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The distribution of activating transcription factor 6 (ATF6) and nerve growth factor (NGF) in the duodenum tissue of diabetic and non-diabetic rats

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ABSTRACT

Objective: This study was conducted with the purpose of investigating the distribution of the Activating Transcription Factor 6 (ATF6) and the Nerve Growth Factor (NGF) in the duodenum tissue of diabetic and non-diabetic rats.

Material and Method: Eighteen female *Sprague dawley* rats were randomly divided into three groups as the control, sham and diabetes groups. Routine histological and immunohistochemical methods were applied on the duodenum tissues collected at the end of the study.

Results: It was determined that the villus length measurements showed a statistically significant difference between the control and diabetes groups. There was NGF immunoreactivity which was moderate and diffuse cytoplasmic in the villus intestinalis and muscularis layer in all groups, weak in the crypts and glands in the control and sham groups and moderate and diffuse cytoplasmic in the diabetes group. ATF6 immunoreactivity was determined moderate in the villus intestinalis, crypts, glands and muscularis layer in the control and sham groups and strong diffuse cytoplasmic in the diabetes group. **Conclusion:** It was determined that both NGF and ATF6 immunoreactivity increased in the duodenum tissue of the rats on which diabetes was induced experimentally.

Keywords: *ATF6, Diabetes, Duodenum, NGF*

INTRODUCTION

In diabetes, which is a chronic metabolic disease, lack of insulin or problems in insulin utilization occur, and the organism cannot sufficiently utilize carbohydrates, fats and proteins (Irak et al., 2018). As a result of long-term continuation of diabetes, complications such as diabetic diarrhea or constipation, urinary incontinence and sexual dysfunctions in men and women occur (Oztekin Kazancıbaşı, 2014). Patients experiencing diabetes-related intestinal problems encounter issues such as constipation (affecting about 60% of patients) as a result of reduced intestinal motility, diarrhea as a result of an increase in the bacteria in the small intestines or fecal incontinence. The development mechanism of these intestinal diseases usually resembles the upper GIS (gastrointestinal system) involvement of diabetes. Other causes of diarrhea include insufficient pancreatic enzymes, excess fat in the excrement, bile salt absorption problems and drugs (Ohlsson et al., 2006; Gangula et al., 2007; Lee and Lee, 2013). Small intestines; It is one of the parts of the digestive system that has an important role in meeting the energy and building block needs of living things. For this reason, changes that may occur in the morphological structure of the small intestine also affect its functions negatively (Koca,

1993; Koca, 1996). The mucus in the content of the intestine provides a natural defense line by preventing pathogenic bacteria from clinging to the intestinal epithelium (Allen et al., 1993; Gu et al., 2002). The Activating Transcription Factor 6 **(**ATF6) is a transcription factor related to the membrane of the endoplasmic reticulum (ER) (Yoshida et al., 1998). As a result of induction of ER stress, ATF6 is transferred from ER to the Golgi apparatus, and here, it is separated by site-1 and site-2 proteases (Haze et al., 1999; Ye et al., 2000; Chen et al., 2002). The structure of ER is highly developed in pancreatic β cells. It was reported that this is associated with their excessive participation in insulin secretion. For this reason, proper functioning of ER is an important factor for the survival of β cells, and it was reported that insulin secretion is directly influenced in the case of any disruption in ER functions (Harding and Ron, 2002; Oyadomari et al., 2002). The Nerve Growth Factor (NGF) is one of the first discovered members of the neurotrophin family (Bayar et al., 2010). NGF has functions such as neuroblast multiplication, dorsal root ganglion maturation and axon growth. Additionally, it is a trophic protein which has a message recipient role between tissue showing a reaction to peripheral stimulation and the nerves stimulating this tissue (Friess et al., 1999; Faydacı et al., 2004; Berker, 2015). In islet cell cultures performed in diabetes, the insulin secretion in β cells decreased by 80%, while NGF and glucose secretion increased 10-fold. The increase in the synthesis of NGF may be an endogenous reaction for the survival of cells and prevention of diabetes formation (Larrıeta et al., 2006). Exogenous neurotrophic factors may induce intestinal myoelectric activities in rats, and NGF expression increases in invasive and acute watery diarrhea (Chai et al., 2003; Sarker et al., 2010).

This study was conducted with the purpose of investigating the distribution of the Activating Transcription Factor 6 (ATF6) and the Nerve Growth Factor (NGF) in the duodenum tissue of diabetic and non-diabetic rats.

MATERIALS and METHODS

Material

A total of 18 female *Sprague-Dawley* rats were used in the study. The rats were kept at 22±2°C, in standard cages under 12-h light-12-h dark conditions and fed *ad libitum* using standard rodent chow and tap water. The rats were divided into 3 groups including 6 animals in each group.

Method

The rats were randomly divided into three groups:

1. Control Group (n=6): No intervention was made on the rats in this group.
2. Sham Group (n=6): Sodium citrate solution was applied to the rats in this group by 50 mg/kg intraperitoneally (i.p.).
3. Diabetes Group (n=6): Streptozotocin (STZ) (50 ml citric acid + 40 ml disodium hydrogen was dissolved in a phosphate buffer solution, and the pH was adjusted as 4.5) was applied by 50 mg/kg i.p. as a single dose to the rats in this group.

Experimental Induction of Diabetes

Blood was collected from the tail vein of the rats after 8 hours of fasting, measured by a glucometer (On Call Plus), and the blood glucose levels were determined (day 1). On the same day, STZ application was made. Three days later, fasting blood sugar values were measured by collecting blood from the rats that were kept fasting again for 8 hours. The rats with a fasting blood glucose value of 250 mg/dL were accepted as having type I diabetes. Likewise, also at the end of the study (day 17), blood was collected from the tail vein of the rats after 8 hours of fasting, and the blood glucose levels were determined. At the end of the study, the rats were sacrificed under deep anesthesia, and their duodenum tissues were collected.

Histological Examinations

The collected duodenum tissue samples were fixed in a 10% formaldehyde solution for histological and immunohistochemical examinations. They were embedded in paraffin blocks by passing through graded alcohols, methyl benzoate and benzol. The 5-μ cross-sections obtained from the paraffin blocks were subjected to Crossman's triple staining, Hematoxylin-Eosin staining (Luna, 1968), and to determine the goblet cells that secrete neutral mucin in the intestines, PAS (Periodic acid - Schiff) staining (Bancroft et al., 1994).

Statistical Analyses

The SPSS (20.0) package software was used to analyze the data obtained in the study. In the analysis of villus lengths and goblet cell counts, one-way ANOVA was used to determine the differences between the groups. To compare the

significant differences between the groups, Duncan's test was used.

Immunohistochemical Examinations

The cross-sections taken on slides coated with chrome alum gelatin were subjected to the indirect method of Streptavidin-biotin peroxidase (Hsu et al., 1981). After deparaffination and rehydration processes, the cross-sections were shaken in PBS (0.1 M, pH 7.2), and to prevent endogenous peroxidase activity, they were incubated for 15 min. in 3% H_2O_2 prepared in 0.1 M PBS. After washing with PBS, to reveal antigens, heat was applied in a microwave oven at the highest power for 10 min. in a citrate buffer solution. They were then washed again with PBS. To prevent non-specific bonding, they were incubated for 10 min. with a Large Volume Ultra V Block solution. Afterwards, at room temperature, for 1 hour and in a humid environment, the anti-ATF6 (Bioss-Bs1634R) (1/200 dilution) and anti-NGF (Abcam-AB6198) (1/600 dilution) primary antibodies were applied on the cross-sections. The cross-sections were washed with PBS, Biotinylated Goat Anti B Polyvalent solution which corresponded to the species where the primary antibody was produced from was dripped onto the cross-sections and incubated at room temperature for 30 min. After this, the cross-sections were washed with PBS, Streptavidin Peroxidase solution was dripped, and they were incubated at room temperature for 30 min. After washing with PBS again, for chromogen application, DAB-H_2O_2 (Diaminobenzidine hydrogen peroxide) (Shu et al., 1988) solution was added, and modified Gill III hematoxylin solution was used for counterstaining. For the purpose of determining whether the ATF6 and NGF primary antibody immunoreactivity was specific or not, all procedures were exactly applied without adding primary antibodies to the cross-sections (negative control). Immunohistochemical assessment was made based on the staining properties of the target cells and the staining intensity in these cells. The assessment was made by two independent observers by assigning values from 0 to 3 for no staining (0), weak staining (1), moderate staining (2) and strong staining (3) (Zhu, 1989; Seidal et al., 2001).

The cross-sections prepared for histological and immunohistochemical analyses were assessed and photographed under a light microscope (Olympus BX51; Olympus Optical Co. Osaka, Japan). In the duodenum tissue of all groups, villus length measurements and goblet cell counts were made by using the image-j (vI. 50i) software. Villus length measurements on the duodenum tissue were made from a total of 33 areas on 6 different cross-sections in each group. Goblet cell counts were made in each group on 6 areas on 6 different cross-sections (Akgül et al., 2015).

RESULTS

Blood Glucose Results

The blood glucose values of the rats were statistically analyzed, and the results are presented in Table 1. It was determined that the blood glucose results of the diabetes group were significantly increased in comparison to the control group on the days 3 and 17 of the study ($p<0.05$).

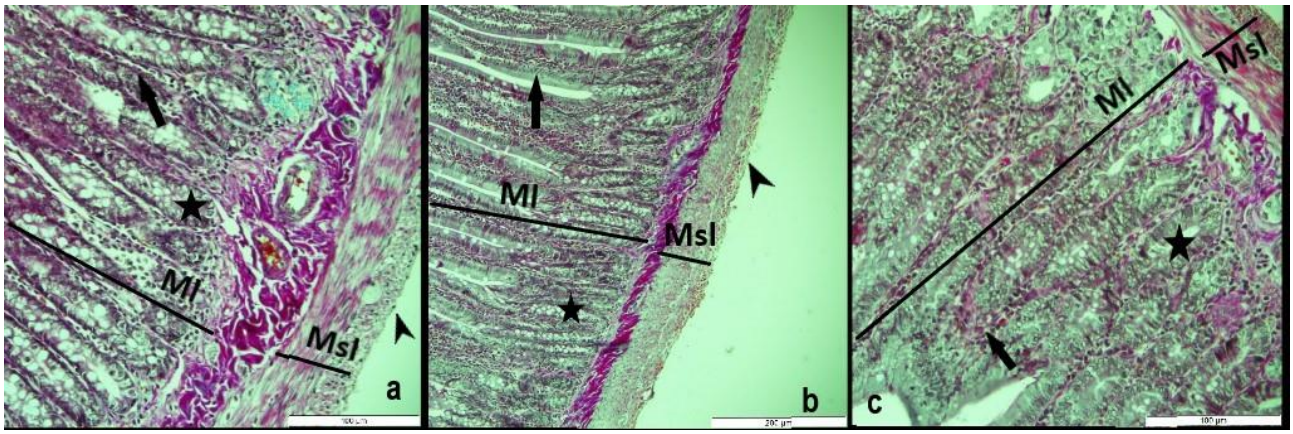

Figure 1. Rat duodenum tissue. a: Control group, b: Sham group, c: Diabetes group. Ml: Mucosa layer, Msl: Muscularis layer, Arrowhead: Serosa layer. Arrow: Villi intestinalis, Star: Crypts, Triple staining.

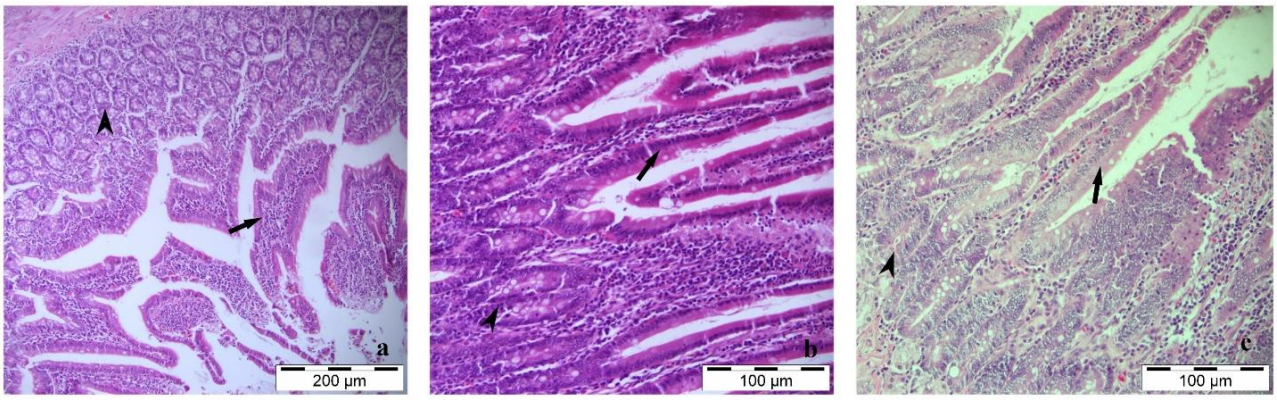

Figure 2. Rat duodenum tissue. a: Control group, b: Sham group, c: Diabetes group. Arrow: Villi intestinalis, Arrowhead: Crypts. H-E staining

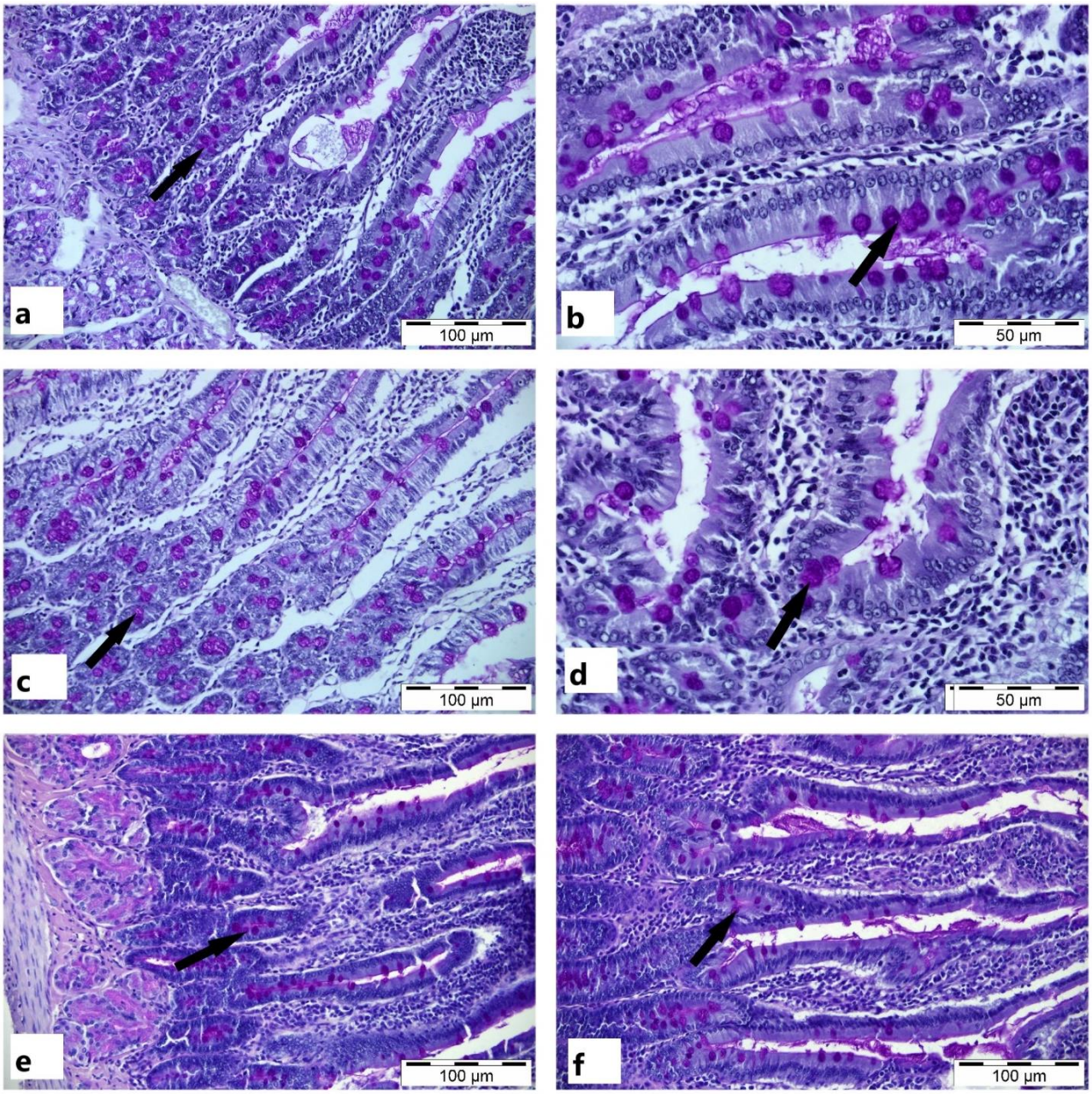

Figure 3. Rat duodenum tissue. a, b: Control group, c, d: Sham group, e, f: Diabetes group. Arrow: Goblet cells. PAS staining.

Table 1. Statistical analysis of blood glucose results.

Days	Control (mg/dl)	Sham (mg/dl)	Diabetes (mg/dl)	p
1st day	88± 3.68 ^{a}	101.67± 0.67^{ab}	95.50± 3.26^{b}	0.014
3rd day	83.17± 1.38^{a}	78± 1.93^{a}	414.67±40.12^{b}	0.000
17th day	95± 1.37^{a}	95± 4.60^{a}	270.67±19.38^{b}	0.000

$^{a, b}$: The difference between the mean values is statistically significant shown with different letters in the same row ($p<0.05$).

Table 2: Histomorphometric results obtained from the duodenum tissues of all groups.

Group	Villus length (µm)	Goblet cells/100 (µm)
Control (n=6)	197.57^{a} ± 4.90	161.50 ± 15.36
Sham (n=6)	204.59^{ab} ± 5.42	161.17 ± 18.45
Diabetes (n=6)	218.58^{b} ± 6.13	165.67 ± 16.83
p	0.02	0.978

$^{a, b, c}$: The difference between the groups is statistically significant shown with different letters in the same row ($p<0.05$).

Histomorphometric Results

There was a significant difference between the control and diabetes groups in terms of the villus lengths ($p<0.05$). There was no significant difference among the groups in terms of the goblet cell counts ($p>0.05$) (Table 2).

Histological Results

The duodenum tissue in all groups consisted of the mucosa, submucosa, muscularis and serosa layers. The villi intestinalis in the lamina epithelialis were almost the same size, and the epithelium was not interrupted. The crypts in the lamina propria, glands in the submucosa and tunica muscularis layer had a normal histological structure (Figure 1-2). The goblet cells that were localized on the epithelium layer covering the surfaces of the villi intestinalis and crypts showed a PAS-positive reaction (Figure 3).

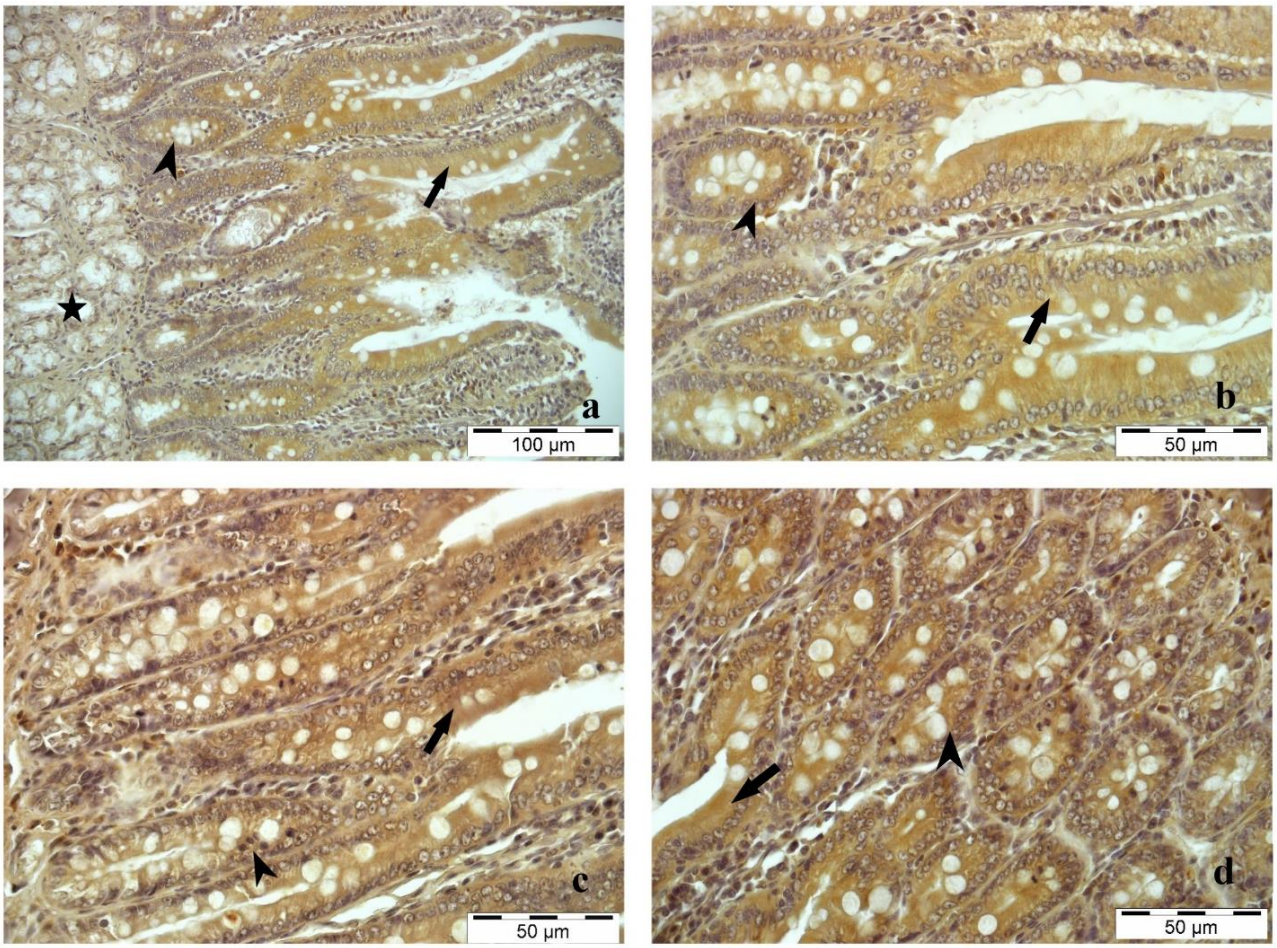

Figure 4. ATF-6 immunoreactivity in the rat duodenum tissue. a, b: Control group, c, d: Diabetes group. Arrow: Villi intestinalis, Arrowhead: Crypts, Star: Glands.

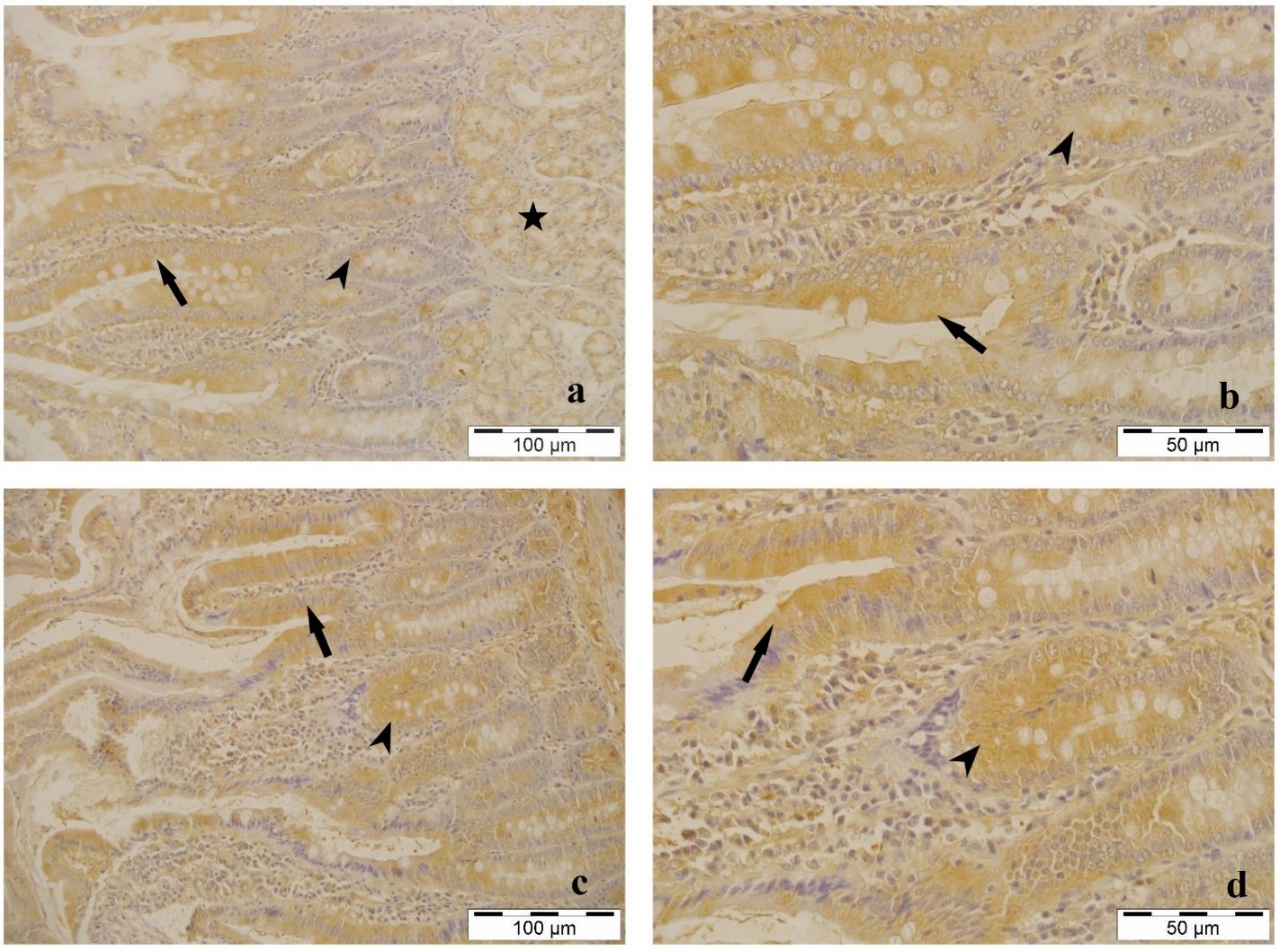

Figure 5. NGF immunoreactivity in the rat duodenum tissue. a, b: Control group, c, d: Diabetes group. Arrow: Villi intestinalis, Arrowhead: Crypts, Star: Glands.

Immunohistochemical Results

There was ATF6 immunoreactivity in the duodenum tissues of the rats in all groups. The ATF6 immunoreactivity was moderate in the villi intestinalis, crypts, glands and muscularis layer in the control and sham groups, while it was strong diffuse cytoplasmic in the diabetes group (Figure 4). There was NGF immunoreactivity in the duodenum tissues of the rats in all groups. The NGF immunoreactivity was moderate and diffuse cytoplasmic in the villi intestinalis and muscularis layer in the duodenum tissue of the rats in all groups, weak in the crypts and glands in the control and sham groups, and moderate and diffuse cytoplasmic in the diabetes group (Figure 5).

DISCUSSION

While the symptoms in the problems of the gastrointestinal system observed in diabetes are not specific, they may sometimes be strong enough to reduce the quality of life of patients. The pathophysiological mechanisms of symptoms are highly complicated, whereas it is believed that multiple factors could be effective. Similarly, it was stated that sensorimotor dysfunctions that are frequently seen in diabetes patients may be closely related to diabetic autonomic neuropathy (DAN) (Frokjaer et al., 2007; Brock et al., 2013; Yarandi and Srinivasan, 2014). Moreover, it was reported that enteric nervous system (ENS) disorders have started to be included in diabetic autonomic neuropathy (Yarandi and Srinivasan, 2014). Intestinal mucins are negatively charged and large-structured glycoproteins. They are synthesized and stored by goblet cells (Kemper and Specian, 1991). Goblet cells firstly form in the crypts, and then, they mature and migrate to the villi. It was reported that goblet cell count and volume are associated with villus size, diet, microbial flora and environmental factors (Miller et al., 1981; Yunus et al., 2005; Brown et al., 2006; Gersemann et al., 2009). Goblet cells in intestinal villi decreased in rats on which experimental ischemia was induced (Dağ et al., 2010). It was also determined that goblet cell accumulation took place on the tops of villi after superficial ischemia-reperfusion (IR) damage in the

small intestines, and this could be effective in the repair of intestinal damage by increasing mucin production and secretion in connection to the increase in goblet cell counts (Chang et al., 2005). A noticeable reduction was seen in goblet cell counts in the case of fasting, and it was stated that this could be related to disruption in the surface epithelium and crypts (Gül et al., 2004). It was determined that there was a statistically significant difference in the blood glucose levels of the diabetes group on the 3rd and 17th days of the study ($p<0.05$). Our results suggested that the experimental diabetes continued until the end of the study. Moreover, in our study, no pathological finding was observed in the duodenum tissue of the rats on which diabetes was induced experimentally, and it was determined that the villi intestinalis were almost the same size, and the epithelium was not interrupted. The finding that there was also no significant difference among the groups in terms of the goblet cell counts suggested that experimentally induced diabetes does not lead to a negative effect on the duodenum tissue.

Endoplasmic reticulum (ER) stress is a process that involves the signaling system known as the unfolded protein response (UPR). In this process, expression of mutant proteins that disrupt normal protein folding occurs in ER (Hetz, 2012). ER stress plays a role in the pathogenesis of diabetes by causing not only the loss of functional beta cells but also development of insulin resistance (Eizirik et al., 2008). In addition to this, UPR affects beta cells in two ways: while it is useful for cells under physiological conditions or mild stress, it damages cells in pathological conditions or under chronic stress by triggering beta-cell dysfunction and apoptosis (Rabhi et al., 2014). Considering the effects of ATF6 in diseases of the digestive system, it was reported that there is ATF6 expression in lesions experiencing pre-cancer atypical changes in cancers related to ulcerative colitis and those that are not related to it (Hanaoka et al., 2018). Looking at the effects of ATF6 on chronic pancreatitis, it was stated that ATF6 signaling regulates the progression of chronic pancreatitis (CP) by modulating pancreatic acinar cell apoptosis, and this may have positive effects in the diagnosis and treatment of CP based on ER stress (Zhou et al., 2019). It was also documented that ATF6 creates a natural immune response in regulation of intestinal dysbiosis and colorectal tumorigenesis (Coleman et al., 2018). In our study, it was determined that the ATF6 immunoreactivity increased in the duodenum tissues of the rats in the diabetes group in comparison to the control group. Considering the association between ATF6 and diseases of the digestive system (Coleman et al., 2018; Hanaoka et al., 2018), it was thought that ATF6 levels could be affected in connection to changes that may occur in the duodenum in diabetes.

It has been reported that diabetes is effective in the histomorphological and biomechanical reshaping of the small intestine and the colon in type I diabetes patients and diabetic animals, and this shaping is closely associated with sensorimotor dysfunctions (Zhao et al., 2003; Zhao et al., 2006; Frokjaer et al., 2007; Zhao et al., 2009). NGF is a nerve-specific growth factor, but later studies showed that NGF also has roles outside the nervous system (Okada et al., 2004). NGF distributions were checked in various segments of the intestinal tissues of rats on which colitis was experimentally induced, and it was determined that the NGF levels in the rats with colitis increased in comparison to the control group. Based on these results, it was stated that NGF may have roles in limiting or solving inflammation that occurs in the intestinal tissue (Barada et al., 2007). It was reported that NGF treatment has positive effects in early intestinal stress and prevention of diseases that may occur in the gastrointestinal system in relation to early intestinal stress (Wong et al., 2019). In our study, it was determined that the NGF immunoreactivity increased in the crypts and glands of the duodenum tissues of the rats on which diabetes was experimentally induced in comparison to the rats in the control group, and these results suggested that experimentally induced diabetes may affect NGF levels in the duodenum tissue.

CONCLUSION

Consequently, several organs and systems may be affected in the long term in diabetes, which is a chronic disease. Several complications also occur in relation to diabetes in the digestive system. In our study, it was determined that the villus length measurements in the duodenum tissues of the rats in the diabetes group and those in the control group had a statistically significant difference, while there were also increased NGF and ATF6 immunoreactivities in the diabetes group. These results that we obtained would provide positive contributions to the literature in terms of the treatment of diabetes and prevention of complications that may occur in relation to diabetes.

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Approval for the research was received from Kafkas University Animal Experimentation Local Ethics Committee (KAÜ-HAYDEK No: 2020-090/ Date: 23.06.2020).

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Author's Contributions: ŞYA and EKS designed the study. ŞYA collected tissue samples, performed immunohistochemical and histological analyzes. SD made histopathological evaluations. ŞYA and EKS evaluated and comment all the data.

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