

Exenatide reduces oxidative stress and cell death in testis in iron overload rat model

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Received October 16, 2017; Accepted April 6, 2018

DOI: 10.3892/etm.2018.6795

Abstract. Glucagon-like peptide-1 (GLP-1) has been demonstrated to affect the oxidative stress status in several *in vitro*, *in vivo* and clinical studies. The aim of the present study was to evaluate the effect of a GLP-1 analogue, exenatide, on oxidative stress parameters and apoptotic markers in testicular cells in an iron overload rat model. To obtain this model, the animals were randomly divided into three groups (n=6/group). Rats in the control group received intraperitoneal injections of saline. Intraperitoneal iron dextran (60 mg/kg/day) was given to Group FE for 5 days a week for 4 weeks. The third group (Group Fe +E) was given subcutaneous injections of 10 µg/kg exenatide in two divided doses for 4 weeks in addition to iron dextran. Testes of all rats were immediately removed for immunohistochemical staining and to measure the malondialdehyde level and superoxide dismutase enzyme activity. A significant reduction was observed in caspase-8 and -3 enzyme staining in testicular stromal and endothelial cells in exenatide injected iron overloaded rats when compared with controls. Oxidative stress markers malondialdehyde levels and superoxide dismutase enzyme activities were also significantly lower in exenatide-injected rats when compared with controls. These findings indicate that exenatide may be protective against the harmful effects of iron accumulation in testis. Further studies are required to evaluate how exenatide reduces oxidative stress and cell death in iron overloaded testis tissue.

Introduction

Iron is one of the most important minerals in an organism, and is crucial for electron transport, the synthesis of nucleic acids and proteins, and proliferation and differentiation of cells (1). The majority of iron in the body iron is found in red blood cells, whereas a lower amount is incorporated into the structure of myoglobin and enzymes. Iron absorption and utilization is regulated by a series of processes involving molecules, including ferroportin and hepcidin (2). These processes are fine tuned to maintain the iron balance in an organism. Iron deficiency and iron overload may lead to different problems in the body. Iron overload may lead to iron accumulation in different organs and tissues including the liver, brain and endocrine glands (3). Diseases characterized by iron accumulation, including hereditary hemochromatosis and thalassemia, may damage testicular tissue in humans leading to hypogonadism and decreased fertility (4,5). Iron overload has previously been demonstrated to induce oxidative damage in the testes in a number of animal and human studies (6-9). Iron toxicity results in morphological changes in the seminiferous tubules, epididymes and sertoli cells (10). Iron toxicity may also damage DNA in sperm, which means there is a risk that offspring may inherit gene mutations (11). To date, certain agents, such as α-tocopherol (12) or growth hormone/insulin like growth factor-1 (13) have been evaluated in the prevention of iron induced oxidative stress in the testes.

Glucagon-like peptide-1 (GLP-1) has been demonstrated to affect the oxidative stress status in several *in vitro* (14,15), *in vivo* (16,17) and clinical (18,19) studies. GLP-1 and its agonists are well known to improve glycemic control, decrease food intake, increase insulin release and increase insulin sensitivity which may contribute to reduced oxidative stress, but direct effects on reactive oxygen species (ROS) and antioxidant capacity have also been suggested to serve a role (20).

Exenatide (active ingredient, exendin-4) is a GLP-1 receptor agonist (GLP-1RA) that is used in the treatment of type 2 diabetes (21). The aim of the present study to evaluate the effect of exenatide on oxidative stress parameters and apoptotic markers in the testicular cells of an iron overload rat model.

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Key words: iron, exenatide, caspase-3, caspase-8, superoxide dismutase, malondialdehyde

Materials and methods

Animals and experimental protocol. The present study was carried out in the Physiology Laboratory of the Gazi University Medical Faculty (Ankara, Turkey), and was approved by the Gazi University Ethics Committee of Experimental Animals. All methods were in accordance with the Guide for the Care and Use of Laboratory Animals (22).

In the present study, 18 male Wistar Albino rats weighing between 250 and 300 g and aged 9-10 weeks, raised under the same environmental conditions, were used. The rats were kept at 20-21°C 50±10% humidity, in a 12-h light/dark cycle and had free access to food until 2 h prior to the anesthesia procedure. Rats were randomly divided into the three groups (n=6/group). Rats in the control group (Group C) received intraperitoneal injections of saline. Intraperitoneal iron dextran (Cosmofer®; 50 mg/ml; Assos Pharmaceuticals İlaç, Istanbul, Turkey), was administered at a dose of 60 mg/kg/day to the second group (Group Fe), 5 days a week for 4 weeks. The third group (Group Fe + E) was administered subcutaneous injections of 10 µg/kg exenatide (Byetta®; Eli Lilly and Company, Indianapolis, IN, USA) in two divided doses for 4 weeks in addition to intraperitoneal iron dextran (60 mg/kg/day). All rats were administered intramuscular ketamine hydrochloride (100 mg/kg; Ketalar; Parke-Davis; Pfizer, Inc., New York, NY, USA) and xylazine hydrochloride (Alfazyne, 2%; Ege Vet, Ltd., Izmir, Turkey), and intracardiac blood samples (≤10 ml) were obtained. The rats were sacrificed and all rat testes were immediately removed for immunohistochemical analyses and sera were used for biochemical experiments.

Immunohistochemical evaluation. Tissues were fixed in 10% formaldehyde for 12 h at room temperature. Sections (3-4 µm thick) were cut from the fixed tissue samples, embedded in paraffin blocks and mounted on poly-L-lysine-coated slides (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The sections were left overnight at 45°C. The sections were held for 20 min at 75°C, followed by tap fixation and paraffin extraction. Deparaffinization was performed with a Leica Bond-Max automatic immunohistochemical/*in situ* hybridization stainer (Leica Microsystems GmbH, Wetzlar, Germany). Citrate buffer was applied for antigen retrieval for 30 min at 75°C and washed with bond wash solution (Leica Microsystems GmbH). Sections were blocked with 0.3% hydrogen peroxide for 5 min at room temperature. Sections were then incubated with primary antibodies against caspase-3 (1:400; p11, C-6; cat. no. sc-271759; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and caspase-8 (1:200; D-8; cat. no. sc-5263; Santa Cruz Biotechnology, Inc.) for 15 min at room temperature. The secondary antibodies (Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK) were incubated with cells for 8 min at room temperature. The Bond™ Polymer Refine Detection system (cat. no. DS9800; Leica Biosystems Newcastle Ltd.) was then added as a horseradish peroxidase polymer (a secondary antibody substitute) at room temperature for 8 min at room temperature. DAB (Leica Microsystems GmbH) was applied to the cells for 6 min at room temperature and the marking became visible. Hematoxylin counterstaining was also performed at 6 min at room temperature.

The stained samples were covered with balsam following washing in water and alcohol and cleared in xylene. The density and intensity of cytoplasmic caspase-3 and caspase-8 staining were evaluated in seminiferous tubules, stromal cells and endothelia using a light microscope (Nikon Eclipse E600; Nikon Corporation, Tokyo, Japan) at a magnification of x40 and x100. The density and intensity were assessed by a pathologist. Staining intensity was scored as: 0, no staining; 1, mild and 2, intense. Samples were assessed using the microscope. If >50% of the seminiferous tubules, stromal and endothelial cells on the section were stained.

Perls Prussian Blue (NovaUltra; IHC WORLD, LLC., Woodstock, MD, USA) was applied to visualize the accumulation of iron in the tissue. Briefly, the 4-µm sections were deparaffinized and dehydrated. Solutions of 2% potassium ferrocyanide and 2% hydrochloride solution were mixed at a ratio of 1:1, and 200 µl was applied to each section for 20 min at room temperature. Sections were then washed in tap water for 2-3 min, and fast red was applied for 5 min at room temperature. Sections were subsequently washed again in tap water, alcohol and xylene. A mounting medium was added to the sections, which were then covered with a coverslip. The concentration of iron accumulation in stromal cells was evaluated in hematoxylin and eosin staining as follows: 0, no accumulation; 1, mild accumulation; 2, intensive accumulation.

For hematoxylin and eosin staining, slides were kept in and oven at 72°C for 20 min, deparaffinized in xylene solution and washed with alcohol three times. Sections were then incubated in hematoxylin for 4 min at room temperature, washed and exposed to acid-alcohol and ammonia solutions for a few second. The slides were then incubated in eosin for 6 min at room temperature, and then immersed in a descending alcohol series and xylene. Stained slides were covered with slip, then evaluated with a light microscope at a magnification of x100.

Serum superoxide dismutase (SOD) enzyme activity. Total SOD enzyme activity was measured as previously described (23), based on measuring the absorbance increase at 560 nm caused by nitro blue tetrazolium reduction to diformazan precipitate (NBTH2). One unit of SOD activity was the amount of enzyme that led to 50% inhibition in the reduction rate of NBTH2. Data were expressed in units/ml.

Measurement of serum malondialdehyde (MDA) levels. A thiobarbituric acid (TBA) reactive substances assay was performed, with a minor modification, as previously detailed by Van Ye *et al* (24). The modification was as follows: proteins were precipitated to remove the adverse effects of protein residues on the experiment. The sample was mixed with 20% (w/v) trichloroacetic acid and the precipitate was then centrifuged at room temperature for 10 min at 1,100 x g. The reaction with TBA at 90-100°C was used to determine the MDA level, as MDA or similar substances react with TBA and produce a pink pigment (25) that has an absorption maximum of 532 nm (26). To ensure protein precipitation, the sample is mixed with 4°C 20% (wt/vol) trichloroacetic acid and the precipitate is then centrifuged for 10 min at 1,100 x g and room temperature to form a pellet. An aliquot of the supernatant is then placed into an equal volume of 0.6%

Table I. Caspase-8 staining in testis tissue.

Staining	Group C	Group Fe	Group Fe+E	Kruskal-Wallis P-value
Seminiferous tubules	0.00±0.00	0.00±0.00	0.00±0.00	-
Stromal cells, density	0.00±0.00	2.00±0.00 ^a	1.33±0.21 ^{a,b}	<0.05
Stromal cells, intensity	0.00±0.00	1.67±0.21 ^a	1.50±0.22 ^a	<0.05
Endothelial cells, density	0.00±0.00	1.33±0.21 ^a	0.67±0.21 ^{a,b}	<0.05
Endothelial cells, intensity	0.00±0.00	1.33±0.21 ^a	0.83±0.31 ^a	<0.05

Data are presented as the mean + standard error of the mean (n=6/group). ^aP<0.05 vs. Group C; ^bP<0.05 vs. Group Fe. Group C, control group; Group Fe, iron overloaded group; Group Fe+E, iron overloaded and exenatide-treated group.

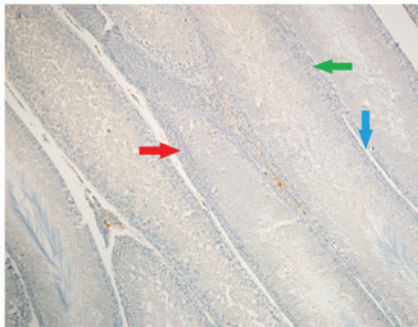


Figure 1. In the control group caspase-8 density staining was not observed in seminiferous tubules (red arrow), stromal cells (green arrow) or endothelium (blue arrow). Original magnification, x100.

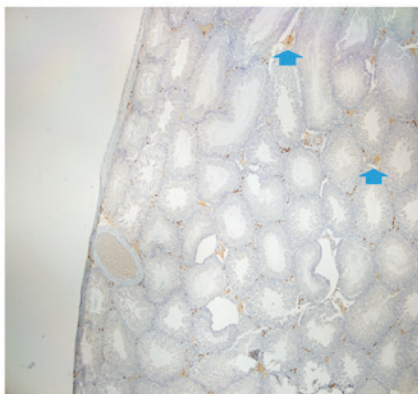


Figure 2. In the iron group, caspase-8 density was <50% (score 1), whereas strong staining intensity (score 2; arrow) was observed in vessel endothelium. Original magnification, x100.

(wt/vol) TBA in a boiling water bath for 30 min. Following cooling, sample and blank absorbance were read at 532 nm and the results were expressed as nM/ml, based on a graph by our group where 1,1,3,3-tetramethoxypropane was used as the MDA standard (27).

Statistical analysis. SPSS (IBM Corp., Armonk, NY, USA; version 20.0) was used for statistical analysis. Data were assessed using Kruskal-Wallis test with a post hoc Bonferroni-adjusted Mann-Whitney U test. Data are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

Caspase-8 staining. Density of stromal cell caspase-8 enzyme activity was significantly different among groups (P<0.05). Stromal cell caspase-8 staining density was significantly higher in Group Fe and Group Fe+E when compared with Group C (both P<0.05) (Table I; Figs. 1-3). In addition, stromal cell caspase-8 staining density was notably higher in Group Fe compared with Group Fe+E (P<0.05) (Table I; Figs. 1-3).

Intensity of caspase-8 staining in stromal cells was significantly different among groups (P<0.05). Stromal cell caspase-8 staining intensity was significantly higher in Group Fe and Group Fe+E when compared with Group C (P<P<0.05) (Table I; Figs. 1-3).

Caspase-8 staining density in endothelial cells was significantly different among groups (P<0.05). Caspase-8 staining density in endothelial cells was significantly higher in Group Fe and Group Fe+E when compared with Group C (both P<0.05). Caspase-8 staining density in endothelial cells was significantly lower in Group Fe+E when compared with Group Fe (P<0.05) (Table I; Figs. 1-3).

Caspase-8 staining intensity in endothelial cells was significantly higher in Group Fe and Group Fe+E when compared with Group C (both P<0.05) (Table I; Figs. 1-3). Caspase-8 enzyme activity in seminiferous tubules was similar among all groups (Table I; Figs. 1-3).

Caspase-3 staining. Density of caspase-3 staining in stromal cells was significantly different among groups (P<0.05). Stromal cell caspase-3 staining density was significantly higher in Group Fe and Group Fe+E when compared with Group C (both P<0.05). Stromal cell caspase-3 staining density was significantly lower in Group Fe+E when compared with Group Fe (P<0.05) (Table II; Figs. 4-6).

Intensity of caspase-3 staining in stromal cells was significantly different among groups (P<0.05). Stromal cell caspase-3 staining intensity was significantly higher in Group Fe and Group Fe+E when compared with Group C (both P<0.05). Stromal cell caspase-3 staining intensity was significantly lower in Group Fe+E when compared with Group Fe (P<0.05) (Table II, Figs. 4, 5A-C, and 6A, B).

Caspase-3 staining density in endothelial cells was significantly different among groups (P<0.05). Caspase-3 staining density in endothelial cells was significantly higher in Group

Table II. Caspase-3 staining in testis tissue.

Staining	Group C	Group Fe	Group Fe+E	Kruskal-Wallis P-value
Seminiferous tubules, density	0.00±0.00	0.00±0.00	0.00±0.00	-
Seminiferous tubules, intensity	0.00±0.00	0.00±0.00	0.00±0.00	-
Stromal cells, density	0.00±0.00	2.00±0.00 ^a	1.50±0.22 ^{a,b}	<0.05
Stromal cells, intensity	0.00±0.00	2.00±0.00 ^a	1.33±0.21 ^{a,b}	<0.05
Endothelial cells, density	0.00±0.00	2.00±0.00 ^a	1.50±0.22 ^{a,b}	<0.05
Endothelial cells, intensity	0.00±0.00	2.00±0.00 ^a	1.33±0.21 ^{a,b}	<0.05

Data are presented as the mean + standard error of the mean (n=6/group). ^aP<0.05 vs. Group C; ^bP<0.05 vs. Group Fe. Group C, control group; Group Fe, iron overloaded group; Group Fe+E, iron overloaded and exenatide-treated group.

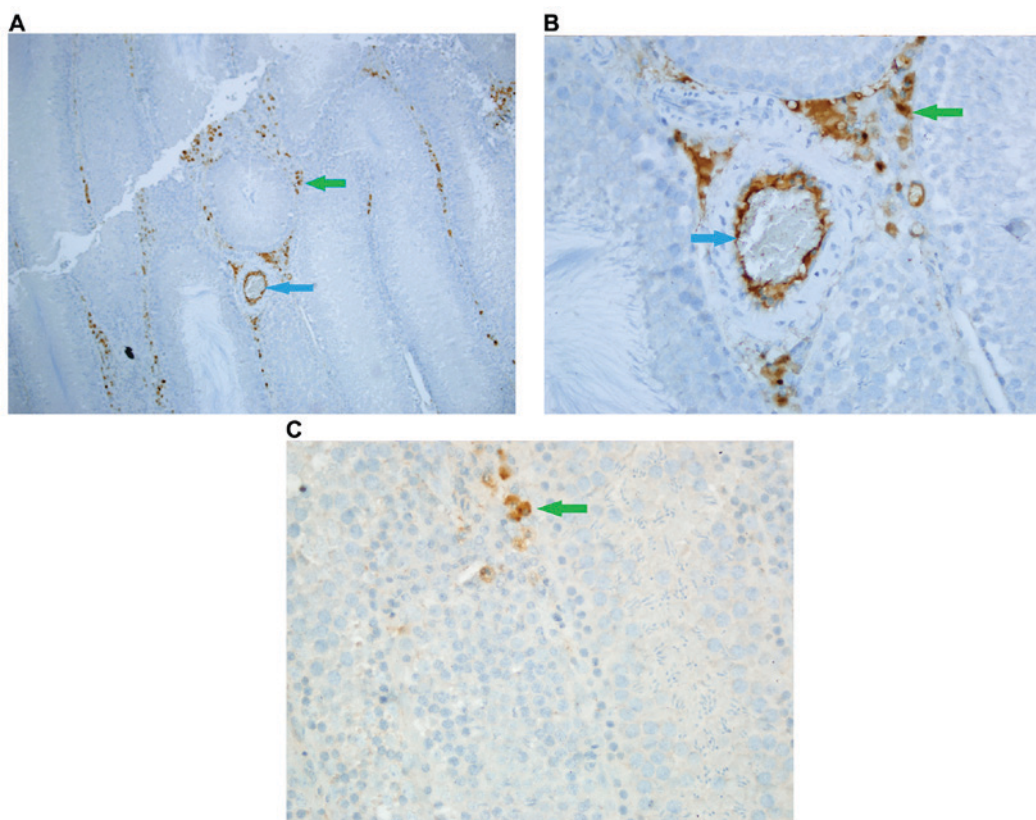


Figure 3. (A) In the group given exenatide, caspase-8 density staining was observed in terms of prevalence and severity in endothelial (blue arrow; score 2) and stromal (green arrow; score 2) cells. Original magnification, x100. (B) Score 2 staining was present in the endothelium (blue arrow) and stromal cells (green arrow). Original magnification, x400. (C) Score 1 staining was observed in stromal cells (green arrow). Original magnification, x400.

Fe and Group Fe+E when compared with Group C (both $P<0.05$). Caspase-3 staining density in endothelial cells was significantly lower in Group Fe+E when compared with Group Fe ($P<0.05$) (Table II; Figs. 4-6).

Caspase-3 staining intensity in endothelial cells was significantly different among groups ($P<0.05$). Caspase-3 staining intensity in endothelial cells was significantly higher in Group Fe and Group Fe+E when compared with Group C (both $P<0.05$). Caspase-3 staining intensity in endothelial cells was significantly lower in Group Fe+E when compared with Group Fe ($P<0.05$) (Table II; Figs. 4-6). Caspase-3 enzyme activity in seminiferous tubules was similar among all groups (Table II; Figs. 4-6).

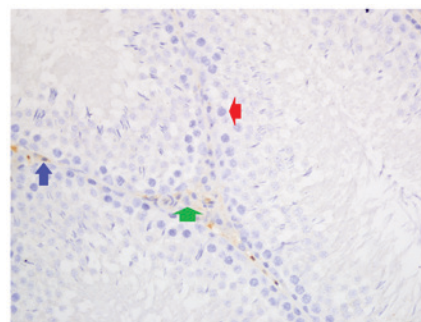


Figure 4. No caspase-3 staining was observed in seminiferous tubules (red arrow), stromal cells (green arrow) or endothelium (blue arrow) in the control group. Original magnification, x400.

Table III. H&E and Iron staining density in testis tissue.

Staining	Group C	Group Fe	Group Fe+E	Kruskal-Wallis P-value
Seminiferous tubules, H&E staining density	0.00±0.00	0.00±0.00	0.00±0.00	-
Stromal cells, iron staining density	0.00±0.00	2.00±0.00 ^a	1.50±0.22 ^{a,b}	<0.05

Data are presented as the mean + standard error of the mean (n=6/group). ^aP<0.05 vs. Group C; ^bP<0.05 vs. Group Fe. H&E, hematoxylin and eosin; Group C, control group; Group Fe, iron overloaded group; Group Fe+E, iron overloaded and exenatide-treated group.

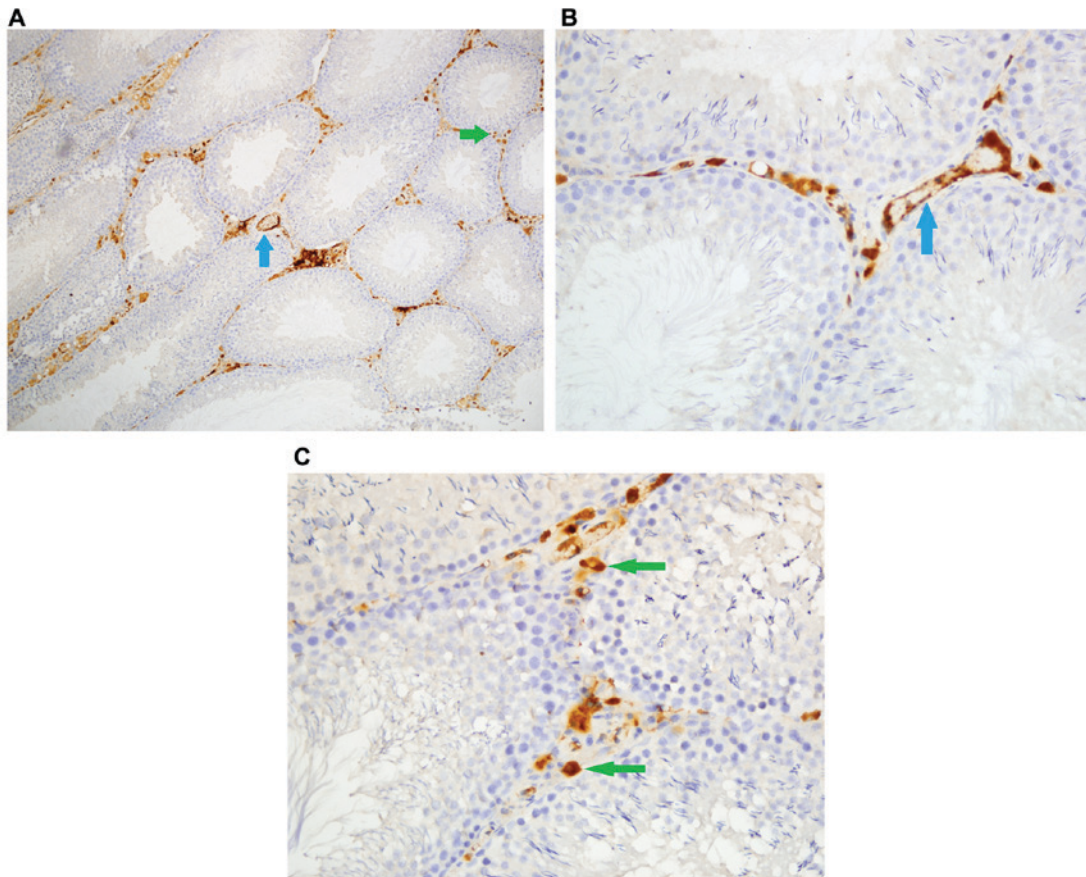


Figure 5. (A) In the iron group, caspase-3 staining was highly prevalent in both prevalence and staining intensity in in endothelial (blue arrow; score 2) and stromal (green arrow; score 2) cells. Original magnification, x100. (B) Staining was also observed in the vascular endothelium (blue arrow; score 2). Original magnification, x400. (C) Stromal cells (green arrows) exhibited strong caspase-3 staining (score 2). Original magnification, x400.

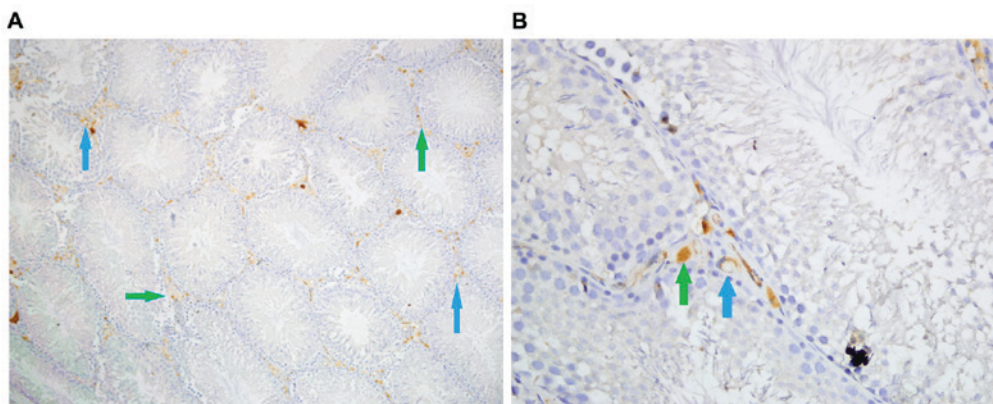


Figure 6. (A) In the group given exenatide, caspase-3 staining was observed in terms of density and intensity in endothelial (blue arrow; score 1) and stromal (green arrow; score 1) cells. Original magnification, x100. (B) Poor staining (score 1) was observed in the endothelium (blue arrow) and stromal cells (green arrow). Original magnification, x400.

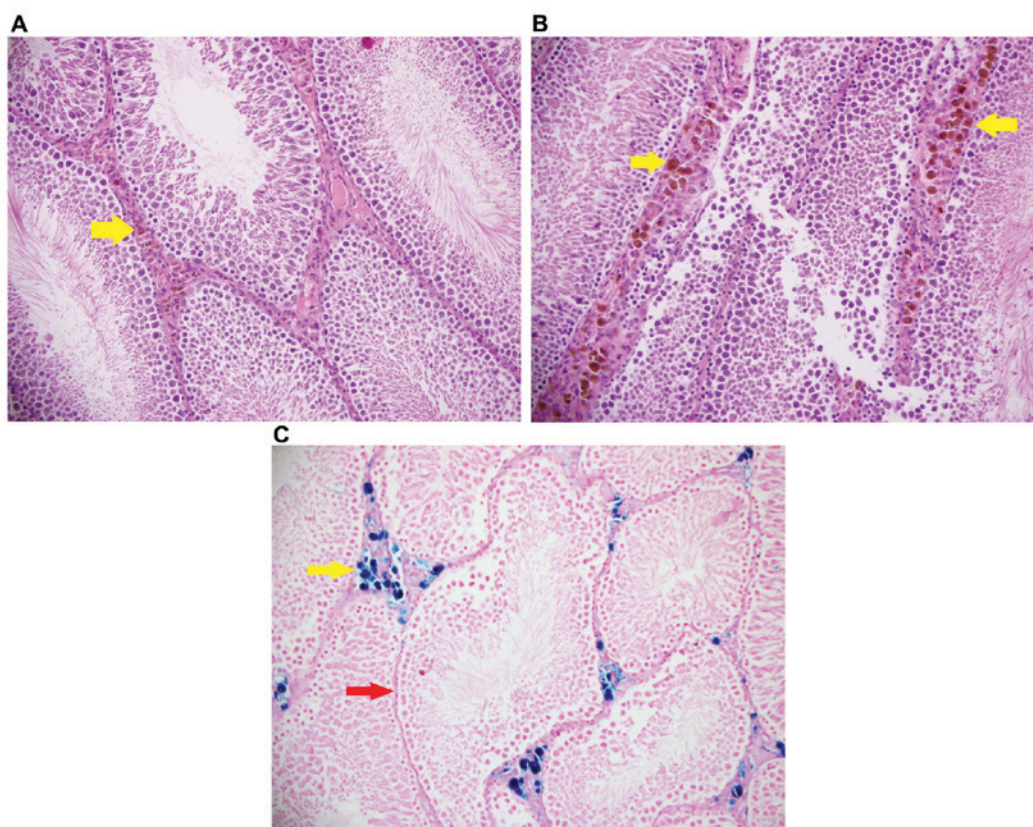


Figure 7. (A) In the stromal cells (yellow arrow), brown colored iron accumulation was observed (score 1). Original magnification, x200. (B) Brown colored iron accumulation is observed in the stromal cells (yellow arrows; score 2). Original magnification, x200. (C) Iron deposits in the histiocytes (red arrow) observed in the stroma were stained blue. There was no staining observed in seminiferous tubules (yellow arrow). Original magnification, x400.

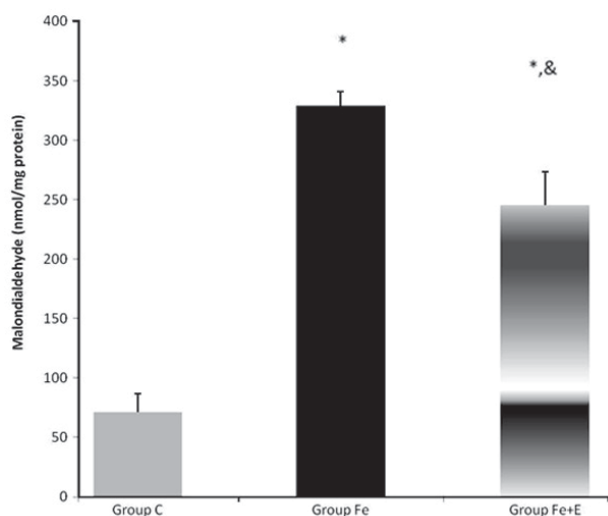


Figure 8. Serum malondialdehyde enzyme activity. Data are presented as the mean + standard error of the mean (n=6/group). *P<0.05 vs. Group C; &P<0.05 vs. Group Fe.

Iron, and hematoxylin and eosin staining. Iron staining density in stromal cells was significantly different among groups (P<0.05). Iron staining intensity in Group Fe and Group Fe+E was significantly higher when compared with Group C (both P<0.05). Iron staining density in stromal cells was significantly lower in Group Fe+E when compared with Group Fe (P<0.05) (Table III; Figs. 4-6). H&E

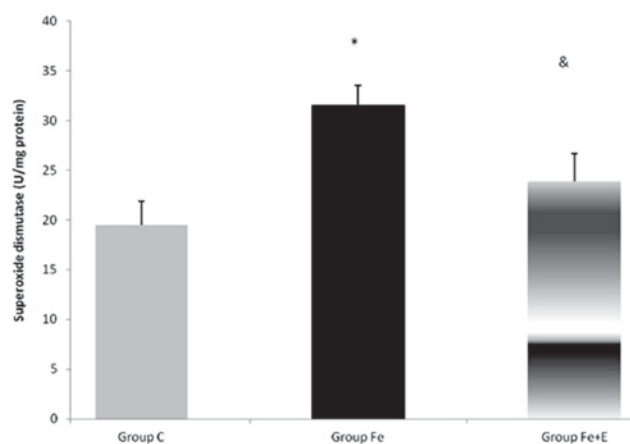


Figure 9. Superoxide dismutase enzyme activity. Data are presented as the mean + standard error of the mean (n=6/group). *P<0.05 vs. Group C; &P<0.05 vs. Group Fe.

staining in seminiferous tubules was not different among groups (Table III; Fig. 7).

Serum MDA and SOD activity. Serum MDA enzyme activity was significantly different among groups (P<0.05). MDA enzyme activity was significantly higher in Group Fe and Group Fe+E when compared with Group C (both P<0.05). MDA enzyme activity was significantly lower in Group Fe+E when compared with Group Fe (P<0.05; Fig. 8).

Serum SOD enzyme activity was significantly different among groups ($P < 0.05$). SOD enzyme activity was significantly higher in Group Fe when compared with Group C ($P < 0.05$). SOD enzyme activity was significantly lower in Group Fe+E when compared with Group Fe ($P < 0.05$; Fig. 9).

Discussion

The beneficial effects of exenatide and exendin-4 include reverting ischemia reperfusion injury (28) and increasing antioxidant enzyme activity (29). Exenatide has also been demonstrated to increase sperm motility and quality, and improve sperm mitochondrial activity and sperm integrity possibly by reducing the expression of proinflammatory cytokines (30).

Excessive production of reactive oxygen species causes testicular damage, which can be reverted by administration of antioxidants (31). Antioxidants have also been demonstrated to enhance testicular function and sperm count in rats (32).

Ahangarpour *et al* (33) created an aging mouse model with D galactose injections and evaluated the effect of exendin-4 on age-related changes on the testes. The aforementioned study demonstrated that the testis weight and volume were decreased as well as the sperm count. Serum luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels were increased in the D-galactose group. Exendin-4 was demonstrated to increase testis volume and weight. Exendin-4 (1 nmol/kg) also decreased LH and FSH levels, and increased the serum testosterone level. Exendin-4 also increased the sperm count in both normal and aging animals. The authors suggested that exendin-4 administration increased testicular weight and volume via decreasing free radicals and increasing antioxidant enzyme activity. In the present study, it was observed that administering exenatide in an iron overload model in rats significantly reduced oxidative stress markers in the testes. This may be associated with the prevention of iron accumulation, as demonstrated via iron staining, and the stimulation of antioxidant enzyme activity by exenatide.

Activation of caspases is a crucial step in apoptosis (34). Caspase-3 is essential for the terminal or execution pathway of apoptosis, which results in dismantling of the cell (35), whereas caspase-8 has a role in the extrinsic pathway of apoptosis (36). In the present study a significant reduction was observed in caspase-8 and -3 enzyme staining in testicular stromal and endothelial cells in exenatide injected iron overloaded rats. This suggests that exenatide may reduce cell death in testicular tissue. This may be related to a preventive effect of exenatide against iron accumulation. Further studies are required to clarify whether exenatide has other contributory effects in the prevention of testicular cell death. Further studies with terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling are planned to evaluate the histopathologic changes in detail.

In conclusion, the present study has demonstrated that exenatide somehow reduces iron accumulation in testis tissue in an iron overload model in rats. Apoptotic markers caspase-3 and -8 are lower in iron overload rats that were administered exenatide at the same time. Oxidative stress markers MDA and SOD enzyme activities were also lower in exenatide-injected rats. These findings suggest that exenatide may be protective

against iron accumulation and its harmful effects on testis tissue. Further studies are required to evaluate how exenatide iron accumulation reduces oxidative stress and cell death in testis.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MA and AKu conceived and designed the study. SY and SMK wrote the manuscript. NS and AKi analyzed hematoxylin and eosin staining and immunohistochemistry results. MK analyzed superoxide dismutase and malondialdehyde activity results. FP and HK reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was performed with the approval of the Gazi University Ethics Committee of Experimental Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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