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Protective effects of royal jelly against testicular damage in streptozotocin-induced diabetic rats

Turan KARACA¹*, Selim DEMİRTAŞ³, İhsan KARABOĞA¹, Süleyman AYVAZ²
¹Department of Histology and Embryology, Faculty of Medicine, Trakya University, Edirne, Turkey
²Department of Pediatric Surgery, Faculty of Medicine, Trakya University, Edirne, Turkey

Background/aim: To examine the effects of royal jelly (RJ) on testicular damage in streptozotocin (STZ)-induced diabetic rats.

Materials and methods: Eighteen adult Wistar albino rats were used, 6 in each of the 3 treatment groups: Group A: control, Group B: STZ-induced diabetes (untreated), Group C: STZ-induced diabetes plus RJ (400 mg/kg daily for 4 weeks). Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg). Four weeks after the onset of diabetes, testicular apoptotic cell death was examined using immunohistochemical staining for caspase-3 and Ki67 staining for localization of proliferative cells.

Results: Compared with the control, the body and testicular weights of the RJ-treated and untreated diabetic rats were decreased (P < 0.05). The histopathological examination showed a significant increase in degenerative changes in the seminiferous tubules and in spermatogenesis of the STZ-treated rats. In contrast, the RJ treatment group showed near-normal morphology, in addition to an increased intensity of immunohistochemical staining for Ki67-positive cells.

Conclusion: Diabetes induced a significant increase in testicular apoptotic cell death (caspase-3–positive cells). Caspase-3–positive cells were significantly decreased in the STZ plus RJ-treated group compared with the untreated STZ-induced diabetic group (P < 0.05).

Key words: Diabetes, testes, apoptosis, proliferation, royal jelly

1. Introduction

Diabetes is a common health problem and leads to decreased sexual function in both males and females (e.g., sexual disinclination, adverse effect on pregnancy outcomes, infertility, penile erection loss, and reduction of clitoral sensitivity) (1,2). Male reproductive dysfunction is a common complication in diabetics. Diabetic testicular dysfunction may be temporary or persistent depending on the duration and the degree of the disease (3,4). Tissue injury induced by free radicals is thought to be a significant factor in the pathogenesis of diabetes and its complications (5).

Previous studies showed that testicular weight, sperm number, and sperm motility are significantly reduced in diabetic subjects and also showed decreased testosterone levels and vacuolization in spermatogonia and spermatocytes (6,7). Another study pointed to increasing seminiferous tubule wall thickness, germ cell depletion, and Sertoli cell vacuolization in diabetic human testicular biopsies (8) and in diabetic rats (9).

Experimental studies suggested that testicular and erectile dysfunction and spermatogenic disruption in the testis were responsible for the observed low testosterone levels in experimental diabetic animals (9). Control of apoptosis is essential for healthy spermatogenesis in the adult testes (10). Significant induction of apoptotic cell death may occur during nonphysiological stresses, such as diabetes, ischemia, and hyperthermia (11).

Royal jelly (RJ) is a traditional product commonly used to supplement the medical treatment of various diseases. RJ includes many important compounds, such as sugars, free amino acids, fatty acids, minerals (e.g., calcium), and vitamins with biological actions (12). Studies showed that RJ exerts protective effects on different systems and tissues, including antiinflammatory effects and lowering of serum cholesterol and glucose levels (13,14).

The aim of the present study was to investigate the effect of diabetes on rat testicular tissue and to determine the effects of RJ on the diabetic rat using immunohistochemical analyses.
2. Materials and methods

2.1. Animals
Eighteen adult male Wistar albino rats (n = 6 × 3) weighing about 250–300 g were obtained from the Laboratory of Animal Science, Medical School, Trakya University, Edirne, Turkey. These rats received free accessible water ad libitum and a standard laboratory diet under a 12/12 h light/dark cycle and were acclimatized to the experimental conditions for at least 1 week prior to the start of the experiment. The study was approved by the Institutional Animal Ethical Committee of the Trakya University, Edirne, Turkey (Permission number: TUHDYEK-2013/23).

2.2. Induction of diabetes
The rats were injected intraperitoneally (i.p.) with streptozotocin (STZ) 60 mg/kg body weight (bw) (Sigma, USA) after fasting overnight. The STZ was dissolved in 0.1 M citrate buffer (pH 4.5) immediately prior to injection. The control rats received the same volume of intraperitoneal citrate buffer. Three days later, a blood sample was collected from the tail vein, and hyperglycaemia was confirmed by a blood glucose level ≥250 mg/dL. Glucose was determined using a commercial glucometer (IME-DC, Germany).

2.3. Experimental design
The male rats were randomly divided into 3 groups. Group A, control; Group B, untreated diabetic (60 mg/kg bw, STZ i.p.); and Group C, diabetic plus RJ (400 mg/kg/day bw; RJ was given orally). The rats were sacrificed 4 weeks after the induction of diabetes. Royal jelly was purchased from İstanbul.

2.4. Histopathological analysis
Testes tissue samples were obtained from sodium pentobarbital (50 mg/kg, i.p.) anesthetized rats. For light microscopic observation, the testes samples were fixed in 10% buffered formaldehyde, dehydrated in ethanol, and embedded in paraffin. The testes tissues were cut into 5-µm-thick sections. The sections were then deparaffinized with xylene and rehydrated with alcohol and water. The rehydrated sections were stained with hematoxylin and eosin, mounted with entellan, and examined under a microscope (Olympus BX51, Tokyo, Japan).

In the sections, mean seminiferous tubule diameter (MSTD) was calculated in micrometers. Testicular damage and spermatogenesis were evaluated histopathologically using Johnsen’s mean testicular biopsy score (MTBS) criteria (15). A score of 0–10 was given to each tubule according to epithelial maturation (Table 1). For these evaluations, the MSTD and MTBS scores were calculated in 80 tubules of each testis using a Olympus CX31 microscope (Olympus, Japan). At the same time, the testes sections were observed and the mean numbers of spermatogonia, spermatocytes, spermatids, and Sertoli cells in each tubule were calculated. The number of Sertoli cells and germ cells were quantified in 15 tubules/animal (6).

2.5. Immunohistochemistry
Sections of testes were deparaffinized with xylene, followed by antigen retrieval by heating in citrate buffer (10 mM, 20 min). This was followed by endogenous peroxidase blocking in 3% H2O2 for 10 min and incubation with rabbit antimouse Ki67 monoclonal antibody (1:300; Millipore, AB9260) and anti-caspase–3 (1:100; Abcam, Ab4051). After washing the slides with phosphate-buffered saline, the sections were incubated with their related secondary antibodies at room temperature for 1 h, followed by detection with 3-amino-9-ethylcarbazole, a chromogen. The slides were counterstained with hematoxylin and mounted in Faramount aqueous mounting medium. Cellular proliferation was assessed by immunohistochemical detection of the Ki67 nuclear antigen. Cells that stained positive for Ki67 and caspase-3 were counted in each sample. One hundred tubules were counted in each group, and the percentage of positive cells in the tubules was determined.

2.6. Chemical immunoassay of testosterone levels
Testosterone in the serum was measured in triplicate using 100-µL samples of each serum with commercially available chemical immunoassay kits (Siemens, Immulite 2000).

2.7. Statistical analysis
A statistical comparison of differences between groups was performed by means of analysis of variance and Fisher’s multiple comparison tests. Differences were considered significant at P < 0.05. The statistical analyses of histological scores were performed by means of the Kruskal–Wallis test and the Mann–Whitney U test with Bonferroni correction.

Table 1. Testicular biopsy scores (MTBS).

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No cells</td>
</tr>
<tr>
<td>2</td>
<td>Sertoli cells without germ cells</td>
</tr>
<tr>
<td>3</td>
<td>Only spermatogonia</td>
</tr>
<tr>
<td>4</td>
<td>Only a few spermatocytes</td>
</tr>
<tr>
<td>5</td>
<td>Many spermatocytes</td>
</tr>
<tr>
<td>6</td>
<td>Only a few early spermatids</td>
</tr>
<tr>
<td>7</td>
<td>Many early spermatids without differentiation</td>
</tr>
<tr>
<td>8</td>
<td>Few late spermatids</td>
</tr>
<tr>
<td>9</td>
<td>Many late spermatids</td>
</tr>
<tr>
<td>10</td>
<td>Full spermatogenesis</td>
</tr>
</tbody>
</table>
3. Results
The blood testosterone levels and the mean body and testicular weights of the groups are given in Table 2. Compared with the control, body weight was decreased in the untreated diabetic and the RJ-treated diabetic rats (P < 0.05). There was a significant difference between the RJ-treated diabetic group and the untreated diabetic group with respect to the means for body and testicular weights (P < 0.05).

3.1. Histopathological evaluations
The control rats showed histologically healthy seminiferous tubules and spermatogenesis. In contrast, the spermatogenic cells (spermatogonia, spermatocytes, and spermatids) of the diabetic rats exhibited significant damage (Figure 1). In the untreated diabetic group, there was a significant decrease in the Johnsen score. The Johnsen score for the testis was significantly increased in the RJ treatment group compared to that of the untreated diabetic group (Figure 2).

3.2. Testosterone level
Serum testosterone concentrations in the untreated diabetic group were significantly lower compared to those in the control group. They were increased in the RJ-treated group compared to the untreated diabetic group (P < 0.05; Table 2).

Table 2. Body and testis weights and testosterone level in the groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Untreated diabetic</th>
<th>Diabetic + RJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>First body weight (g)</td>
<td>248.4 ± 12.3</td>
<td>293.6 ± 13.5</td>
<td>296.0 ± 10.2</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>298.0</td>
<td>172.5 ± 9.7a</td>
<td>192.0 ± 8.2b</td>
</tr>
<tr>
<td>Testicular weights (g)</td>
<td>1.27 ± 0.4</td>
<td>0.68 ± 0.08a</td>
<td>0.74 ± 0.04b</td>
</tr>
<tr>
<td>Testosterone (ng/mL)</td>
<td>2.39 ± 0.4</td>
<td>0.036 ± 0.01a</td>
<td>0.046 ± 0.01b</td>
</tr>
</tbody>
</table>

* P < 0.05 comparison to control group.

b P < 0.05 comparison to control and untreated diabetic groups.

Figure 1. Testicular histological results. A. Control group: normal seminiferous tubular and germ cell layers. B. Untreated diabetic group: diabetic group showed seminiferous tubular atrophy, a decline of germ cell and number of germ cell layers, and spermatogenous arrest. C. Diabetic + royal jelly group: treatment group showed nearly healthy histological structure of the seminiferous tubules and spermatogenesis. Hematoxylin–eosin, ×400. D. Mean seminiferous tubule diameter for each group. a: P < 0.05 comparison to control; b: P < 0.05 comparison to control and untreated diabetic groups.
3.3. Apoptosis in seminiferous tubules
Few caspase-3–positive cells were observed in the seminiferous epithelium or in the interstitium of the testis sections of the control rats. Compared to the controls, there was a significant increase in the number of caspase-3–positive cells observed in the seminiferous epithelium of the STZ-induced diabetic rats and in the STZ-induced diabetic plus RJ-treated rats. However, caspase-3–positive cells were significantly decreased in the STZ plus RJ-treated group compared with the untreated STZ-induced diabetic group (P < 0.05) (Figure 2).

3.4. Cell proliferation activity in seminiferous tubules
The activity of Ki67 was markedly lower in the STZ-induced diabetic rats compared with the control group (P < 0.05). In the RJ-treated diabetic group, the activity of Ki67 was significantly increased compared with the untreated diabetic group. Ki67-stained cells were mainly localized in the spermatogonial germ cells in seminiferous tubules. Some Ki67-positive cells were observed in the interstitium (Figure 3).

4. Discussion
In the present study, we investigated the protective effects of RJ against STZ-induced diabetic damage of the testes tissue, evaluated histological changes, identified apoptosis by caspase-3 staining in testicular tissue, and examined levels of serum testosterone.

Diabetes is a common health problem that results in decreased sexual function in both females and males. Hyperglycemia can cause testicular dysfunction and reduce fertility in the diabetic rat (16). Both reactive oxygen and nitrogen species are known to alter intracellular macromolecules, such as lipids, proteins, nucleic acids, and carbohydrates, in diabetes-induced hyperglycemia (17). Kanter et al. (2012) reported a reduction in the diameters of seminiferous tubules and spermatogenic cells and damage to the morphology of the epithelium in diabetic rats.

The body weight of the STZ-induced diabetic rats was significantly decreased in comparison to that of the control group in the present study. The RJ treatment increased the body weight, the testis weight, and the testosterone level in the diabetic plus RJ (400 mg/kg) group compared to the untreated diabetic group. However, the increase in body weight was not significant. Previous studies have demonstrated a reduction in body and testis weight and a decrease in the testosterone level in the serum and/or testes tissues, as well as testicular dysfunction, in diabetic male rats (6). Elnagar (2010) suggested that RJ significantly (400 mg/kg) increases the serum testosterone levels, ejaculate volume, seminal fructose, sperm motility, and...
total sperm output in bucks. Other studies confirmed that RJ treatment increases the ejaculate volume and seminal plasma fructose (18,19), in addition to sperm motility (20) and total sperm counts (21). In the present study, observed severe tubular damage was strongly correlated with the decreased level of serum testosterone.

The present results showed that the diameter of the seminiferous tubules and the Johnsen score were increased in the diabetic plus RJ treatment group compared with the untreated diabetic group. These values were significantly decreased in the untreated diabetic group when compared with the control group. Karacal and Aral (2008) suggested that RJ treatment improves abnormal sperm concentrations based on a study of the testes of mice exposed to heat stress. Orally administered RJ was reported to exert estrogenic effects in adult male rats (22). However, another study found that high-dose RJ treatment caused histopathological and sperm morphology changes and a poor sperm count (22).

The expression of the Ki67 protein is associated with cell proliferation. Interphase stage Ki67 protein is mainly found within the nucleus, whereas most of the protein is relocated to the surface of the chromosomes in mitosis (23). In the present study, Ki67-positive cell counts were substantially increased in the spermatogonia and early-stage spermatocytes of the healthy rats compared to those of the untreated diabetic and diabetic plus RJ groups. The numbers of Ki67-positive cells were significantly elevated in the RJ-treated diabetic group compared with the untreated diabetic group. These results demonstrate the beneficial effects of RJ treatment on the diabetic testis during oxidative stress.

Previous work reported declines in germ cell proliferative activity and increases in germ cell apoptosis in testes tissue of STZ-induced diabetic rats (6,24,25). Diabetes is associated with increased oxidative stress, which damages sperm and the nuclear DNA of oocytes. Antioxidant agents have been shown to be helpful in alleviating oxidative damages related to diabetes mellitus. In the present study, significant increases in the number of caspase-3–positive cells were a common feature in all the samples, suggesting continuing oxidative disturbance of the diabetic testis. By the end of the present study, the number of caspase-3–positive cells in the germinal epithelium was significantly increased in the untreated diabetic group. In contrast, the number of caspase-3–positive cells was markedly reduced following the RJ treatment.

In conclusion, the present study confirmed male reproductive irregularities associated with diabetes and the ability of RJ treatment to ameliorate diabetes and testicular dysfunction in diabetic rats. RJ has a significant protective effect on testicular damage in STZ-induced diabetes.
References


