Changes in rat ovary with experimentally induced diabetes and the effects of lycopene on those changes

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Abstract

Free radicals increase in the presence of diabetes. Lycopene is a powerful antioxidant. The goal of the present study was to determine the effect of diabetes on rat ovaries and the protective role of lycopene in that context. Experimental diabetes was induced with 50 mg/kg streptozotocin. Rats were randomly separated into four groups, as follows: control + corn oil, control + lycopene, diabetes + corn oil and diabetes + lycopene. The histological and histometric evaluations were performed using Crossman’s triple staining method. The immuno-histochemical connexin-43 expression was identified and the apoptotic cell density was determined using the terminal deoxynucleotidyl transferase dUTP nick-end labeling method, while the malondialdehyde levels were measured using the enzyme-linked immunosorbent assay technique in the ovaries. Vacuolization of the corpus luteum, hydrosis degeneration in the interstitial regions, and the number of corpora lutea increased in the ovary as effects of diabetes while the diameter of the corpora lutea decreased. The intensity of connexin-43 expression decreased in the primordial and atretic follicles, interstitial cells and luteal cells of the corpora lutea in the diabetes + corn oil group. The ovarian malondialdehyde levels and the number of apoptotic cells in the granulose layers of the large antral follicles increased in the presence of diabetes. Lycopene increased the expression of connexin-43 in the primordial, secondary and large antral follicles in the ovaries of diabetic animals. The changes caused by diabetes in the ovaries and the protective role of lycopene in some but not all parameters was revealed.

Keywords: experimental diabetes, connexin-43, lycopene, ovary, rat.

Introduction

Diabetes mellitus is a disease causing high blood sugar levels, requiring constant medical care with high treatment costs and leading to serious complications [1]. Ovarian mass [2], the number of primary and secondary follicles [3], follicular diameters [4] and ovulation rate are known to decrease with the effect of diabetes [5]. On the other hand, the number of atretic follicles [4, 6] and degenerative oocytes [7] and the oocyte aneuploidy rate all increase [5]. Furthermore, diabetic women experience polycystic ovary syndrome and hirsutism more often than the general population [8].

Serum malondialdehyde (MDA) level [9, 10] and erythrocyte membrane lipid peroxidation both increase significantly as effects of diabetes [11]. By contrast, the plasma levels of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) decrease significantly in diabetic patients [12]. It has also been reported that diabetic animal ovaries show a marked decrease in copper/zinc superoxide dismutase (Cu/Zn SOD) activity [13].

The gap junctions are shaped by proteins coming from the connexin gene family [14]; intercellular communication established by connexin is important for numerous cellular functions such as the regulation of cell growth, differentiation and development [15]. In particular, the connection between granulosa cells is established by connexin-43 and is required for the continuation of follicular growth [16]. Connexin expression in oocytes surrounded by cumulus decreases because of diabetes, and thus it has been reported that oocyte maturation can be disrupted [17, 18].

Less-developed follicles with many apoptotic foci due to diabetes have been found in the ovaries [18]. An increase has been observed in the number of oocytes expressing annexin V, Bax and caspase-3 [19], and it has been reported that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptor KILLER expression increase in cumulus cells [18].

Lycopene, a carotenoid-type pigment, has a strong antioxidant effect [20] and is effective in sweeping reactive oxygen radicals, thus preventing lipid peroxidation and DNA damage [21]. It has been reported that lycopene usage is effective against weight loss and hyperglycemia in diabetic rats [22], and that lycopene increases serum insulin levels [23] and decreases neuropathic pain [24]. On the other hand, lycopene is effective against hypoxia-and reoxygenation-induced cardiomyocyte apoptosis [25] and increases gap junction communication during the carcinogenesis phase [21].

In the present study, the researchers examine the histological, histopathological, histometric, immunohistochemical, enzyme-linked immunosorbent assay (ELISA) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) observations of ovarian changes in adult female rats with experimental diabetes and assess the effects of orally administered lycopene on those changes.
Materials and Methods

Animals

In the study, 42 adult Wistar albino female rats (four months old) were used; the rats were obtained from the Department of Laboratory Animals, Ege University in Izmir, Turkey. The study was performed with the permission (No. 2011/042) of the Ethics Committee of Adnan Menderes University, Aydin, Turkey. The rats were housed in a room with a temperature of 24 ± 1°C and kept under conventional conditions, with a 12-hour light/dark cycle and supplied water and feed ad libitum.

Induction of diabetes

A single dose of 50 mg/kg of streptozotocin (BioShop, STR 201.1) in a 0.01 M citrate buffer (pH 4.5) was intraperitoneally administered to 30 rats to induce experimental diabetes [26]. To measure fasting blood glucose values [27], blood samples were taken three days later from the tails of the rats using a glucometer (Bayer, Contour TS). The animals included in the study had fasting blood glucose levels of 250 mg/dL or more [24, 28]. The animals in the control groups received an equal dose of 0.01 M citrate buffer (pH 4.5) intraperitoneally. Experimental diabetes has not developed in five animals. Therefore, they were removed from the study. Seven animals died at the experiment term in diabetes groups. Therefore, the study was carried out with 30 animals.

Experimental design

Three weeks after diabetes was induced using streptozotocin, the control and diabetic rats were randomly separated into control + corn oil (n=6), control + lycopene (n=6), diabetes + corn oil (n=8) and diabetes + lycopene (n=10) groups. The control + lycopene and diabetes + lycopene groups of rats were fed 4 mL/kg corn oil. In the last 4 weeks (DSM Inc., 10% FS, Redivivo™, Code 7803). The control + corn oil and diabetes + corn oil groups of rats were fed 4 mL/kg corn oil. In the last week of the lycopene administration, all animals in all groups were checked for estrous cycles using the vaginal smear method. At both the beginning and end (week 8) of the study, all rats in all groups were weighed. The fasting blood glucose values were measured at the beginning of the study, at the start of the lycopene administration (week 4) and at the end of the study (week 8).

Sample collection and preparation

At the end of the study, all animals in all groups were checked for estrous cycles using the vaginal smear method. When the animals were in the diestrus period, they were sacrificed by cervical dislocation under a light ether anesthetic. The right ovaries of the animals were removed and fixed for 24 hours at +4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). After routine histological processing, the tissues were embedded in paraplast and 5-μm thick cross-sections were taken. The left ovaries were frozen in liquid nitrogen and stored at 80°C.

Histological, histopathological and histometrical analyses

All tissue cross-sections were prepared using Crossman’s triple staining method [29]. Two cross-sections from each animal were used to assess variations from normal histological appearance of the tissues, numerical values and histometric measurements. The classification of ovarian follicles was carried out according to conventional follicle classification [30], taking into account both the granulosa cell form and the number of cell layers.

The number of follicles and corpora lutea per group was identified in the sections of each animal [31]. The corpus luteum in each cross-section was also examined for vacuolization, while interstitial areas were examined histopathologically for hydropic degeneration. The variations observed were assessed semi-quantitatively and scored (0 – negative; 1 – low; 2 – medium; 3 – strong) following the lead of Rømer et al. [32]. The diameters of the follicles and corpora lutea in the ovaries were determined by measuring all follicles and corpora lutea in the sections [33].

Immunohistochemistry

The 5-μm cross-sections obtained from each animal’s ovary blocks were placed on slides covered in organosilane, with the Avidin–Biotin Peroxidase method used to identify connexin-43 expression. The cross-sections were processed using deparaffinization and dehydration, then boiled three times for five minutes each time in a microwave oven at 98°C in 0.01 M pH 6 sodium citrate. Then, the cross-sections were treated with a 3% H2O2 distilled water solution for 15 minutes to stop endogenous peroxidase activity. The cross-sections were placed in a blocking solution for one hour (Invitrogen, Broad Spectrum Ref. 859043, Batch 1018708A). After this process, the sections were incubated overnight at 4°C with a primary antibody diluted to 1:200 (Bioss, rabbit anti-connexin 43 polyclonal antibody, unconjugated, bs-0651P). Negative control sections were processed using tris-buffered saline (TBS, pH 7.4) instead of the primary antibody. The following day, the cross-sections were incubated in biotinylated secondary antibody for one hour (Invitrogen, Broad Spectrum Ref. 859043, Batch 1018708A), then incubated in Streptavidin–HRP (Horseradish peroxidase) (Invitrogen, Broad Spectrum Ref. 859043, Batch 1018708A) and processed with 3,3'-diaminobenzidine (DAB) for two minutes. Finally, all sections were counterstained with Harris’s Hematoxylin and closed using entellan.

Connexin-43 expression was assessed in two cross-sections from each animal; connexin-43 immunoreactivity was evaluated in all primordial, primary, secondary, small antral, large antral, atretic follicular, corpus luteal and interstitial cells in the ovaries using a subjective scoring system: 0 to +3 [0 – no immunoreactivity (-); 1 – weak immunoreactivity (+); 2 – moderate immunoreactivity (++; 3 – strong immunoreactivity (+++)] [34].

TUNEL

The apoptotic cell density in the ovaries was identified using the TUNEL method, specifically the GenScript TUNEL Apoptosis Detection Kit (Cat. No. L00297). The cross-sections were processed through deparaffinization and dehydration, after which the samples were incubated
in proteinase K for 15 minutes, a 3% H$_2$O$_2$ methanol blocking solution for 10 minutes, in the TUNEL reaction solution (equilibration buffer, biotin-11-DUTP and TdT) for 60 minutes and a Streptavidin–HRP solution for 30 minutes. The sections were then processed using DAB chromogen for two minutes, before being counterstained with Methyl green and closed using entellan.

The apoptotic cell counts were identified in the granulosa layer of large antral follicles and corpora lutea. All large antral follicles and corpora lutea in the sections were assessed. For this purpose, apoptotic cell numbers were obtained from five different 1764 μm$^2$ unit areas in the granulosa layer of large antral follicles [35]. The apoptotic cell density in corpora lutea was determined through a semi-quantitative apoptosis scoring system (0 – no staining; 1 – low staining; 2 – moderate staining; 3 – strong staining) [36].

**Lipid peroxidation measurement**

Each ovary taken from the animals was weighed and broken into pieces mechanically. The tissue fragments were homogenized using a Teflon-glass stirrer (IKA Overhead Stirrer) in 150 mM phosphate buffer (pH 7.4; 1/10, v/v) for two minutes at 2000 rpm in an ice bath. The homogenates were centrifuged using a Nüve-Benc Top Centrifuge (NF 800 R) at 8000 g (11 300 rpm) for 10 minutes at 4°C. The homogenized samples were then placed in Eppendorf tubes. The protein content of the homogenized samples was identified using the Lowry protein assay; a standard curve resulted. Finally, the MDA levels in the ovaries were identified with the ELISA method (Cell Biolabs, Oxiselect Adduct ELISA Kit STA-332).

The BSA (bovine serum albumin) standards and protein samples were placed on a plate and incubated for two hours at 37°C, after which assay diluent was added to the plate and left for two hours. Then, an anti-MDA antibody and secondary antibody conjugated with HRP were successively added to the plate and incubated for one hour each. A substrate solution was added to the plate and left for 30 minutes. Finally, the enzyme reaction was stopped using the stop solution and the results measured immediately with a spectrophotometer at 450 nm. The absorbance values resulting from the ELISA protocol were calculated using the prepared standard curve. The tissue MDA values were compared to the protein values and given as pmol/mg.

**Statistical analysis**

SPSS 17.00 (IBM) was used for statistical analysis. To identify whether there were any differences among group means for body weight, fasting blood glucose and ovarian MDA levels, Kruskal–Wallis variance analysis was used [37]. The group mean values of the histopathological changes, follicle and corpus luteum numbers and diameters, the TUNEL-positive cell numbers and connexin-43 immunoreactivity densities were compared using a one-way analysis of variance (ANOVA) [17, 33], while Duncan’s test was used as a post hoc test. The body weight values that belonged to the same groups at different times were compared with a repeated measures Wilcoxon test [38]. Similarly, the fasting blood glucose values that belonged to the same groups at different times were analyzed with repeated measurements ANOVA and Friedman tests [39]. The analysis of significant differences was performed using a Bonferroni-corrected Wilcoxon test. The data were reported as means ± standard errors, and values of $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (****) were considered to be statistically significant.

### Results

The examination of body weight and blood glucose values

In the present study, there was no significant difference among the groups in terms of the animals’ body weights at the end of the study (week 8). A comparison of the body weight data from the beginning and end of the study shows a significant difference between the body weights of the control + corn oil group and control + lycopene groups at the end of the study when compared with initial measurements ($p<0.05$, Table 1). The fasting glucose values of the rats at weeks 4 and 8 were significantly higher in the diabetes groups compared to the control groups ($p<0.001$). A comparison of intra-group data showed that the glucose values of the diabetes + corn oil group and diabetes + lycopene group showed significant increases at weeks 4 and 8 ($p<0.01$, Table 2).

| Table 1 – Body weight [g] values in control and diabetes groups |
|------------------|------------------|------------------|------------------|------------------|------------------|
| Week              | Control + corn oil | Control + lycopene | Diabetes + corn oil | Diabetes + lycopene | $P$              |
|                   | $n=6$            | $n=6$            | $n=8$            | $n=10$           |                  |
| Initial           | 134.33 ± 4.50$^b$| 153.00 ± 1.03$^b$| 169.60 ± 1.99  | 184.50 ± 6.16    | –                |
| $g^{th}$          | 162.50 ± 6.94$^a$| 184.50 ± 4.59$^b$| 167.20 ± 3.91  | 174.40 ± 8.31    | NS               |
| $P$               | *                | *                | NS              | NS               |                  |

$^a$Different superscripts in the same column indicate the significant difference. NS: Non-significant, $^p<0.05$.

| Table 2 – Fasting blood glucose [mg/dL] values in control and diabetes groups |
|------------------|------------------|------------------|------------------|------------------|------------------|
| Week              | Control + corn oil | Control + lycopene | Diabetes + corn oil | Diabetes + lycopene | $P$              |
|                   | $n=6$            | $n=6$            | $n=8$            | $n=10$           |                  |
| Initial           | 65.83 ± 3.38     | 58.50 ± 4.41     | 49.60 ± 1.83$^b$| 64.80 ± 5.67$^b$ | –                |
| $4^{th}$          | 77.33 ± 5.89$^b$| 61.00 ± 3.16$^b$| 511.00 ± 40.15$^{a,b}$ | 519.10 ± 40.27$^{a,b}$ | ***              |
| $8^{th}$          | 73.50 ± 3.21$^a$| 66.33 ± 4.72$^a$| 476.90 ± 41.13$^{a,b}$ | 512.90 ± 38.47$^{a,b}$ | ***              |
| $P$               | NS               | NS               | **               | **               |                  |

$^a$ and $^b$Different superscripts in the same row and column indicate the significant difference. NS: Non-significant, $^{*}p<0.01$, $^{**}p<0.001$.

### Follow-up of the estrous cycle and histopathological assessment

An examination of the estrous cycle of the animals shows that the control group rats had a regular estrous cycle, while in the diabetes groups it was irregular and many animals were held in the diestrous phase; even the diabetic group animals fed with lycopene had irregular cycles. In terms of ovarian histopathology, the corpus luteal vacuolization was significantly higher in the diabetes + corn oil and diabetes + lycopene groups compared to the control + corn oil group ($p<0.01$). Similarly, the hydropic degeneration in interstitial areas was significantly higher in the diabetes + corn oil group compared with control + corn oil ($p<0.05$, Table 3, Figure 1).
Table 3 – Intensity values of histopathological ovarian structures of control and diabetes groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control + corn oil</th>
<th>Control + lycopene</th>
<th>Diabetes + corn oil</th>
<th>Diabetes + lycopene</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=6</td>
<td>n=6</td>
<td>n=8</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>Vacuolization in the corpus luteum</td>
<td>0.20 ± 0.05</td>
<td>0.38 ± 0.08</td>
<td>0.46 ± 0.04</td>
<td>0.59 ± 0.06</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>0.05c</td>
<td>0.08b</td>
<td>0.04a</td>
<td>0.06a</td>
<td></td>
</tr>
<tr>
<td>Hydropic degeneration in the interstitial regions</td>
<td>0.50 ± 0.15b</td>
<td>0.25 ± 0.13c</td>
<td>1.06 ± 0.23a</td>
<td>0.81 ± 0.16a</td>
<td>*</td>
</tr>
</tbody>
</table>

**Different superscripts in the same row indicate the significant difference. *p<0.05, **p<0.01.

Histological and histometric examination

An assessment of the follicular and corpus luteal numbers among groups showed no significant difference in the primordial, primary, secondary, small antral, large antral and atretic follicle numbers, while the number of corpora lutea was significantly higher in the diabetes + corn oil group than in the control + corn oil group (p<0.01, Table 4). An examination of the ovarian follicular and corpus luteal diameters among groups showed no significant difference in the primordial, primary, secondary, small and large antral follicle diameters. The corpus luteum diameters were significantly smaller in the diabetes + corn oil and diabetes + lycopene groups than in the control + corn oil group (p<0.01, Table 5, Figure 2).

![Figure 1 – Histopathological changes in the ovary. The corpora lutea (A and C) and interstitial regions (B and D) that belong to control + corn oil group (A and B) and diabetes + corn oil group (C and D) and the vacuolization in the corpora lutea (C, arrowheads) and the hydropic degeneration in the interstitial regions (D, asterisk) all increased in the diabetes + corn oil group compared with the control + corn oil group. Crossman’s triple staining method. Scale bars: (A and C) 30 μm; (B and D) 20 μm.](image)

Table 4 – Number of follicles and corpora lutea in the ovaries of control and diabetes groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control + corn oil</th>
<th>Control + lycopene</th>
<th>Diabetes + corn oil</th>
<th>Diabetes + lycopene</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>n=6</td>
<td>n=6</td>
<td>n=8</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>Primordial follicle</td>
<td>8.36 ± 1.65</td>
<td>7.33 ± 1.47</td>
<td>7.33 ± 1.22</td>
<td>6.47 ± 0.87</td>
<td>NS</td>
</tr>
<tr>
<td>Primary follicle</td>
<td>5.55 ± 1.07</td>
<td>8.42 ± 1.66</td>
<td>6.08 ± 0.74</td>
<td>5.42 ± 0.65</td>
<td>NS</td>
</tr>
<tr>
<td>Secondary follicle</td>
<td>1.36 ± 0.41</td>
<td>2.00 ± 0.53</td>
<td>2.06 ± 0.37</td>
<td>1.21 ± 0.16</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Different superscripts in the same row indicate the significant difference. NS: Non-significant, **p<0.01.
Table 5 – Follicle and corpus luteum diameters [μm] in the ovaries of control and diabetes groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control + corn oil</th>
<th>Control + lycopene</th>
<th>Diabetes + corn oil</th>
<th>Diabetes + lycopene</th>
<th>P</th>
</tr>
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<td>n=6</td>
<td>n=6</td>
<td>n=8</td>
<td>n=10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primordial follicle</td>
<td>10.03 ± 0.19</td>
<td>9.92 ± 0.16</td>
<td>9.72 ± 0.12</td>
<td>9.50 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td>Primary follicle</td>
<td>20.14 ± 0.77</td>
<td>18.40 ± 0.51</td>
<td>19.48 ± 0.42</td>
<td>19.29 ± 0.73</td>
<td>NS</td>
</tr>
<tr>
<td>Secondary follicle</td>
<td>73.83 ± 6.17</td>
<td>66.43 ± 4.52</td>
<td>68.59 ± 3.36</td>
<td>67.22 ± 3.65</td>
<td>NS</td>
</tr>
<tr>
<td>Small antral follicle</td>
<td>159.99 ± 10.67</td>
<td>180.97 ± 11.29</td>
<td>181.82 ± 9.50</td>
<td>166.81 ± 8.27</td>
<td>NS</td>
</tr>
<tr>
<td>Large antral follicle</td>
<td>396.71 ± 16.73</td>
<td>423.77 ± 22.87</td>
<td>461.24 ± 15.44</td>
<td>405.66 ± 21.37</td>
<td>NS</td>
</tr>
<tr>
<td>Corpus luteum</td>
<td>802.14 ± 32.40</td>
<td>746.58 ± 41.76</td>
<td>685.03 ± 16.23</td>
<td>705.01 ± 21.76</td>
<td>**</td>
</tr>
</tbody>
</table>

*a,b Different superscripts in the same row indicate the significant difference. NS: Non-significant, **p<0.01.

Immunohistochemical examination

The expression of connexin-43 in the ovaries was identified in the germinative epithelium and in cytoplasm of primordial and primary follicle epithelial cells. The expression was also observed in the granulosa cell layers of secondary, small and large antral follicles and the theca of follicles, in the oocyte and interstitial cells, and in the corona radiata cells of the large antral and atretic follicles. In the corpora lutea, connexin-43 expression was determined in the luteal cells. By contrast, in the primordial and atretic follicles and interstitial cells and the luteal cells in the corpora lutea, the diabetes + corn oil group had a lower rate of connexin-43 immunoreaction compared to the control + corn oil group (p<0.01, p<0.001, respectively; Figure 3, Figure 4). In the primordial, secondary and large antral follicles, connexin-43 expression was higher in the diabetes + lycopene group than in the diabetes + corn oil group (p<0.01, Table 6, Figure 3, Figure 5).

Table 6 – Intensity values of connexin-43 immunoreactivity in the ovaries of control and diabetes groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control + corn oil</th>
<th>Control + lycopene</th>
<th>Diabetes + corn oil</th>
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<td>n=6</td>
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<td></td>
</tr>
<tr>
<td>Primordial follicle</td>
<td>2.57 ± 0.50</td>
<td>2.44 ± 0.60</td>
<td>2.27 ± 0.60</td>
<td>2.50 ± 0.05</td>
<td>**</td>
</tr>
<tr>
<td>Primary follicle</td>
<td>2.55 ± 0.06</td>
<td>2.45 ± 0.06</td>
<td>2.40 ± 0.06</td>
<td>2.60 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Secondary follicle</td>
<td>2.47 ± 0.13</td>
<td>2.75 ± 0.09</td>
<td>2.38 ± 0.11</td>
<td>2.87 ± 0.07</td>
<td>**</td>
</tr>
<tr>
<td>Small antral follicle</td>
<td>2.36 ± 0.20</td>
<td>2.48 ± 0.13</td>
<td>2.19 ± 0.12</td>
<td>2.48 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td>Large antral follicle</td>
<td>2.16 ± 0.12</td>
<td>2.46 ± 0.08</td>
<td>2.08 ± 0.10</td>
<td>2.45 ± 0.08</td>
<td>**</td>
</tr>
<tr>
<td>Atretic follicle</td>
<td>1.44 ± 0.06</td>
<td>1.57 ± 0.07</td>
<td>1.06 ± 0.02</td>
<td>1.02 ± 0.10</td>
<td>***</td>
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<tr>
<td>Interstitial cells</td>
<td>1.36 ± 0.15</td>
<td>1.46 ± 0.14</td>
<td>1.00 ± 0.00</td>
<td>1.16 ± 0.08</td>
<td>**</td>
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<tr>
<td>Corpus luteum</td>
<td>2.22 ± 0.06</td>
<td>2.17 ± 0.10</td>
<td>1.47 ± 0.04</td>
<td>1.52 ± 0.05</td>
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</table>

*a,b Different superscripts in the same row indicate the significant difference. NS: Non-significant, **p<0.01, ***p<0.001.

Examination of apoptotic cell density

In the examination performed with the TUNEL method, apoptotic cells were frequently seen in the large antral follicle granulosa layer, the atretic follicles and the corpora lutea. The data showed that the number of apoptotic cells in the granulosa layer of the large antral follicles and corpora lutea was higher in the diabetes + corn oil group than in the control + corn oil group (p<0.05), but the increase in the corpora lutea was not statistically significant (Figure 6). Additionally, the number of apoptotic cells in the ovaries of diabetic animals appeared to decrease with the effect of lycopene, though this finding was not statistically significant (Table 7).

Table 7 – Number of apoptotic cells in the unit area in the granulosa layer of large antral follicles and density values of apoptotic cells in the corpora lutea in the ovaries of control and diabetes groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control + corn oil</th>
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<th>Diabetes + corn oil</th>
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<td>n=6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Large antral follicle</td>
<td>0.86 ± 0.18</td>
<td>1.14 ± 0.17</td>
<td>1.68 ± 0.21</td>
<td>1.44 ± 0.20</td>
<td>a</td>
</tr>
<tr>
<td>Corpus luteum</td>
<td>0.68 ± 0.10</td>
<td>0.67 ± 0.15</td>
<td>0.81 ± 0.05</td>
<td>0.58 ± 0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>

*a,b Different superscripts in the same row indicate the significant difference. NS: Non-significant, *p<0.05.
Examination of the MDA level

MDA analysis of the ovary tissues showed that the MDA level was higher in the diabetes + corn oil group compared to the control + corn oil and control + lycopene groups ($p<0.01$). Furthermore, the MDA level of the diabetes + lycopene group was lower than that of the diabetes + corn oil group, even though this difference was not statistically significant (Table 8).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control + corn oil</th>
<th>Control + lycopene</th>
<th>Diabetes + corn oil</th>
<th>Diabetes + lycopene</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovary</td>
<td>12.85 ± 1.16c</td>
<td>22.39 ± 3.51b,c</td>
<td>35.67 ± 4.85a</td>
<td>30.78 ± 4.12b,a</td>
<td>**</td>
</tr>
</tbody>
</table>

Different superscripts in the same row indicate the significant difference. **$p<0.01$.

Figure 3 – Connexin-43 expression in primordial (A–C) and atretic (D–F) follicles. An increase in intensity of connexin-43 expression was seen in the primordial follicles of the control + corn oil (A) and diabetes + lycopene groups (C) compared with the diabetes + corn oil group (B) (arrows). It was also observed that expression decreased in the atretic follicles of the diabetes + corn oil (E) and diabetes + lycopene (F) groups compared with the control + lycopene group (D, arrows). Avidin–Biotin–Peroxidase method. Scale bars: (A–C) 5 μm; (D–F) 20 μm.
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Figure 4 – Connexin-43 expression in the interstitial cells (A and C, arrows) and luteal cells of the corpora lutea (B and D, arrowheads). Connexin-43 expression decreased in the interstitial cells and luteal cells of the corpora lutea in the diabetes + corn oil group (C and D) when compared with the control + corn oil group (A and B). Avidin–Biotin–Peroxidase method. Scale bar: 20 μm.

**Discussion**

There is a widely attested, marked decrease in body weights with the effect of diabetes [24, 28, 40–42]. In the present study, there was no difference in body weight among groups at the end of the study. A comparison of the beginning and ending body weights of the animals showed that the body weights of the control groups increased. Although the body weights of the diabetes groups decreased, it was not statistically significant. The administration of lycopene to the diabetic animals significantly increases body weight, according to some researchers [24, 28, 40, 41], while others have not found any significant variation in body weights [42, 43]. In the present study, we found that lycopene increased the average body weight of the diabetic animals, though this finding was not statistically significant. Based on this data, we can say that weight loss among diabetic animals may have been prevented by the corn oil. On the other hand, different lycopene doses have been administered to diabetic animals and lycopene has decreased high blood glucose, depending on dose levels [24, 28, 40, 41, 43–45]. In our study, there was no statistically significant difference between fasting blood glucose values in the diabetes + corn oil and diabetes + lycopene groups, which is not consistent with the findings of other studies. It is thought that this unexpected result of the study may have been caused by the specific lycopene dosage and the experiment’s duration.

Irregular menstrual cycles have been reported by 27.9% to 33% of diabetic women [46, 47], and oligomenorrhea and amenorrhea are more frequently observed among type 1 diabetic women [48] while late menarche, early menopause, lower fertility and higher rates of stillbirth have also been reported [49]. Diabetic rats display constant diestrus and ovulation rates significantly lower than control groups [50]. In our study, the diabetic rats displayed irregular estrous cycles and many rats remained in the diestrous phase, confirming that diabetes causes disruptions in the estrous cycles of rats and that the lycopene administered to diabetic animals had no role in regulating their cycles.

The ovaries of diabetic animals show significant stromal and follicular degeneration and marked follicular atrophy [51]. In this study, we observed that the diabetes groups had higher vacuole levels in the corpora lutea and increased areas of hydropic degeneration in the interstitial areas, compared to the control groups. These findings confirm that diabetic ovaries show various structural deformities. The present study also determined that lycopene has no effect on the improvement of histopathological deformities in the ovaries.

It has been reported that diabetes causes a decrease in ovarian corpora lutea [18] and primordial, secondary and tertiary follicles and an increase in atretic follicles [33]. In the present study, the number of follicles did not change significantly among groups, while the number of corpora lutea increased significantly in the diabetes +
corn oil group in comparison with the control + corn oil group. We posit that this situation is caused by the disruption of the estrous cycle in diabetic rats, especially the frequency of the diestrous phase.

It has been observed that some follicle diameters have decreased in the ovaries of diabetic animals [4, 18, 33]. In the present study, a decrease was observed in the diameters of the corpora lutea and primordial, primary and secondary follicles in the diabetes + corn oil group in comparison with the control + corn oil group, but this decrease was only statistically significant in corpora lutea. This finding suggests that diabetes may have a negative effect on follicular and corpus luteal development.

Diabetes decreases connexin-43 expression in rat heart [52, 53] and bladder [54]. It has also been reported that oocytes surrounded by cumulus cells obtained from diabetic mice showed a decrease in connexin expression [17]. In the present study, connexin-43 immunoreactivity displayed a decrease in some parameters of the diabetes + corn oil group when compared with the control groups. It has also been reported that high glucose levels suppress gap junction activity through excessive phosphorylation of connexin-43 in vascular smooth muscle cells [55]. Based on these findings, we can postulate that diabetes may suppress the expression of connexin-43 in the ovaries and that oocyte maturation and follicle development may be negatively affected.

Figure 5 – Connexin-43 expression in secondary (A–C) and large antral (D–F) follicles. The intensity of connexin-43 expression was observed more in the secondary and large antral follicles of the control + lycopene (A and D) and diabetes + lycopene groups (C and F) than in the diabetes + corn oil group (B and E, arrows). Avidin–Biotin–Peroxidase method. Scale bars: (A–C) 20 μm; (D–F) 30 μm.
Lycopene is known to have significantly increased connexin-43 expression in cancer cell lines [56, 57], and carotenoids have been identified as increasing connexin-43 mRNA levels [58]. In our study, we observed that the administration of lycopene to diabetic rats increased connexin-43 expression in all parameters except the atretic follicle, with the increase in the primordial, secondary and large antral follicles showing statistical significance. It has also been reported that lycopene decreases lipid peroxidation related to diabetes [40, 42]. Based on this data, we postulate that lycopene administered to diabetic animals may protect cell membranes from oxidative damage and increase connexin-43 transcription and translation.

The ovaries of diabetic animals show a higher level of TUNEL-positive cells than controls, and it has been reported that TRAIL and its receptor KILLER expression display an increase in oocytes surrounded by cumulus cells [18]. Similarly, it has been observed that the rate of apoptotic cells in cumulus cells in diabetic mice increased significantly [59]. In the present study, the diabetes + corn oil group had a higher rate of apoptotic cells in the granulosa layer of large antral follicles and corpora lutea than the control + corn oil group; the increase was statistically significant for the large antral follicles. Based on this data, we have confirmed that diabetes causes an increase in the ovarian apoptosis rate. However, although it was observed that lycopene was effective in decreasing the number of apoptotic cells in the ovaries, this finding was not statistically significant.

Both plasma MDA concentration and erythrocyte lipid peroxidation in diabetes patients increase in comparison with healthy individuals [60, 61]. In studies in which experimental diabetes was induced, it was reported that MDA levels increased in the testes [62], hippocampus, brain cortex, cerebellum and spinal cord [63], liver and kidneys [64]. In the present study, MDA levels increased in ovarian tissues in the diabetes + corn oil group in comparison with the control groups. While lycopene administration in diabetes has been reported to decrease MDA levels in blood samples [65] and the corpora cavernosa [41], some studies have reported that lycopene administration in diabetic animals did not lead to a significant difference in MDA levels in blood [42] or tissue samples [40]. In the present study, the administration of lycopene to diabetic rats decreased MDA levels in the ovaries, though the decrease was not statistically significant.

**Conclusions**

The certain important changes in the ovaries due to diabetes have been determined. Also, it was revealed as different from the previously described researches that lycopene might provide the continuations of follicular growth and oocyte maturation during diabetes by increasing connexin-43 expression in the ovary. On the other hand, we believe that further studies increasing the lycopene dosage and the administration period will demonstrate even more effectively that lycopene has protective effects against diabetes in the ovaries.
Conflict of interests

The authors declare that they have no conflict of interests.

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References


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