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Article

Diabetogenic Effects of Ochratoxin A in Female Rats

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Abstract: In this study, diabetogenic effects of long term Ochratoxin A (OTA) administration in rats were investigated and its role in the etiology of diabetes mellitus (DM) was examined utilizing 42 female Wistar rats for these purposes. The rats were divided into 3 different study and control groups according to the duration of the OTA administration. Rats received 45 µg OTA daily in their feed for 6, 9 and 24 weeks study groups. Three control groups without any treatment were also used in the same periods. Blood and pancreatic tissue samples were collected during the necropsy at the end of 6, 9 and 24 weeks. Plasma values of insulin, glucagon and glucose in study and control groups were determined. Pancreatic lesions were evaluated by histopathological examination; then insulin and glucagon expression in these lesions were determined by immunohistochemical methods. Statistically significant decrease in insulin levels in contrast to increases in glucagon and glucose levels in blood were observed. Slight to moderate degeneration in Langerhans islet cells were observed at the histopathological examination in all OTA treated groups. Immunohistochemistry of pancreatic tissue revealed decreased insulin and increased glucagon expression. This study demonstrated that OTA may cause pancreatic damage in Langerhans islet and predispose rats to DM.

Keywords: Ochratoxin A; insulin; glucagon; glucose; rat plasma; pathology; immunohistochemistry

1. Introduction

Ochratoxin A (OTA) is a mycotoxin that naturally occurs as a fungal metabolite and is the most toxic product of *Aspergillus ochraceus* and *Penicillium verrucosum* [1]. Its widespread occurrence in human and animal food and some preliminary cytotoxic, carcinogenic data suggest a possible role for dietary OTA in the development of numerous organ damages and occurrence of different tumors [2-5]. In addition to carcinogenic, mutagenic and cytotoxic effects, OTA also known to evoke a decrease of food intake and body weight gains [6]. OTA has been shown to be nephrotoxic, immunotoxic, and teratogenic to a variety of animal species [7]. It is a ubiquitous mycotoxin produced by these fungi in improperly stored food products. Various commodities including corn, peanuts, wheat, maize, rye, barley, coffee beans, flour, rice, spices, bread and animal feed may contain OTA. The highest amounts of OTA in food of plant origin were found mainly in Eastern Europe [8-12].

Diabetes mellitus (DM) is an autoinflammatory syndrome that is a collection of many disorders such as hyperglycemia, dyslipidemia, insulin resistance, impaired beta-cell functioning, and insulin secretion [13-16]. DM is associated with disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both [17]. It is considered among the major life-threatening diseases worldwide, particularly in developing countries [13-16]. DM is one of the most crippling diseases that man has ever had to deal with, and its prevalence has risen dramatically over the past two decades [18]. Currently, there are over 150 million diabetics worldwide, and this is likely to increase to 300 million or more by the year 2025 due to increased sedentary lifestyle, consumption of energy-rich diet and obesity [19].

There are few studies investigated diabetogenic effects of OTA and the extent of diabetogenic effects of OTA is far beyond clear. Especially increased DM incidence in animals necessitates the clarification of any possible relation between OTA consumption and occurrence of DM. Because of the increased incidence of OTA in contaminated foods and DM in underdeveloped countries, possible relations between OTA and diabetes toughed and this study designed. The aim of this study was to examine OTA toxicity in pancreas and possible diabetogenic effect of this toxication in a rat model.

2. Results

All pancreases had normal gross appearance during necropsy in all groups. Histopathological examinations of rats revealed vacuolization, megalocytosis and karyomegaly in some islets of Langerhans in the OTA-treated groups (OTA6, OTA9 and OTA24) while no pathological lesions were observed in control groups (Ctrl6, Ctrl9 and Ctrl24) (Fig.1a and 1d).

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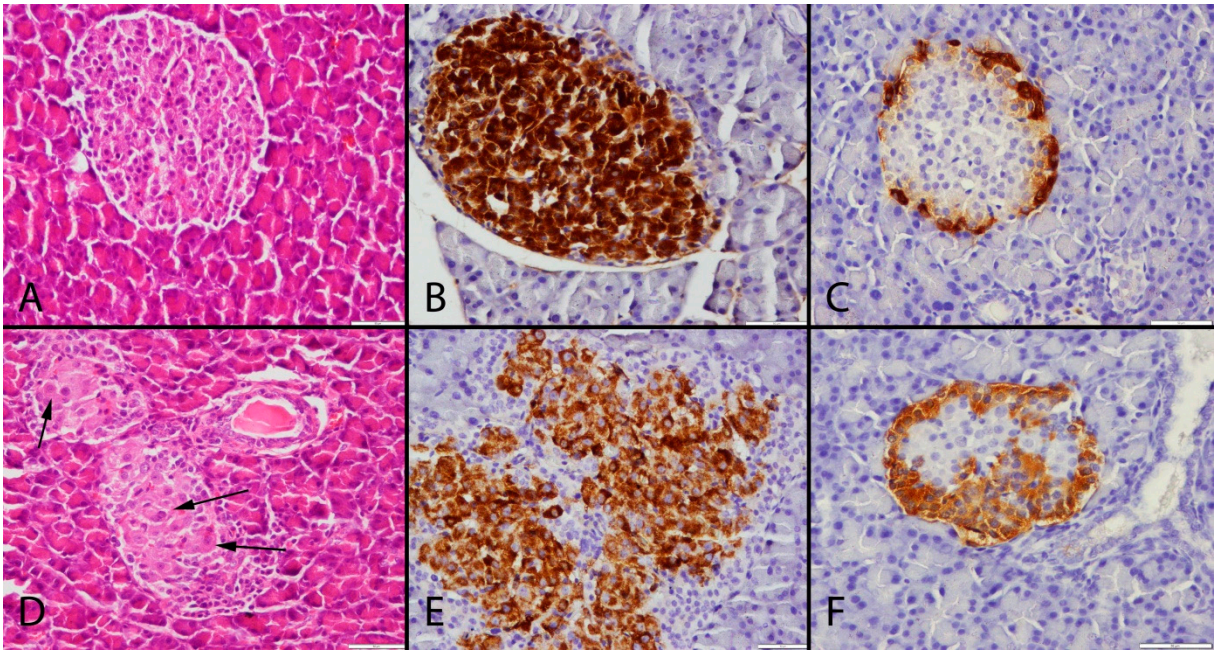

Fig.1: Histopathological and immunohistochemical appearance of the pancreas of 24-week period. (A) normal histology, HE, (B) severe insulin and (C) glucagon immunoexpression in control group, streptavidin biotin peroxidase method, (D) degenerative and karyomegalic cells in Langerhans islet (arrows), HE, (E) decreased insulin (F) and increased glucagon expression, streptavidin biotin peroxidase method, Bar= 50μm.

Marked increases in blood glucose and glucagon levels in contrast to decrease in insulin levels in the study groups compared to the control groups in 6, 9 and 24-week study periods were observed. Statistical analysis results of plasma glucose, insulin and glucagon levels are shown in Fig. 2-3-4. Statistically significant differences were observed in all study periods between study and control groups. This study showed that OTA cause damages in endocrine pancreatic functions even during a 6-week exposure period.

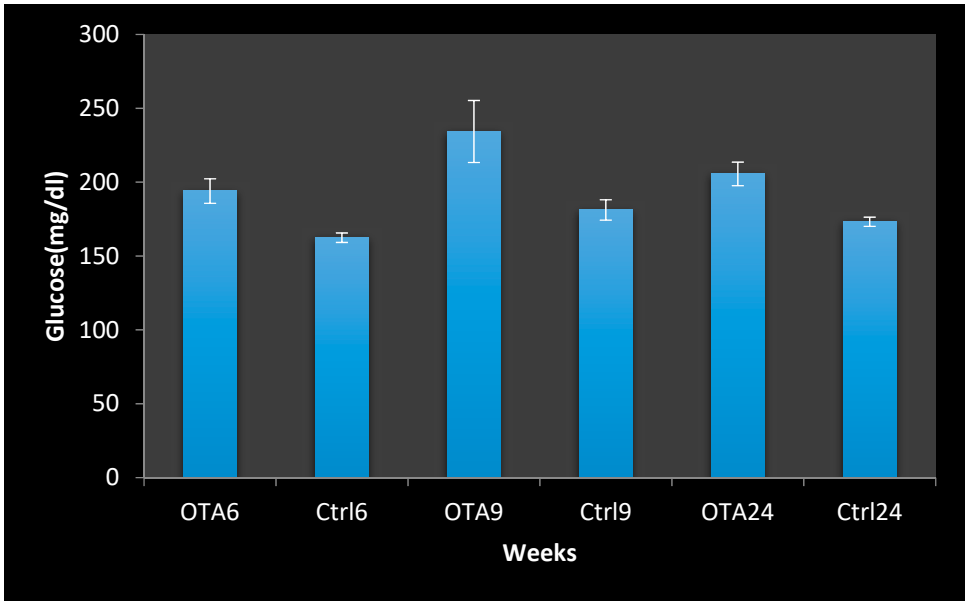

Fig.2: Statistical analysis of results of plasma glucose levels between study and control groups. Statistically significant increase was seen in study group results comparing the same period in control groups (P=0.009 in OTA6 and Ctrl6; P= 0.048 in OTA9 and Ctrl9; P=0.007 in OTA24 and Ctrl24).

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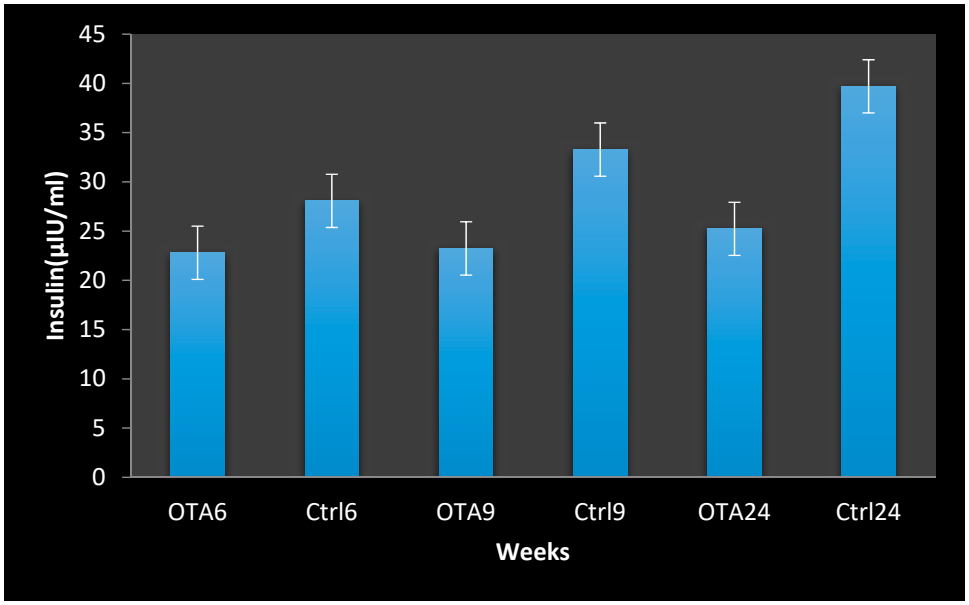

Fig.3: Statistical analysis of results of insulin levels between study and control groups. Statistically significant decrease was seen in study group results comparing the same period in control groups (P=0.048 in OTA6 and Ctrl6; P= 0.010 in OTA9 and Ctrl9; P=0.035 in OTA24 and Ctrl24).

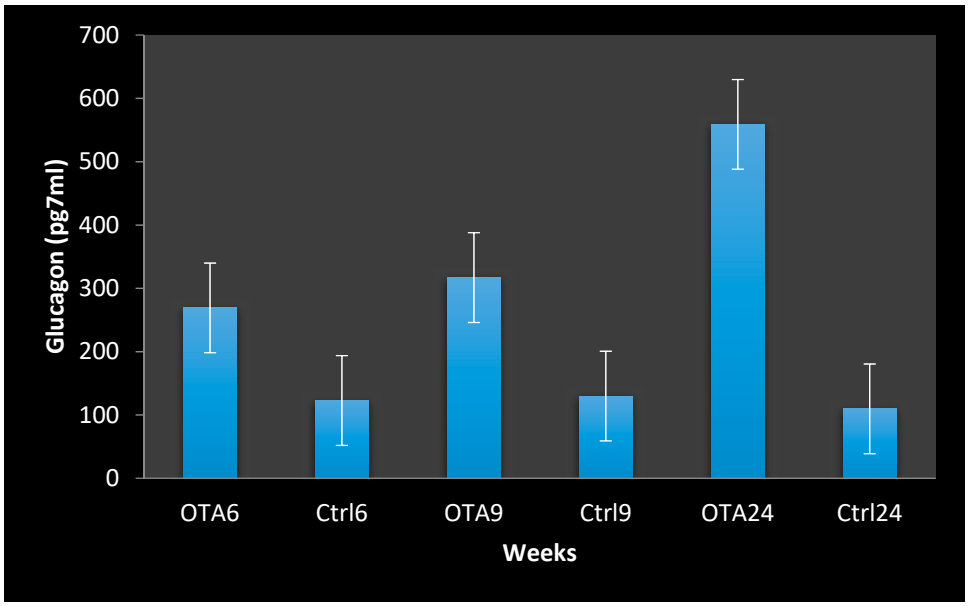

Fig.4: Statistical analysis of results of plasma glucagon levels between study and control groups. Statistically significant increase was seen in study group results comparing the same period in control groups (P=0.007 in OTA6 and Ctrl6; P= 0.035 in OTA9 and Ctrl9; P=0.004 in OTA24 and Ctrl24).

The immunohistochemical examinations showed that the insulin-secreting cells were localized in the central area of the islets of Langerhans. Marked insulin secretion was observed in the control groups, and the OTA treatment caused a slight to moderate decrease in the number of insulin-secreting cells together with the degree of insulin expression in some Langerhans islets. Furthermore, the decrease in insulin expression is augmented with the increased duration of OTA exposure (Fig 1 b and Fig 1 e)

Similar levels of glucagon expression were observed in the control groups during the study, whereas a slight increase occurred in the OTA-treated groups (Fig. 1c and 1f). However, glucagon

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positive cells were decreased with the increased duration of OTA exposure. The results of the statistical analysis results of the numbers of insulin and glucagon expressing cells are shown in Fig 5-6.

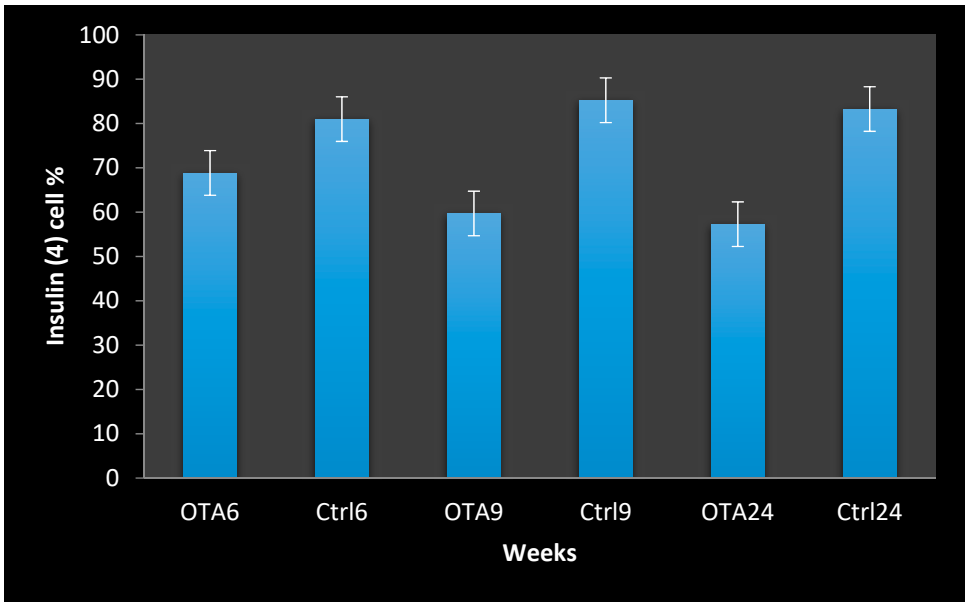

Fig.5: Statistical analysis of results of insulin positive cell percentage between the groups. Statistically significant decrease was seen in study group results comparing the same period control groups (P=0.004 in OTA6 and Ctrl6; P< 0.001 in OTA9 and Ctrl9; P<0.001 in OTA24 and Ctrl24).

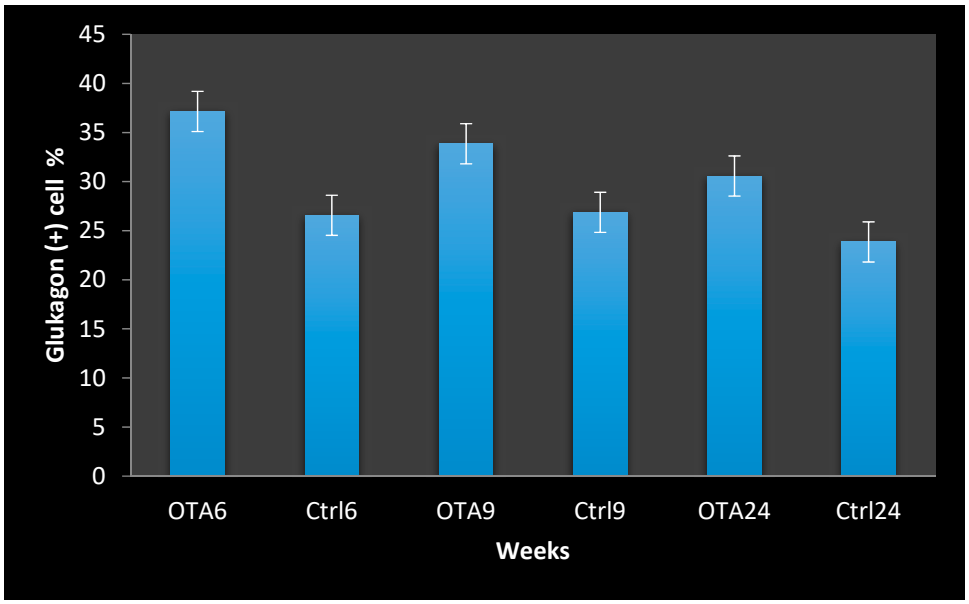

Fig.6: Statistical analysis of results of glucagon positive cell percentage between the groups. Statistically significant increase was seen in study group results comparing same period in control groups (P<0.001 in OTA6 and Ctrl6; P= 0.025 in OTA9 and Ctrl9; P=0.048 in OTA24 and Ctrl24).

3. Discussion

OTA toxication and DM are increasing problems in both human and animal health. Because of the both OTA toxication and DM are related food consumption any possible relation thought to be in these problem. Only small number studies reported OTA can cause endocrine pancreas problems but diabetogenic effects of OTA is not clear. The purpose of this study was to examine OTA toxicity in pancreas and possible diabetogenic effect of this toxication in a rat model.

DM is a common illness with high morbidity and an early mortality rate, causing vascular, renal, retinal, or neuropathic disorders in the long term as well as acute metabolic complications [20]. Diet along with lifestyle modification is believed to play an important role in the management of this disease [21,22). Polydipsia and polyphagia are the most common symptoms of diabetes [17]. OTA is a ubiquitous mycotoxin produced by fungi of improperly stored food products [23]. Because both OTA-related toxicity and DM are food intake related diseases, the idea of any possible relation between these conditions was the main objective of this study.

There is a little knowledge about relationships between mycotoxins and DM [24-26]. These authors reported increase in glucose levels after mycotoxin exposure in rats and chickens. Similar increase in blood glucose levels were observed in OTA treated groups in this study. But there is no report about detailed pathological and histopathological finding in OTA toxication. This is the first study about the effect of OTA on pancreas in a rat model.

Suseela et al., 1986 reported on increase in glucose and decrease in insulin levels after mycotoxin administration in rats [26]. Subramanian et al.1985, also reported similar results in calves after OTA exposure [25]. In a recent study, after 30 days OTA exposure liver and kidney damages were also reported in addition to increase in blood glucose levels [27]. The results of our study are in agreement with previous studies that OTA caused increase in blood glucose and glucagon levels, but decrease in blood insulin levels in groups that are fed with OTA contaminated food in all durations.

It has been reported that OTA exposure results in changes in blood glucose levels in pigs (28-31), rabbits (32) and rats (27,33). Studies with pigs about blood glucose levels after OTA toxication are contradictory (28-31). When rabbits fed with 1 and 2 ppm OTA contaminated food, a decrease in blood glucose level observed after 3 weeks and at week 8, blood glucose level was half of the control group (32). This rather seems to be contradicting and inconclusive results could be related to wide range of OTA doses used in these studies, exposure period as well as species specific effect. On the other hand, there have been two studies reporting that OTA can cause to increase in blood glucose levels in rats. In the first study, OTA (100 µg/rat/day) is given orally by gavage for 8 weeks; it is found that blood glucose level increases while insulin level decreases (33). Similarly, the second study showed that 30 days of OTA treatment results in blood glucose level increase (27). As both studies indicated that OTA is a possible diabetogenic toxin, here in its direct effect to pancreas and pancreatic cells first time revealed as well as its effect to blood glucose, insulin and glucagon levels. In fact, a relationship with OTA and blood glucagon level and a marked glucagon decrease after 6 weeks OTA exposure is also first time reported in this study. In addition to all these new findings, diabetogenic effect of OTA exposure were also first time followed in time dependent manner, from 6 weeks to 24 weeks. At the same time, this is the first study shows that OTA do not only alter blood glucose and insulin levels in rats, it also leads to increase in blood glucagon level and cause pancreatic lesions.

A decrease in insulin secretion or diminished activity of insulin causes a rise in glucagon concentrations and pancreatic expression of the hormone [32]. Although some reports are available about insulin and glucose levels in mycotoxin administered rats, there is no knowledge about glucagon levels in OTA-related rat studies in the literature. This is the first study that glucagon levels are examined in a rat model of OTA toxication together with blood results by ELISA and pancreas results by immunohistochemistry. In agreement with Ozmen et al.'s study [34], the results of this study demonstrated that OTA causes increase in glucagon blood levels and expression in pancreas. Possible cause of this result may be related to decrease insulin secretion and relative increase in glucagon secreted cells. Very sensitive insulin and glucagon ELISA kits were used and assay results supported immunohistochemical findings.

OTA can cause degenerative, necrotic and cancerogenic effects in cells. Karyomegaly is the most common and characteristic pathological findings in liver and kidney cells after OTA exposure [35]. However, there is no knowledge about pancreatic cell reactions after OTA treatment. This study demonstrated that OTA caused degeneration, karyomegaly and megalocytosis in pancreatic cells. Insulin secreted cells were found to be more sensitive to OTA toxication in this study, comparing to glucagon secreted cells.

DM is the most challenging metabolic disorder as it cannot be cured but needs only to be managed. Numerous people and animals suffer from DM throughout the world [36,37]. Numerous studies have been performed about etiology, pathogenesis or treatment of this important and common disease. OTA is the main cause of the numerous organ and tissue damages in exposed human and animals [2-5,27]. The results of this study suggest that OTA at the same time may be a candidate to be involved in diabetogenic agents. In this study, none of our three control group exhibited any abnormality in both plasma levels and pancreatic tissues, for that reason all of the abnormal plasma and tissue results attributed to the OTA toxicity.

The main potential flaw of the study was small number of rats included the study. But because of the long duration and 6 groups the number of the rats reduced. The other limitation may be only immunohistochemistry used for molecular analysis. Further and expanded research may be planned for better evaluation. Because of this study is first extensive study, it will be preliminary study for further studies.

The results of this study demonstrated that OTA can cause toxic effects in endocrine pancreas in a rat model. The toxicity can be seen even in 6-week period exposure; however longer term OTA exposure may be more effective. Our results demonstrated that OTA damage in exocrine pancreas may be an etiologic factor for DM in human and animals. In addition, our study suggests a possible relation between OTA exposure period and severity of pancreatic damage. This is the first detailed research about diabetogenic effect. Further and expanded studies are needed for the clarification of the relation between OTA and DM.

4. Material and Methods

4.1 Animals, Housing Conditions and Experimental Procedure

In this study, pancreas and plasma samples were collected from our previous study that approved by Akdeniz University Local Animal Research Committee (No:2011.12.02). The study was performed in accordance with the National Institutes of Health Guidelines for the Care and Handling of Animals. Rats were kept in the experimental animal laboratory of Akdeniz University. This laboratory has an international accreditation certificate attesting suitable conditions for such work.

Female Wistar rats (16 weeks old), from the Akdeniz University Animal Experiment Unit, were housed on sawdust bedding in groups of 4–5 in polycarbonate cages with stainless steel covers. Room temperature was maintained at 22 ± 2 °C with a relative humidity of 55% ± 10% and a day/night cycle of 12 h. Rats were fed with diet (20 g/rat/day) in stainless steel containers, and water was available *ad libitum*. The physical condition of each rat was assessed daily for any obvious clinical signs.

Forty-two rats, weighing 125-150 g at the start of the experiment, were randomly allocated into six groups (n=7). Experimental groups (OTA6, 9 and 24) were fed with OTA-contaminated diet for 6, 9 and 24 weeks, respectively. Control groups (Ctrl6, 9 and 24) were fed with standardized powdered rat diet for the same period of time. The feeding methods and OTA doses were essentially the same as Mor et al. (2014). Rats were fed with 3 mg OTA kg^{-1} food and the initial OTA dose of rats was approximately 45 µg OTA/rat/day.

At the end of study periods, blood (1-1.5 ml) was collected from a tail vein under ether anesthesia, centrifuged immediately and the plasma stored -40 °C. After blood collection, rats were euthanatized to collect tissue samples.

4.2 Plasma insulin, glucagon and glucose determination

Plasma glucose levels were analyzed by using an Autoanalyzer (Gesan chem 200 Gesan Production srl, Campobello, Italy). Plasma insulin and glucagon levels were analyzed by using an ultrasensitive rat insulin ELISA kit (Cat. No: Rab 3050; Sigma Aldich Chemie GmBH, St.Louis, USA) and glucagon ELISA kit (Cat. No: Rab 0202; Sigma Aldich Chemie GmBH, St.Louis, USA) with a multiplate ELISA reader (EPOCH microplate reader; Bio-Tek, Inc., Winooski, Vt). Results are expressed as mg/dl for glucose, µIU/mL for insulin and pg/mL glucagon.

The minumum detectable amount (MDA) for rat insulin was 5 μIU/mL. Recovery was performed by spiking plasma samples with rat insulin (10-50 μIU/mL) and the recovery was 67-129 % with an average of 94.68 %.

The minumum detectable amount (MDA) of glucagon was 6.37 pg/mL. The recovery of glucagon spiked to levels throughout the range of the assay in rat plasma was evaluated. Mean recovery is 93 % and range is 86-101%.

For plasma insulin and glucagon levels methods were used according to the manufacturer's instructions.

4.3 Histological Examination

During the necropsy, pancreatic tissue samples were collected and fixed in 10% buffered formalin. After routine processing, tissues were embedded in paraffin and sectioned to 5-μm thickness. Tissue sections were stained with hematoxylin-eosin and examined microscopically.

Pancreas samples were then immunostained with insulin antiserum (Anti-insulin + Proinsulin antibody, [D6D4] Abcam (ab8304), 1/100 dilution; Abcam, Cambridge, United Kingdom) and glucagon antiserum (antiglucagon antibody, Abcam (ab8055), 1/100 dilution; Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions. All the slides were analyzed for hormone positivity and a semiquantitative analysis was carried out as detailed later. The overall number of positive cells in 1 high-power field (40X), as well as the number of cells per islet that were positive for each hormone, were noted and compared with normal pancreatic tissue counts. An attempt was made to quantify the percentage positivity of each hormone-producing cell in each of the islets. All islets in at least 5 high-power fields were selected, the total number of nuclei in each islet was counted, and the average number of nuclei/islet was calculated. The average percentage of cells positive for each hormone was then calculated for the islets. Morphometric evaluation was made by using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan).

4.4 Statistical analysis

One-way ANOVA analysis of variance tests was used to determine whether there were any significant differences between the groups with regard to their blood glucose, glucagon and insulin values. In the determination of differences between groups, the Bonferroni Dunn test was used. Calculations were made using the MiniTab (2011) program pack.

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