

The apoptotic effects of Streptozotocin in different dose and administration time on pancreatic islet cells of rats

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Abstract

The loss of islet cells by apoptotic cell death plays a central role in the pathogenesis of experimental animal models such as hyperglycemia and diabetes. Streptozotocin (STZ) is widely used for the induction of diabetes in animals by destruction of pancreatic β cells. The aim of the present study is to determine the effects of different dose and administration time of STZ on pancreatic islet cells. With this aim, four different experimental groups consist of the animals treated with citrate buffer (0.01 M, pH: 4.5) only, the animals sacrificed after 5 hours of the 40 mg/kg STZ injection, the animals sacrificed after 6 hours of the 60 mg/kg STZ injection, and the animals sacrificed after 4 hours of the 75mg/kg STZ injection were composed. While the apoptotic cells were observed by TUNEL staining method and according to morphologic criteria of the cells, insulin synthesized β cells were shown by immunohistochemical technique. As the result it was determined an increase in blood glucose levels and apoptotic islet cells in 40 and 75 mg/kg STZ-injected rats while islet and beta cell areas were decreased in all groups. These results indicate that 40 and 75 mg/kg STZ injection at the end of 5 and 4 hours, respectively, causes hyperglycemia by triggering apoptosis in islet cells of adult rats.

Keywords: Apoptosis, hyperglycemia, islet cell, rat, streptozotocin.

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Abbreviations: DNA, deoxyribonucleic acid; H&E, Hematoxyline and Eosin; NAD, nicotinamide dinucleotid; PARP, poly-(ADP-ribose) polymerase; STZ, streptozotocin; TUNEL, TdT-mediated dUTP nick end labelling.

Farklı doz ve uygulama zamanlarında Streptozotocin'in sıçanların pankreatik ada hücreleri üzerine apoptotik etkileri

Özet

Ada hücrelerinin apoptotik hücre ölümü ile kaybedilmesi hiperglisemi ve diyabet gibi deneysel hayvan modellerinin patogenezinde merkezi bir yol oynar. Streptozotocin (STZ) pankreatik β hücrelerinin tahrip edilmesi yolu ile hayvanlarda diyabetin uyarılması için sıklıkla tercih edilmektedir. Bu çalışmanın amacı farklı doz ve uygulama zamanlarında STZ'nin pankreatik ada hücreleri üzerine olan etkilerinin araştırılmasıdır. Bu amaçla sitrat tamponu (0.01 M, pH: 4.5) uygulanan, 40 mg/kg STZ enjekte edildikten 5 saat sonra sakrifiye edilen, 60 mg/kg STZ enjekte edildikten 6 saat sonra sakrifiye edilen ve 75 mg/kg STZ enjekte edildikten 4 saat sonra sakrifiye edilen hayvanlar olmak üzere 4 farklı deney grubu oluşturuldu. Apoptotik hücreler TUNEL işaretleme tekniği ve hücrelerin morfolojik özellikleri göz önüne alınarak belirlenirken, insulin üreten β hücreleri immünohistokimya tekniği kullanılarak gösterildi. Sonuç olarak, 40 ve 75 mg/kg STZ uygulanan sıçanların kan

glukoz miktarında ve apoptotik ada hücre sayısında bir artış gözlenirken, tüm gruplarda ada ve β hücre alanlarında azalma olduğu tespit edildi. Bu bulgular, 40 ve 75 mg/kg STZ enjeksiyonunun sırasıyla 5 ve 4. saatin sonunda erişkin sıçanların ada hücrelerinde apoptozu uyarak hiperglisemiye neden olduğunu göstermektedir.

Anahtar Kelimeler: Apoptoz, hiperglisemi, ada hücresi, sıçan, streptozotocin.

Introduction

Pancreatic β cell loss by apoptotic cell death is accepted as a most important event in development of hyperglycemia and diabetes. Due to its importance in diabetic mechanism apoptotic islet cell death is under consideration in this research area. Both of surgical and toxin-mediated pancreatic damage is used in the studies of the consequences of hyperglycemia. While pancreatectomy model is used for generating diabetic animals (Kim et al. 2010) non-surgical methods of inducing hyperglycemia by damaging the pancreas also exist. These include the administration of toxins such as streptozotocin (STZ, 2-deoxy-2-[3-methyl - 3 - nitrozoure] - 1 - D - glucopiranoze; Gezginci - Oktayoglu and Bolkent 2009) and alloxan (Makni e al. 2010). As diabetes models selective inbreeding has produced in several strains of animal, such as the nonobese diabetic (NOD; Gordon et al. 2005), the biobreeding (BB; Papaccio et al. 2006) animals. Among these models STZ is the most preferred toxin for inducing hyperglycemia and diabetes through β cell damage.

Streptozotocin is an antibiotic that the 2-deoxy-D-glucose derive of N-metil-N-nitrozoure and it is produced by *Streptomyces achromogenes* (Herr et al. 1967). STZ is diabetogenic because it selectively destroys the insulin producing beta cells by inducing apoptosis or necrosis (Szkudelski 2001; Gezginci-Oktayoglu and Bolkent 2011). This agent is transported to pancreatic β cell by glutathione-2 glucose transporter and it reacts with the different side of deoxyribonucleic acid (DNA). These sides are nitrogen or exosyclic oxygen atoms of DNA bases, especially. As a result of these reaction 7-methylguanin, 3-methyladenin ve O^6 -methylguanin are generated. 7-methylguanin and 3-methyladenin are eliminated by alkylpurin-DNA-N-

glucosylase, and as a result of this reaction apurinic/aprimidinic sides are generated on DNA. These parts are cut by apurinic/aprimidinic endonuclease and DNA is breaked to pieces (Bennett and Pegg 1981; Saffhill et al. 1985). The degeneration of DNA causes decrease in nicotinamide dinucleotid (NAD^+) level by activating poly-(ADP-ribose) polymerase (PARP), is an enzyme that synthesized ADP-riboz from NAD^+ . Insufficiency of NAD^+ causes increase in destruction of DNA as a result of losing DNA repairing enzyme's ability to connect the related sides of DNA (Szkudelski 2001).

Based on these data the aim of the present study was to determine the effects of different dose and administration time of STZ on pancreatic islet cells. In this way, it would be displayed an exact model for investigating of cellular mechanism resulted hyperglycemia and diabetes.

Materials and Methods

Experimental design

All experiments were carried out in accordance with the guidelines of the Istanbul University local ethic committee of experimental animals. The male *Wistar albino* rats at 10 weeks of age were obtained from Experimental Medical Research Institute of Istanbul University. They were kept at a constant temperature ($22 \pm 1^\circ C$) with 12 h light and dark cycles and fed with a standard pellet chow.

All experiments were initiated after fasting glucose concentration was measured in blood samples to ensure that the rats were not hyperglycemic. STZ was dissolved in sterile 0.01 M citrate buffer (pH: 4.5) just prior to use and injected intraperitoneally into overnight fast

rats. The animals were divided to 4 groups consisted of 5 rats:

Group 1 : The control animals treated with sterile 0.01 M citrate buffer (pH: 4.5) only.

Group 2 : Single dose STZ injected into rats at a concentration of 40 mg/kg body weight. They sacrificed after 5 hours of the injection.

Group 3 : Single dose STZ injected into rats at a concentration of 60 mg/kg body weight. They sacrificed after 6 hours of the injection.

Group 4 : Single dose STZ injected into rats at a concentration of 75 mg/kg body weight. They sacrificed after 4 hours of the injection.

The pancreas tissues were taken immediately after sacrificing by cervical dislocation.

Glucose measurement

Fasting blood samples were obtained from tail vein and glucose levels were determined using an automated glucose analyzer (Roche Accu Check Sensor Comfort, Roche Diagnostics, GmbH, Mannheim, Germany). Blood samples of 10 animals were analyzed for each group.

Histopathological Evaluations

The pancreas tissue was cut into small pieces and fixed in Bouin's solution. Following dehydration tissue samples were cleaned and embedded in paraffin. Sections with 5 μ m thickness were stained Hematoxyline and Eosin (H&E). Pancreatic islets were analyzed for observation apoptotic islet cells or necrosis in H&E-stained sections. Cells were identified as apoptotic on the basis of their morphology, using previously defined characteristics such as condensation and margination of nuclear chromatin and membrane-bound cell fragments containing uniformly dense masses of nuclear chromatin (Wyllie et al. 1980).

Immunohistochemistry

The immunohistochemical streptavidin-

biotin-HRP technique was performed on araffin sections for the detection of insulin (Thermo MS-1379, dilution 1:400) by using a broad spectrum kit (Zymed, 85-9942). Staining was developed with 3-Amino-9-Ethylcarbazole (AEC) to obtain a red reaction in the cytoplasm. Islet cells were viewed using a light microscope (Olympus, CX41) at a magnification of 400X. At least 10 islets were assessed for per slides. Islet area was calculated by dividing to total area (Σ islet area/ Σ area x 100). β cell area in islet was denoted as the number of total islet area occupied by insulin (Σ insulin positive area / Σ islet area x 100).

TUNEL Assay

Apoptotic or necrotic cells can be shown by labelling DNA strand breaks with the TdT-mediated deoxyuridine triphosphate nick end labelling (TUNEL) technique. TUNEL reaction can show an affinity for staining some intensely necrotic areas. However, this pattern of staining was easily discernible from truly apoptotic staining as there was no "bleeding" of the chromogen into these areas (Garrity et al. 2003). The DNA strand breaks were stained by using an apoptosis detection kit (Chemicon, S7101). Briefly, sections were deparaffinised and digested with proteinase K (20 μ g/ml for 15 min at 37 °C). Sections were adapted to terminal deoxynucleotidyl transferase and deoxyuridine triphosphate mixture at 37 °C for 1 h. Staining was developed with diaminobenzidine to obtain a brown reaction in the nucleus. The TUNEL positive islet cell number was calculated by using following formula: $TUNEL^+ \text{ Islet Cell} = \frac{\Sigma \text{ TUNEL positive islet cell number}}{\text{Islet}}$.

Statistical analysis

Data were expressed as mean \pm S.E.M. The results were analyzed by Student's t-test to compare differences among groups by using GraphPad Prism software, version 4.00 (San Diego, CA).

Results

Blood Glucose Levels

Fasting blood glucose levels did not change significantly in Group 1 and 3 ($p > 0.05$). On the other hand, there was a significant increase in fasting blood glucose levels in the Group 2 and 4 ($p < 0.01$ and $p < 0.001$, respectively; Fig.1).

Histopathological Changes

Islet cells were normal morphology in the groups of citrate buffer-injected animals (Group 1). On the other hand, STZ injection changed islet morphologies. Some cells shrunk and had condensed chromatin like apoptotic cells in islets of animals from Group 2. While it was come across rarely with cells in apoptotic morphology, necrotic areas were dominant in the islets of animals from Group 3. It was observed a lot of cell have apoptotic morphology in the islet of animals from Group 4. However, there was no necrotic area in islets from this group (Fig. 2).

Alteration in Islet and β Cell Areas

While the islet area was 76.4 ± 1.8 %, it was decreased significantly to 60.6 ± 3.7 % in group 2 ($p < 0.01$), 54.3 ± 3.1 % in group 3 ($p < 0.001$), and 57.2 ± 1.2 % ($p < 0.001$) (Fig 3A and 3B).

Insulin⁺ beta cell areas were examined by immunohistochemistry. Beta cells occupied 76.4 ± 1.8 % of the islet area in the Group 1. The insulin proportion in the islet area decreased significantly to 61 ± 3.4 % in Group 2 ($p < 0.01$), 54.3 ± 3.1 % in Group 3 ($p < 0.001$), and 57.2 ± 1.2 % in Group 4 ($p < 0.001$) by STZ treatment (Figs 3A and 3C).

Alteration in TUNEL⁺ Islet Cells

Apoptotic (TUNEL⁺) cell presence was examined in pancreas. TUNEL⁺ islet cell number was 0.4 ± 0.2 in group 1 and 0.4 ± 0.2 group 3. The prevalence of TUNEL⁺ islet cells increased significantly to 20.2 ± 7.9 in group 2 and 19.8 ± 5.1 in group 4 ($p < 0.05$, $p < 0.01$) (Fig. 4).

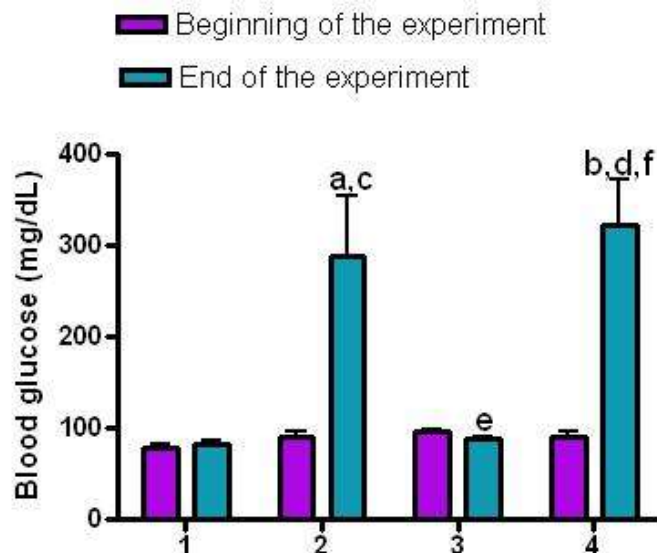


Figure 1. Fasting blood glucose levels (mg/dL) at the beginning and the end of the experiments are following in the groups: Group 1- 77.60 ± 4.20 and 83.0 ± 2.98 ; Group 2- 89.80 ± 6.87 and 288.40 ± 65.33 ; Group 3- 92.20 ± 1.15 and 88.20 ± 2.15 ; Group 4- 89.60 ± 7.56 and 322.00 ± 49.58). Data were expressed as mean \pm S.E.M. ^a $p < 0.01$ vs. at the beginning of the experiment, ^b $p < 0.001$ vs. at the beginning of the experiment, ^c $p < 0.01$ vs. at the end of the experiment of Group 1, ^d $p < 0.001$ vs. at the end of the experiment of Group 1, ^e $p < 0.01$ vs. at the end of the experiment of Group 2, ^f $p < 0.01$ vs. at the end of the experiment of Group 3. The numbers 1-4 indicates the group number.

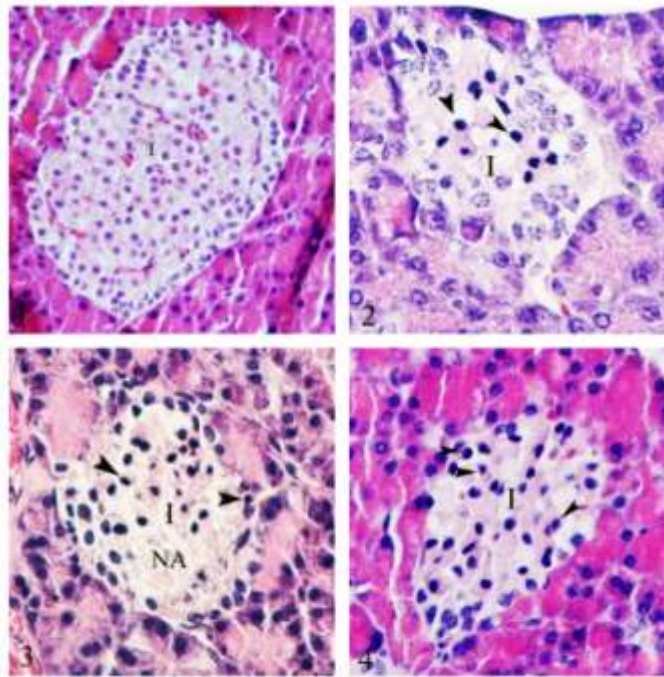


Figure 2. STZ injection changed morphology of the islet cells. Cells have apoptotic morphology (►), necrotic areas (NA), islet (I), H&E, 540X. The numbers 1-4 indicates the group number.

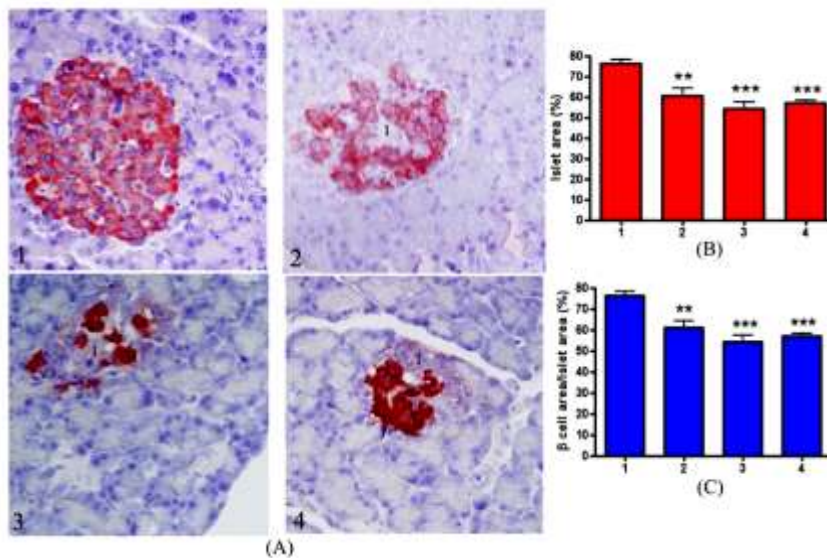


Figure 3. (A) Insulin⁺ beta cells can be seen in the pancreas from all groups. Loss of insulin-produced beta cells in STZ-administered rats is conspicuous. I:islet, 540X. (B) The graph demonstrates the islet area in all groups. (C) The graph demonstrates the beta cell area in islets by insulin immunohistochemistry. ** $p < 0.01$ and *** $p < 0.01$ versus Group 1. Values are means \pm S.E.M. $n = 5$ animals for each group. The numbers 1-4 indicate the group number.

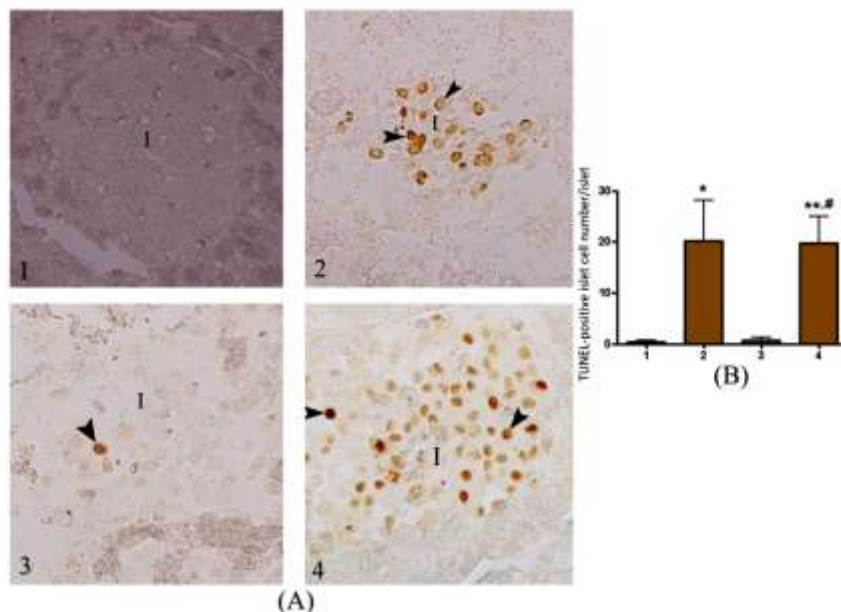


Figure 4. (A) TUNEL⁺ islet cells (►) can be seen in the pancreas in STZ-injected groups. I:islet, 540X. (B) The graph demonstrates the TUNEL⁺ islet cell/islet in all groups. * $p < 0.05$ and ** $p < 0.01$ versus Group1, # $p < 0.05$ versus Group3. Values are means \pm S.E.M. $n = 5$ animals for each group. The numbers 1-4 indicate the group number.

Discussion

Streptozotocin has been frequently used for inducing hyperglycemia and diabetes especially in rats and mice (Gezginci-Oktayoglu and Bolkent 2009; Gezginci-Oktayoglu and Bolkent 2011). It has been reported that STZ induce dose-dependent pancreatic β cell damage and hyperglycemia by intraperitoneal or intravenous injection at a dose ranging from 25 to 100 mg/kg (Junod et al. 1969; Abeeleh et al. 2009). Its effects can be seen within one hour after STZ administration (Junod et al. 1967). Pancreatic β cells die by triggering of apoptotic cell death machinery in hyperglycemic conditions. For this reason, inducing of β cell apoptosis is valuable tool in diabetes research area. It is also very difficult to catch the apoptotic β cells *in vivo*, because of they eliminated by the macrophages or the neighbour cells. We investigated the correct experimental model for

triggering apoptosis of islet cells in adult rats by using STZ in the present study.

The frequently preferred experimental model for inducing of insulin dependent diabetes is the single and high dose between 40-75 mg/kg (Ganda et al. 1976; Gezginci-Oktayoglu and Bolkent 2009) or multiple low dose (Lin et al. 2010) injection of STZ in adult rats. The administration of STZ as multiple low dose is used predominantly in the mouse and the induction of diabetes is mediated by the activation of immune mechanisms (Szkudelski 2001). Single high dose STZ injection causes a lot of DNA fragment generation and cells die by necrosis (Saffhill et al. 1985). On the other hand, it was reported that administration of multiple low dose STZ was induced apoptosis in β cells (O'Brien et al. 1996; Cardinal et al. 2001). However, there are different results in the literature. Some research groups have reported that single dose STZ injection could provoke apoptosis in high

amount of β cell *in vivo* (Morimoto et al. 2005; Le May et al. 2006). Indeed, we observed an increase in DNA strand breaks by TUNEL assay in 40 and 75 mg/kg STZ-injected rats. The reason of these differences could be result of the different administration time and animal strains. On the other hand, although we tried to investigate the effect of multiple low dose injection of STZ to adult rats, as 40 mg/kg for 5 days, we could not keep them alive in this experimental process (data not shown). This result suggested that multiple low dose STZ administration is not usable method for rats. Due to mouse are more resistant, this method is used frequently for them (Szkudelski 2001).

Streptozotocin causes characteristic alterations in blood insulin and glucose concentrations in β cells. Two hours after injection, the hyperglycemia occurs with high levels of blood insulin (West et al. 1996). These changes in blood glucose and insulin concentrations are indicators of abnormalities in β cell function. STZ breaks glucose oxidation (Bedoya et al. 1996), decrease insulin synthesis and secretion (Bolaffi et al. 1987; Nukatsuka et al. 1990). It has been reported that administration of a single dose of STZ (40-65 mg/kg ip or iv) results in hyperglycemia within 72 hours (Gojo et al. 2007; Al-Qattan et al. 2008). We observed an increase in blood glucose levels in 40 and 75 mg/kg STZ-injected rats after 5 and 4 hours of administration, respectively. Interestingly, there was a small amount decrease in blood glucose levels of 60 mg/kg STZ administered rats after 6 hours of treatment. We thought that it is possible to develop hypoglycemia at 6th hours in these rats. Because of the blood glucose level balanced by insulin we also checked the insulin synthesized β cell area in islets and islet areas in the sections. We observed a decrease in β cell and islet areas in STZ-injected rats as we thought.

In conclusion, these results indicate that 40 and 75 mg/kg STZ injection in 5 and 4 hours, respectively, can be used for triggering apoptosis in islet cells of adult rats and

hyperglycemia, *in vivo*. On the other hand, 60 mg/kg STZ causes necrosis in islets after 6 hours. These observations are very important for choosing of experimental model in diabetes researches.

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