular tissue, either as a single agent, or together with vitamin E.

Methods: The present study was carried out at the Faculty of Medicine, Gazi University, Ankara, Turkey from May to August 2011 for 10 weeks. Forty-eight adult male Wistar albino rats, weighting 250-300 g, were divided into 8 groups: control; nondiabetic vitamin E (0.4 mg/kg/NG); nondiabetic green tea (300 mg/kg/NG); nondiabetic vitamin E plus green tea administered groups; diabetic group (60 mg/kg/IV streptozotocin); diabetic vitamin E; diabetic green tea; and diabetic vitamin E plus green tea administered groups. Proliferative and apoptotic indexes were determined using anti-PCNA antibody immunohistochemistry and TUNEL assays respectively. Tubule degeneration was evaluated using the Johnson's score and also seminiferous tubules diameters, epithelial thickness were measured.

Results: Histopathological examination in diabetic group revealed degenerative changes in the seminiferous tubules together with a statistically significant decrease in PCNA positive cells, in epithelial thickness, diameter of the tubules and in Johnson's score, while exhibited an increase in the number of apoptotic cells. When all these findings are considered together, the most successful protective effects in diabetes were obtained in the combined antioxidant group.

Conclusion: Combined therapy of vitamin E and green tea in diabetes was more effective than monotherapy. Therefore, these antioxidants may be use as a supporting therapy for reproductive dysfunction.

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Diabetes mellitus is a chronic metabolic disease associated with many functional and structural complications, arising due to the lack of either free or conjugated insulin.[1] Deteriorative effects of diabetes mellitus on male sexual and reproductive functions has been reported such as decrease in libido and impotence, testicular structural changes and dysfunction associated with sustained hyperglycemia.[2-4] Growing evidence indicates that oxidative stress is increased in diabetes due to overproduction of reactive oxygen species (ROS) and decreased efficiency of antioxidant defenses.[5] Excess amounts of ROS and free radicals have adverse effects on sperm motility and fertility, thus oxidative damage to lipids and DNA of spermatozoa is associated with declining motility and diminished fertility of human sperm.[2] Apoptosis is an active, regulated process of cell death also referred to as "programmed cell death". ROS represent a group of potential modulator of apoptosis as oxidative stress is implicated to be a triggering mechanism for apoptosis.[6,7] To control the flux of ROS, aerobic cells have developed an antioxidant defense system containing enzymatic and non-enzymatic components.[2] Common antioxidants include the vitamins A, C, and E, glutathione, and the enzymes superoxide dismutase, catalase, glutathione peroxidase, and several bioflavonoids with several others to work and fight against different types of free radicals in synergy.[8] Vitamin E is one of the major antioxidants inhibiting a number of ROS-initiated processes in membranes.[4] The primary role of vitamin E is as a lipid peroxyl radical scavenger in vivo, and this role likely mediates the effects vitamin E has on cell signaling pathways.[9] Vitamin E has also been shown to suppress lipid peroxidation in testicular microsomes and mitochondria and to reverse the detrimental effects of oxidative stress on testicular function.[10] Green tea, favored in Japan and China, contains characteristic polyphenolic compounds including; epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin (EC), (+)-gallocatechin (GC) and (+)-catechin (C). Until today, tea catechins have attracted considerable interest due to the potential health-promoting properties of these substances, including strong antioxidant activity with their ability to scavenge reactive oxygen species together with their cancer chemo-protective effects.[11,12] The aim of the present study was to investigate the possible therapeutic or protective effects of green tea in diabetic rat's testicular tissue, either as a single agent or together with vitamin E.

Methods.

Animals and experimental design. The present study was carried out at Gazi University, Faculty of Medicine, Ankara, Turkey from May 2011 to August 2011 following the institutional guide for care and use of laboratory animals. The experimental protocol was approved by the local "Ethics Committee for Animal Studies" of Gazi University Medical Faculty. The experiments were performed on 48 adult male Wistar albino rats weighing 250-300 g. The experimental animals were divided into 8 groups: Group 1 - Control group; Group 2 - Vitamin E (0.4 mg/kg/NG) administered nondiabetic group; Group 3 - Green tea (300 mg/kg/NG) administered nondiabetic group; Group 4 - Vitamin E plus green tea administered nondiabetic group; Group 5 - Diabetic group (60 mg/kg/IV STZ); Group 6 - Diabetic vitamin E administered group; Group 7 - Diabetic green tea administered group; and Group 8 - Diabetic vitamin E plus green tea administered group. Diabetes was induced by a single intraperitoneal injection of streptozotocin (Sigma Chemical Co, USA) dissolved in 0.2 ml of 0.1 M citrate buffer, pH 4.5. Six weeks after diabetes induction, vitamin E and green tea extract was administered orally through the nasogastric gavage tube for 4 weeks. At the end of the 10th week, all the animals were sacrificed.[13,14]

Materials. Green tea extract powder (Martin Bauer Company, Germany), which consist of 66.95% total catechin 4.36 % epigallocatechin (EGC), 1.18% catechin (C), 2.66% epicatechin (EC), 42.10% epigallocatechin-3-gallate (EGCG), 16.65% epicatechin-3-gallate (ECG), and also Vitamin E (D-α-Tocopherol, Sigma-Aldrich, USA) was used for the treatments.

Blood glucose and body weight of animals. At the beginning of the experiment blood glucose levels of all animals were measured using a glucometer (Accu-Chek Go, Roche Diagnostics, Mannheim, Germany) to confirm that the level was between 60-110 mg/dl. The third day after the induction of diabetes, following 6 hour of starvation, blood glucose was measured. Rats were considered diabetic if their blood glucose levels exceeded 250 mg/dl. Blood glucose and body weight were monitored once in a week during the experiment period and the data collected were statistically evaluated (**Table 1**).

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Tissue sampling. At the end of the tenth week all animals were sacrificed as follows; each animal was anesthetized by ketamine hydrochloride (Ketalar, Parke-Davis, Istanbul, Turkey) 30 mg/kg intramuscularly. For muscle relaxation, 2% xylazine hydrochloride (Rompun, Bayer, Istanbul, Turkey) 6 mg/kg was used. Then, they were perfused with 4% paraformaldehyde solution. Following perfusion, the testes were removed and fixed in neutral formalin for 72 hours and processed for paraffin embedding. Sections (4-5 µm thick) were taken on polylysine coated microscope slides for further examination.

Immunohistochemistry. For immunohistochemical evaluation of proliferative cell nuclear antigen (PCNA), polylysin coated microscope slides were stored in a microwave oven in 0.01 M citrate Buffer (Lab Vision, Fremont, CA, USA). Endogenous peroxidase activity was blocked in 3% hydrogen peroxide (Lab Vision, Fremont, CA, USA). Epitopes were stabilized by application of serum blocking solution (Lab Vision, Fremont, CA, USA) and then slides were incubated with PCNA (mouse monoclonal antibody Ab-1, 0.2 mg/ml, NeoMarkers, Fremont, CA, USA), 60 minutes at room temperature. After that, the biotinylated secondary antibody (goat anti-rabbit, Lab Vision, Fremont, CA, USA) was applied. Then, streptavidin peroxidase (Lab Vision, Fremont, CA, USA) was applied to the slides. A 3-amino-9-ethylcarbazole (Lab Vision, Fremont, CA, USA) was used as chromogen. Afterwards, the slides were counterstained with Mayer's hematoxylin and examined with photo-light microscope (DCM4000 Image Analyze System and QWin3 Programme, Leica, Germany).[15]

Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) assay. The apoptotic index in germ cells was determined by the TUNEL assay. The sections were stained with an in situ cell apoptosis detection kit (ApopTag Plus Peroxidase In Situ S7101, Chemicon International, Temecula, CA, USA). The sections were incubated at 60°C overnight. Sections were deparaffinized in xylene for an hour and rehydrated with reduced alcohol series (100, 95, 85, 70, and 60%). The slides were incubated with 20 µg/ml of proteinase K (Invitrogen, Camarillo, CA, USA) for 10 minutes. Washing with phosphate-buffered saline (PBS, İnvitrogen, Camerillo, CA, USA) was performed in every stage. Endogenous peroxidase activity was blocked with 3% H_2O_2. After washing with PBS, the sections were incubated with equilibration buffer for 10-15 minutes, and with terminal deoxynucleotidyl transferase (TdT) enzyme (77 µl reaction buffer + 33 µl TdT enzyme mix [1 µl TdT enzyme]) for 60 minutes at 37°C. Stop/wash buffer (1:10) was applied for 10 minutes at room temperature, and the slices were incubated with antidigoxigenin conjugate for 30 minute. After washing with PBS 3 times for 5 minutes, the sections were stained with DAB components to detect TUNEL-positive cells, and then they were counterstained with methyl green.[16]

Histopathological evaluation of spermatogenetic activity. The spermatogenesis was categorized by using the Johnson's score.[17] It applies a grade from 1 to 10 to each tubule cross section according to the following criteria: 10 = complete spermatogenesis and perfect tubules; 9 = many spermatozoa present and disorganized spermatogenesis; 8 = only a few spermatozoa present; 7 = no spermatozoa but many spermatids present; 6 = only a few spermatids present; 5 = no spermatozoa or spermatids but many spermatocytes present; 4 = only a few spermatocytes present; 3 = only spermatogonia present; 2 = no germ cells but only Sertoli cells present; and 1 = no germ cells and no Sertoli cells present.

Measurement of seminiferous tubule diameter and epithelial thickness. The seminiferous tubules were randomly selected in each section of the testis. The tubule diameters and epithelial thickness of the ten most circular seminiferous tubules were measured with an ocular micrometer using the x10 objective. In each groups, the mean diameter and epithelial thickness were evaluated.[3]

Table 1 - Body weight and blood glucose level of all the groups of rats included in a study conducted at Gazi University, Faculty of Medicine, Ankara, Turkey.

Parameters	Control	Vitamin E	Green tea	Vitamin E plus green tea	Diabetic	Diabetic vitamin E	Diabetic green tea	Diabetic vitamin E plus green tea
Glucose (mg/dl)	92.50±3.27[e]	93.83±3.92[e]	94.17±5.64[e]	90.00±1.41[e]	482.33±58.64[a,b,c,d]	405.00±38.06[a,b,c,d,e]	406.83±40.50[a,b,c,d,e]	319.50±22.02[a,b,c,d,e]
Initial body weight (g)	254.83±2.79	259.67±7.74	262.17±5.46	256.00±4.47	260.67±7.61	262.00±5.29	259.50±6.19	258.83±5.67
Final body weight (g)	294.83±5.53[†]	321.83±11.46[†]	308.67±11.98[†]	321.00±16.07[†]	194.67±6.09[†]	230.17±20.21[†]	248.33±24.11	289.67±21.06*

[a]versus control group (p<0.01), [b]versus Vitamin E group (p<0.01), [c]versus green tea group (p<0.01), [d]versus Vitamin E plus green tea group (p<0.01), [e]versus diabetic group (p<0.01), *p<0.05, [†]p<0.001

Statistical analysis. All statistical analyses were performed with the Statistical Package for Social Sciences software version 17 (SPSS Inc, Chicago, IL, USA). The results of the tests were expressed as the number of observations (n), mean ± standard deviation, median and min-max values. The results of the homogeneity (Levene's Test) and normality tests (Shapiro Wilk) were used to decide, which statistical methods to apply in the comparison of the study groups. Normally distributed and with homogeneous variances groups were compared 2-paired groups by paired t test and compared 3 or more unpaired groups by analysis of variance. According to those tests results parametric test assumptions were not available for some variables, so the comparisons of 2 dependent groups were performed by Wilcoxon test and 3 independent groups were performed by Kruskal Wallis test. Multiple comparison tests, the adjusted Bonferroni test was used. A $p<0.05$ was considered statistically significant. Multiple comparison test results are described in detail the differences between groups (**Table 2**).

Results. *The PCNA immunoreactivity.* The PCNA-positive cells for each group are represented in **Table 2**. Both seminiferous tubules and interstitial tissue compartments were observed to be normal in the control group. The PCNA reactivity was found to be highest in active tubules, particularly in the spermatogonia (**Figure 1A**). Similar findings were determined in the testes samples of vitamin E administered and green tea administered groups (**Figure 1B & 1C**). The histological structure was impaired in vitamin E plus green tea administered group. Significant edema was present. However, it was observed that the lumens were filled with spermatozoa that were either in their spermiogenesis stage or had completed the stage in all sections of the tubules. In some other tubules, immature cells were also released into the lumen. According to the PCNA involvement, it was observed that all of the tubules were active, and the immunoreactivity was seen in spermatogonium and spermatocyte I (**Figure 1D**).

In the diabetic group, the histological structure was also seen to have changed. Normal seminiferous epithelia and mature or submature sperms were present in the lumen of some tubule sections, whereas edema was present in some others. In a group of tubules, the seminiferous epithelia were observed to have thinned, and very few mature sperms were present in the lumen. Diffuse edema was observed in these tubules and interstitial area. In the detailed examination, few cells were PCNA (+) in the tubules with active appearance (**Figure 1E**). In the diabetic vitamin E administered group, tubules with similar structures with the diabetic groups were also remarkable. The seminiferous epithelia were observed to be thinned in a group of tubules and no mature sperms were present in the lumens of these tubules. Edema was observed instead. According to the evaluation of PCNA involvement, immunoreactivity was positive in the seminiferous tubule sections presenting a normal histological structure, especially in the spermatogonia. In rare tubules, the PCNA involvement was noticed throughout the epithelium (**Figure 1F**).

In the diabetic green tea administered group, the histological examination of the testis tissue showed that the spermatogenesis were active in most of the tubules, whereas immature or submature sperms were present in the lumens of the inactive tubules. The general histological structure of most of the tubules was normal, except for edema. Interstitial edema was also present in this group similar to the diabetes group. The PCNA was positive in the majority of the cells within the active

Table 2 - The PCNA, TUNEL, Johnson score, epithelial thickness, testis diameter, and variables found between the groups of rats included in a study conducted at Gazi University, Faculty of Medicine, Ankara, Turkey.

Variables	PCNA*	TUNEL*	Johnson score*	Epithelial thickness*	Testis diameter*
Control	80.33±6.59	2.50±1.05	9.67±0.52	106.42±5.35	357.83±24.58
Vitamin E	73.00±6.10[bcd]	1.83±1.47 [bc]	9.50±0.55 [bc]	89.20±9.17 [a]	334.17±10.76
Green tea	75.00±5.22[bcd]	0.67±0.82 [bc]	9.67±0.52 [bcd]	101.43±7.53 [bc]	331.02±17.77 [d]
Vit. E plus green tea	85.33±4.32[bcd]	4.67±1.21 [b]	8.83±0.75 [bc]	102.60±4.51 [bcd]	381.41±13.46 [bcde]
Diabetic	30.50±6.57 [abcd]	32.50±4.14 [a]	5.67±0.52 [ac]	82.62±7.77 [a]	309.57±5.60 [a]
Diabetic vitamin E	45.17±4.17 [abcd]	27.67±2.88 [abc]	6.83±0.75 [a bd]	77.93±5.56 [a]	331.60±23.11
Diabetic green tea	61.67±6.09 [abc]	0.33±0.52 [bc]	8.50±0.55 [a bc]	89.22±3.09 [a]	342.77±29.72
Diabetic vitamin E plus green tea	84.17±4.75[bcd]	0.67±0.82 [bc]	9.33±0.52 [bc]	99.63±8.04 [bc]	338.15±13.72
Total	**66.90±19.41**	**8.85±12.65**	**8.5±1.51**	**93.63±11.54**	**340.82±26.56**

PCNA - proliferative cell nuclear antigen, TUNEL - [a]versus control group, [b]versus diabetic, [c]versus diabetic vitamin E, [d]versus diabetic green tea, [e]versus diabetic vitamin E plus green tea, *$p<0.01$

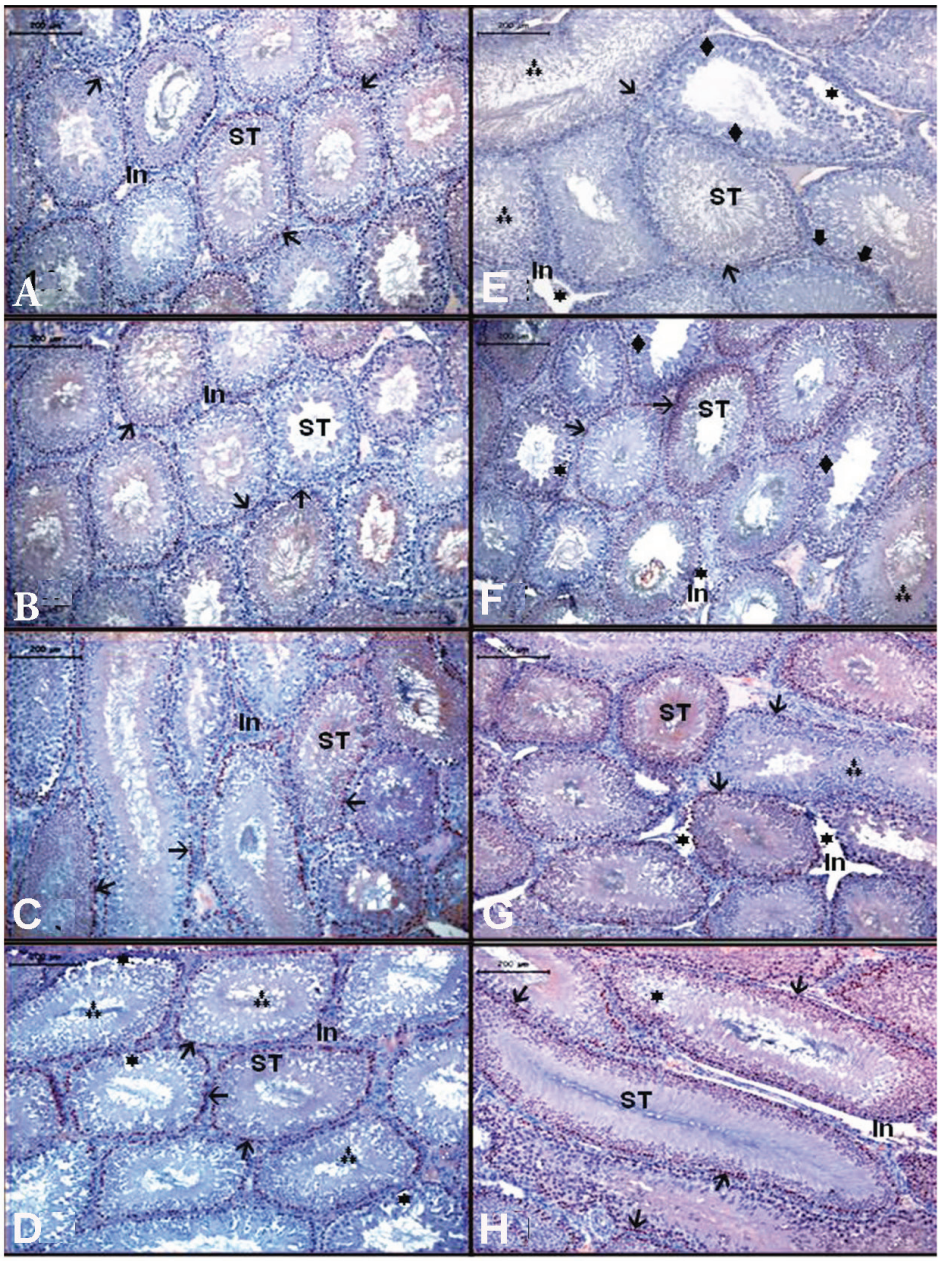

Figure 1 - Images of rat testicular tissue are examined by proliferative cell nuclear antigen (PCNA) immunostaining. The structure of testes is normal in the control (**A**), vitamin E treated control (**B**) and green tea treated control (**C**) groups. Significant edema is present and immature cells are also released into the lumen in some areas in the vitamin E and green tea treated control group (**D**). The PCNA involvement, is extremely common in the active tubules in all of these groups. In diabetic group (**E**), seminiferous epithelia is thinned and diffuse oedema is appeared. In this group PCNA involvement is decreased compared to the other groups. In the diabetic vitamin E treated group (**F**), tubules are similar in structure with the diabetic groups. In this group the number of PCNA (+) cells are decreased compared to the control groups. In the diabetic green tea treated group (**G**), general histological structure is normal. The PCNA is also positive in this group. In diabetic vitamin E plus green tea treated group (**H**) general tubule structure and PCNA (+) cells are similar to the normal control group. ST - seminiferous tubule, In - interstitial area, ✶ - edema, ✶ ✶ - immature sperm in the lumen, ← - degenerated seminiferous tubule, ♦ - thinning seminiferous epithelium, ↑ - PCNA (+) cells (immunoperoxidase - Hematoxylin x100).

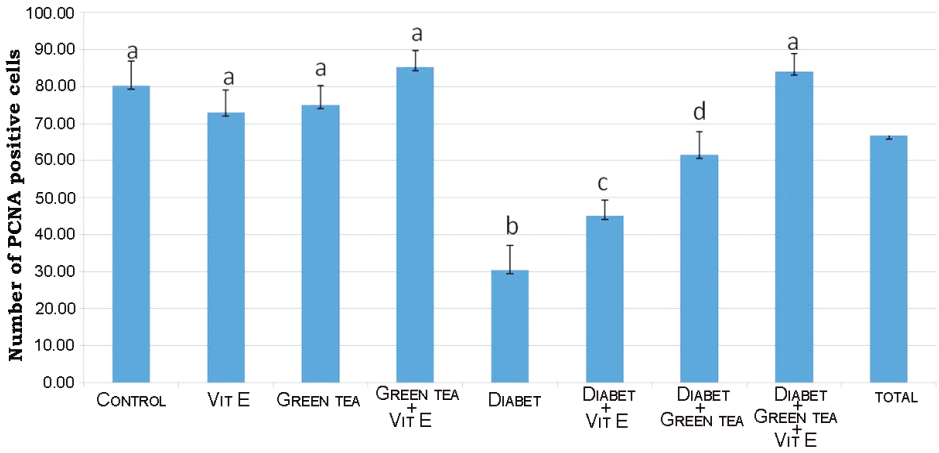

Figure 2 - Distribution of proliferative cell nuclear antigen (PCNA) immunopositive cells all of the groups in rat testes. Different letters depict significant differences among groups ($p<0.01$). [a]versus control group, [b]versus diabetic, [c]versus diabetic vitamin E, [d]versus diabetic green tea.

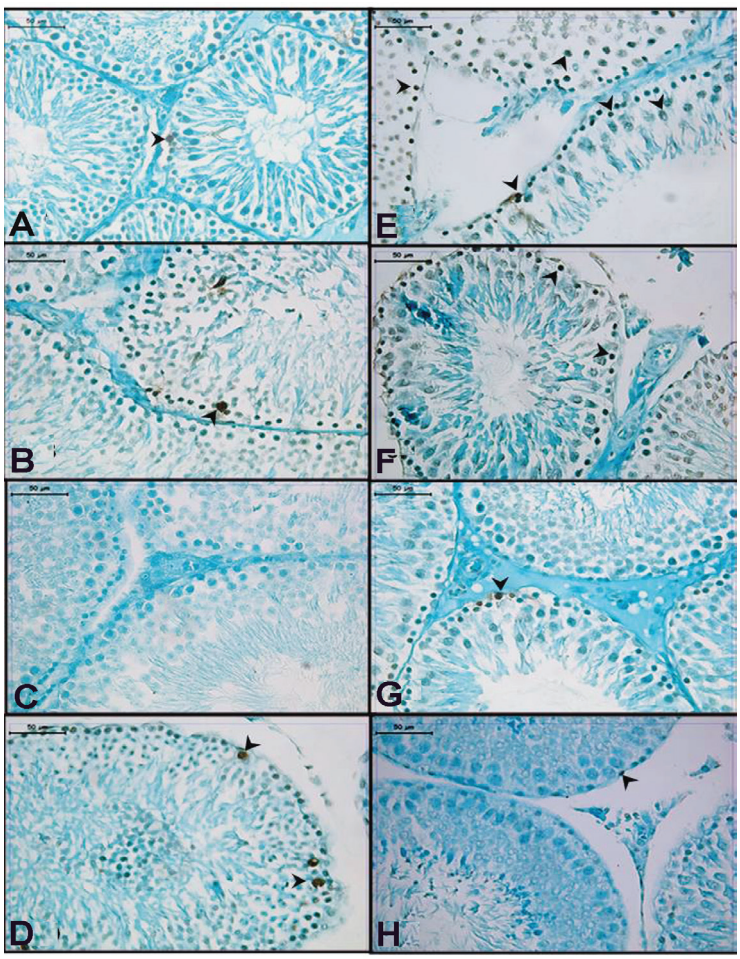

Figure 3 - Images of rat testicular tissue are examined by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) staining. Several TUNEL (+) are observed in: **A**) control; **B**) vitamin E treated; and **C**) green tea treated groups. The number of positive cells are increased in vitamin E and green tea treated rat group (**D**). In the diabetic group (**E**) it demonstrated an increase in the number of TUNEL (+) cells in all tubules. In the diabetic vitamin E treated group (**F**) as determined by TUNEL (+) staining, it decreased compared to that of the diabetic group. In diabetic green tea treated group (**G**) the general appearance is similar to the control groups. In the diabetic vitamin E and green tea treated groups (**H**), are similar to the control group. ➢ - TUNEL (+) cells (TUNEL - methyl green x400).

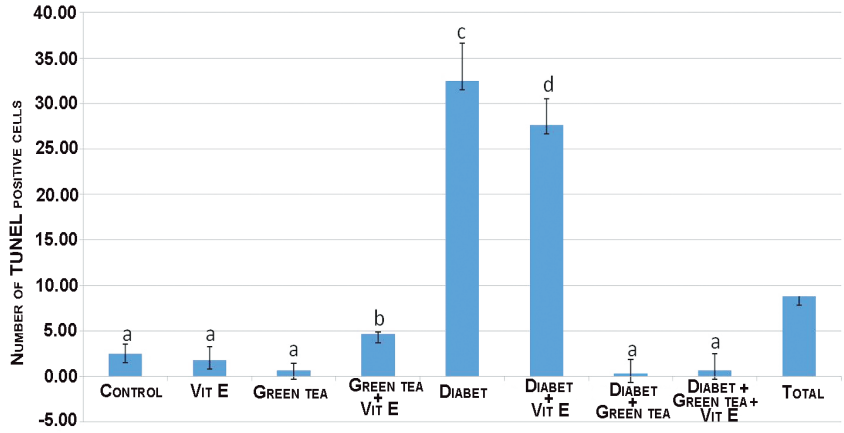

Figure 4 - Distribution of terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) stained positive cells all of the groups in rat testes. [a]versus control group, [b]versus diabetic, [c]versus diabetic vitamin E, [d]versus diabetic green tea.

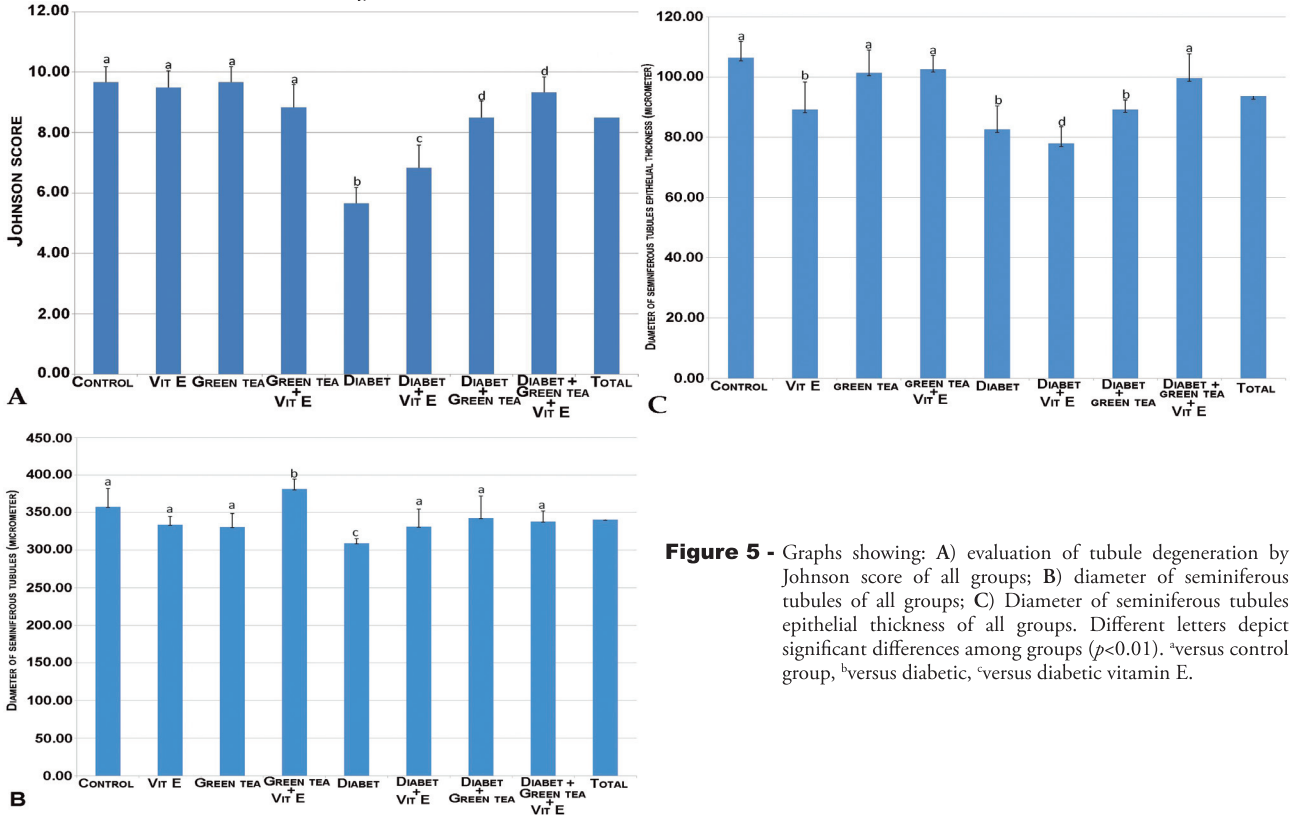

Figure 5 - Graphs showing: **A**) evaluation of tubule degeneration by Johnson score of all groups; **B**) diameter of seminiferous tubules of all groups; **C**) Diameter of seminiferous tubules epithelial thickness of all groups. Different letters depict significant differences among groups (p<0.01). [a]versus control group, [b]versus diabetic, [c]versus diabetic vitamin E.

tubules, whereas several spermatogonia were PCNA (+) within the inactive tubules (**Figure 1G**). The general tubule structure in diabetic vitamin E plus green tea administered group was similar to that of the control group. Most of the tubules were determined to be active with sperms inside their lumens, whereas rare inactive tubules were also observed. The cell proliferation assessment in this group showed the presence of the PCNA (+) cells throughout the seminiferous epithelia of the active tubules, whereas reactivity was observed only at spermatogonium level in the tubules with inactive appearance (**Figure 1H**). In accordance to the statistical analyses, no significant differences was observed in the number of PCNA (+) cells in nondiabetic vitamin E, green tea and vitamin E plus green tea groups compared to the control group (p=0.99). In the diabetic group the evaluation of the PCNA involvement showed that it was significantly decreased compared to the other groups

throughout all the tissues (p<0.01). In the diabetic vitamin E administered group was observed that the number of PCNA (+) cells was decreased compared to the control group (p<0.01), but increased compared to the diabetes group (p<0.01). In the diabetic green tea administered group The PCNA (+) cells in this group were observed to be statistically significantly increased compared to that of the diabetic and diabetic vitamin E administered groups (p<0.01). In the diabetic vitamin E plus green tea administered group, a significant increase was observed in this group compared to that of the diabetic, diabetic vitamin E administered, and diabetic green tea administered groups (p<0.01) No significant difference was observed between this group and the control group regarding the number of PCNA (+) cells (p=0.99) (**Figure 2**).

The TUNEL result. The TUNEL-positive cells for each group are represented in **Table 2**. Several TUNEL-positive spermatogonia were observed in control, vitamin E administered and green tea administered groups whereas the number of positive cells were increased in vitamin E and green tea administered group (p<0.01). The TUNEL staining performed in the diabetes group demonstrated a dramatic increase in the number of positive-stained cells in all the tubules, and in addition to the spermatogonia, many spermatocytes were also positively stained (p<0.01). Most of the spermatogonia in diabetic vitamin E administered group was determined to be TUNEL (+) where the spermatocyte staining was decreased compared to that of the diabetic group (p<0.01). Several spermatogonia were observed to be positive in diabetic green tea administered group and the general appearance was similar to that of the control groups (p<0.01). Likewise, several spermatogonia were TUNEL (+) in diabetic vitamin E plus green tea administered group, whereas a significant difference was observed between this group and the diabetic group (p<0.01). However, it was similar to the control group (**Figure 3A-H**, and **Figure 4**).

Statistical results of Johnson score, seminiferous tubule diameter and epithelial thickness. Johnson Score, seminiferous tubule diameter and epithelial thickness values for each group are shown in **Table 2**. In the scoring system performed according to the Johnson criteria, no significant differences was seen in nondiabetic vitamin E, green tea and vitamin E plus green tea groups compared to the control group (p=0.99). But decrease was observed in the scores of the diabetic group compared to that of the control group (p<0.05), whereas increases were observed in those of the diabetic green tea administered group and diabetic vitamin E and green tea administered group (p<0.01) (**Figure 5A**). The seminiferous tubule diameter was decreased in the diabetic group compared to that of the control group (p<0.05) (**Figure 5B**). The seminiferous epithelial thickness in the diabetes group was significantly decreased compared to the control group (p<0.01), whereas it was increased in the diabetes group with the application of both antioxidants (p<0.05) (**Figure 5C**).

Discussion. The aim of this study was to evaluate the possible effects of mono, or combined use of 2 antioxidants, vitamin E and green tea, on the testes of diabetic rats. Diabetes is a chronic metabolic disease leading to severe complications such as retinopathy, nephropathy, atherosclerosis and reproductive dysfunction.[1,18] Diabetes sequentially causes apoptotic cell death, seminiferous tubule atrophy, a decrease in the diameter of the tubules and the number of spermatogenic cells, and uncontrolled or unnecessary apoptosis during spermatogenesis. Therefore, apoptosis plays a critical role in the pathology of testicular dysfunction in male patients. The most characteristic property of apoptosis is the DNA fractures within the nucleus. The TUNEL method has an informative role in determining the DNA fractures in diabetes-induced reproductive cell apoptosis.[3]

In our study, accordance with the literature, the TUNEL-staining demonstrated an increased number of positive cells in the diabetes group. The PCNA immunoreactivity was showed decreased staining in the diabetes group as well. The Johnson scoring (p<0.05), seminiferous tubule diameter (p<0.05), and seminiferous epithelial thickness parameters (p<0.01) were also significantly decreased in the diabetes group compared to the control group. The mechanism of the increased oxidative stress which has long been accepted to have an important role in the generation and progression of diabetes still remains unclear and believed to include the activation of transcription factors, AGEs and protein kinase C.[8] Naziroglu[4] has mentioned that the aetiology of diabetes may be the oxidative damage caused by the ROS and this may be prevented by antioxidant support. Shrilatha[19] generated diabetes-related experimental oxidative stress in the testes and suggested that this could lead to a testicular degeneration resulting in testicular dysfunction. Amaral et al[2] demonstrated in their experimental study that the sperm concentration and motility were decreased, lipid peroxidation was increased, which was in accordance with the morphological findings, and the activity of glutathione peroxidase and the levels of ATP were decreased. They concluded that diabetes had negatively

affected the production of energy and the oxygen radicals. Diabetes-related hyperglycemia causes the production of free radicals and damages the endogenous antioxidant defence systems by different mechanisms.[8] One of important antioxidant is vitamin E, especially in maintaining the integrity of the cell membrane.[20] Furthermore, vitamin E has been demonstrated to display a protective effect in the testicular damage of rats with experimentally created cryptorchidism,[21] and that was protective against insecticides,[22] and various toxic materials.[23,24] The plasma vitamin E concentrations in diabetics were shown to be lower than that of healthy individuals.[9]

In our study, we evaluated the effects of vitamin E in the testes with experimentally created diabetes, which shows that vitamin E significantly increases the spermatogenic proliferation index, and decreases the spermatogenic apoptosis in diabetic rats. However, no significant result was obtained with regard to the seminiferous epithelial thickness, Johnson score, or tubule diameter data. Green tea is another important antioxidant, which is produced by the enzymatic inactivation of the plant *Camellia sinensis.* The beneficial effects of green tea are related to the catechins, which was demonstrated to have the potential to inhibit the tumor growth in leukemia and breast cancer, to stimulate their degradation, and to inhibit the proliferation of prostate cancer cells, head, neck, and pancreatic carcinoma cells.[11,25] Saussi et al[26] have emphasized the strong antioxidant effects of polyphenols within green tea against metal ions. As a result of the examinations on the kidney, liver and the testes, they demonstrated that the serum oxidant indicator levels were decreased, and the vitamins E and C levels were increased by green tea administration in rats that were given metal ions.

In our study, the findings show that the green tea administration improved the testis structure in diabetes, induced the proliferation index and reduced apoptotic index, but was not sufficient for improvement in the epithelial thickness and tubular diameter. It was concluded that green tea administration was more effective than vitamin E in maintaining the seminiferous tubule structure in diabetes. In recent experimental studies, investigators have generally examined the protective or curative effects of combined vitamin or antioxidant applications.[4,27] In a biochemical study investigating the effects of vitamins E, C, and selenium in rats with experimental diabetes, it was observed that vitamin E monotherapy was insufficient in the protection of the testis against the oxidative stress caused by diabetes, but that its combined therapy was highly effective.[4]

In our study, the histological investigations of the diabetic groups have demonstrated that the combined therapy of vitamin E plus green tea has significantly protected the testis structure compared to the diabetes and vitamin E administered diabetic groups. Similar results were obtained in the PCNA and TUNEL staining's of the control group, but the spermatogenic proliferation and apoptotic findings compared to the diabetic group were statistically significantly different ($p<0.01$). The findings of the combined vitamin E plus green tea administered group were similar to that of the control group, and no significant difference was observed in the proliferation indexes or the TUNEL data ($p=0.99$). As an indicator of the increased proliferation, seminiferous epithelial thickness was determined to be significantly increased compared to the diabetic group ($p<0.05$), whereas no difference was observed in the tubule diameter.

The administration of a single dose green tea and a single type of antioxidant (vitamin E), are the limitation of our experimental procedure. Comparison of the effects of the green tea in diabetic rats with other antioxidants found in the market will be helpful to decide whether they are beneficial or harmful to the patient. Therefore, we will design our future experiment to compare the effects of the different doses of green tea in diabetic rats, together with most prescribed antioxidant such as selenium, vitamin C and resveratrol.

In conclusion, the combined therapy of vitamin E plus green tea in diabetes was more effective than monotherapy. Therefore, in routine clinical trials these antioxidants may be used as supporting therapy for reproductive dysfunction.

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