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ARAŞTIRMA / RESEARCH

Lung injury aggravated in Streptozotocin-induced diabetes: an experimental study

Streptozotosin kaynaklı diyabette akciğer hasarı: deneysel bir çalışma

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Abstract

Purpose: The aim of the study explores the impact and potential mechanisms on the STZ (Streptozotocin)-induced diabetes model histopathologically and immunohistochemically on diabetic lung fibrosis.
Materials and Methods: In this study, 14 adult female Wistar albino rats were divided into groups of seven random animals: the control and STZ induced diabetic groups. In the study, a blood glucose level above 200 mg/dl was accepted as diabetes. Nine days after the experiment, the rats were sacrificed under anesthesia and lung samples were taken from each. The histopathological appearance of the samples was evaluated and histopathologic damage score was performed. Apoptosis and inflammation in tissues were evaluated with caspase-3 and IL-1β immunohistochemically.
Results: In the histopathological examination, the STZ group had a higher histopathologic damage score than the control group, and there were findings such as vascular congestion, thickened alveolar wall, and inflammatory cell infiltration. In the caspase-3 immunohistochemistry, staining of the lung tissues of the STZ group was higher than the control group. This difference was also significant in terms of IL-1β immunoreactivity intensity.
Conclusion: This study determined that lung complications and damage due to diabetes-induced by STZ occurred.
Keywords:. Diabetes mellitus, lung, streptozotocin, apoptosis

Öz

Amaç: Bu çalışmanın amacı, diyabetik akciğer fibrozisi üzerine histopatolojik ve immünohistokimyasal olarak STZ (Streptozotosin) kaynaklı diyabet modeli üzerindeki etki ve potansiyel mekanizmaları araştırmaktır.
Gereç ve Yöntem: Bu çalışmada, 14 yetişkin dişi Wistar albino sıçan, 7 rastgele hayvandan oluşan gruplara ayrıldı: kontrol ve STZ ile indüklenen diyabet grupları. Çalışmada kan şekerinin 200 mg/dl'nin üzerinde olması diyabet olarak kabul edildi. Deneyden dokuz gün sonra sıçanlar anestezi altında sakrifiye edildi ve her birinin akciğer örnekleri alındı. Örneklerin histopatolojik görünümleri değerlendirildi ve histopatolojik hasar skoru yapıldı. Dokulardaki apoptoz ve inflamasyon, immünohistokimyasal olarak kaspaz-3 ve IL-1β ile değerlendirildi.
Bulgular: Histopatolojik incelemede STZ grubunun histopatolojik hasar skoru kontrol grubuna göre daha yüksekti ve damar tıkanıklığı, alveol duvarında kalınlaşma, inflamatuar hücre infiltrasyonu gibi bulgular vardı. Kaspaz-3 immünohistokimyasında, STZ grubunda akciğer dokularının boyanması kontrol grubuna göre daha yüksekti. Bu fark, IL-1β immünreaktivite yoğunluğu açısından da anlamlıydı.
Sonuç: Bu çalışmada STZ'ye bağlı olarak pulmoner komplikasyonların ve diyabete bağlı hasarın oluştuğu belirlendi.
Anahtar kelimeler: Diabetes mellitus, akciğer, streptozotosin, apopitoz

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INTRODUCTION

Diabetes mellitus (DM) is an endocrine disease characterized by hyperglycemia and glucagon elevation result from absolute or relative insulin insufficiency (Type I) or insulin resistance in target organs (Type II). Further, DM is a metabolic syndrome characterized by impaired carbohydrate, protein, and lipid metabolism, which causes acute metabolic and chronic degenerative complications. More than 400 million people live with diabetes today worldwide. The prevalence of Type II DM is rapidly increasing with malnutrition and lifestyle changes. It used to occur nearly entirely among adults but now appears in children[1].

DM has microvascular and macrovascular complications. In addition to microvascular complications such as frequently observed retinopathies, neuropathies, and nephropathies, lung function disorders have also been reported[2]. DM patients have an increased risk of chronic obstructive pulmonary disease, pulmonary fibrosis, and asthma. Moreover, it has been reported that lung capacity decreases and pulmonary dysfunction develops[3]. It has been stated that collagen accumulation in lung tissue and thinning of alveolar basement membranes may cause this dysfunction. It is also known that high levels of insulin cause contraction and proliferation in airway smooth muscles[4]. As a result of this dysfunction that develops in the lungs, ventilation is impaired, and hypoxia occurs. Hypoxia also causes inflammation, oxidative stress, and damage to organs[5]. Therefore, hypoxia may contribute to the progression of these lung injuries caused by DM. In streptozotocin (STZ)-induced diabetic mice, there is a significant increase in pulmonary neutrophil infiltration and production of proinflammatory cytokines (such as interleukin 1 beta (IL-1β) and interleukin 6 (IL-6)[6]. IL-1β is an important pro-inflammatory cytokine that increases acute hypoxia[7]. In addition, it has been claimed that patients exposed to hyperglycemia overproduce reactive oxygen species (ROS), and their antioxidative cell defense is insufficient. Organ damage also begins with the formation of inflammatory agents such as tumor necrosis factor-alpha (TNF-α)[8]. Specific genes and protein products of these genes are regulated by the apoptosis mechanism. Caspases are the primary drivers of apoptotic cell death. Caspase-3, which is an effector caspase, determines the first stage of apoptosis by taking part in chromatin condensation and DNA fragmentation. It is well-known that apoptosis is induced by hyperglycemia[9]. DM also induces apoptosis by regulating signaling molecules such as caspase-3 in the apoptosis pathway[10]. However, apoptotic and inflammatory effect of diabet in animal model remain unclear. The main objectives of this study were to evaluate the apoptotic and inflammatory status of the STZ-induced rat lung injury on the basis of histopathologic and immunohistological studies.

MATERIALS AND METHODS

Animals

The current study protocol was approved by the Experimental Animals Ethics Committee of Erciyes University (Decision number; 19/004). Fourteen healthy adults (8-10 weeks old), Wistar albino female rats, were used for the experiment. Female rats were housed in plastic cages with a temperature-controlled and reversed light/dark cycle (22 ± 2 ºC, 12 hours light/dark) during the experiment.

Study design and animal treatment

The 14 adult female rats were randomly assigned to the control and STZ groups, each containing seven rats. Before starting the experiment, glucose levels were measured using a glucometer (Accu-Chek Go (Roche)) in blood samples from the tail vein of rats. Blood glucose levels were measured as 94 mg/dl on average.

For the diabetic model, the rats fasted overnight and then were injected intraperitoneally (ip) with STZ (Sigma-Aldrich, ST. Louis, MO, USA) freshly prepared in citrate buffer at a single dose while control animals received an equal volume of plain citrate buffer. STZ was prepared in citrate buffer (0.1 M, Ph 4.5) before injection into rats and administered 60 mg/kg. The DM was induced by the STZ injection as described previously[7].

72 hr following STZ injection, DM was verified by estimation of fasting blood glucose levels from animal's tail vein using Accu-Check Compact-Plus glucose meter system (Roche Diagnostics, Meylan, France). Those with blood glucose values above 200 mg/dl were considered as having diabetes. The diabetic state was confirmed nine days after the final STZ injection by the presence of hyperglycemia and 7 rats were caged with diabetes.

Histopathology

For histopathological analysis, the rats were sacrificed under anesthesia nine days after being accepted as diabetes[11]. For histopathological analysis, mice were admitted nine days later and after sacrification, the lung tissue was removed rapidly. The samples were fixed in 10% formaldehyde solution. Then, the tissues were dehydrated by passing through increasing graded alcohol series (50%, 70%, 80%, 96%, 3x 100%) and embedded in paraffin by transparent with xylol.

Lung tissue samples were taken in 5 µm sections on slides. Sections were stained with Hematoxylin-Eosin (H&E) and Masson's trichrome for examination under the light microscope. Briefly, the sections were removed from paraffin with xylol and passed through decreasing graded alcohol (100%, 96%, 80%, 70%, 50%) batches and washed in running water. After staining, it was passed through graded alcohol series, passed through xylol, and covered with a coverslip using entellan.

Stained preparations were evaluated under the light microscope (Olympus BX51, Tokyo, Japan). Scoring criteria for lung inflammation, as previously described, were; 0: normal tissue, 1: minimal inflammatory change, 2: no significant damage to the lung structure with leukocyte infiltration, 3: thickening of the alveolar septa with edema and leukocyte infiltration, 4: nodules that disrupt the normal structure, and 5: completely destruction[12].

Immunohistochemistry

Additionally, immunohistochemical staining was performed on sections to evaluate IL-1β (Santa Cruz, sc-52012, 1/500) and caspase-3 (Cell Signaling Technology, 9661S, USA, 1/200) activity using the avidin-biotin-peroxidase method (Thermo Scientific, Waltham, MA). Briefly, sections taken from slides with lysine were incubated in 3% hydrogen peroxide for 10 minutes for endogenous peroxidase activity. After washing the samples with PBS, they were applied microwave process in 0.01 M sodium citrate buffer for antigen retrieval. Sections were incubated with IL-1β and caspase-3 primary antibodies at 4 °C overnight in a humid chamber. Then sections were rinsed with PBS, and biotinylated secondary antibodies were applied. After rinsing with PBS and with 3,3 3, p-diaminobenzidine tetrahydrochloride (DAB) (Thermo Scientific, Waltham, MA) as chromogen, it was incubated for 3–5 minutes. After washing with deionized water, sections were stained with Gill's hematoxylin for counterstaining rinsed with tap water, and covered with entellan. An Olympus BX51 microscope equipped with DP71 imaging was used to examine and photograph the stained samples. Photographs were taken from at least five different areas of each tissue in a 40× magnification. Using the Image J Software program, the immunoreactivity intensity in each area was calculated, and the data were analyzed statistically.

Statistical analysis

The Graphpad PRISM (Graphpad Software Inc., Version 8.0d) program was used for statistical analysis. Raw data are presented as group means ± SEM (standard error of the mean). Scoring and immunohistochemical immunoreactivity densities between groups were analyzed by Student's t-test method. Statistical analysis was considered significant if $p < 0.05$.

RESULTS

In the study, there was an increase in blood glucose levels in diabetic rats according to the control group after DM was induced ($P<0.001$) (Figure 1). The microscopy findings demonstrated the histology of the lungs of each group. We conducted analyzes to evaluate the lung damage caused by DM with lung damage scoring. There were no histologically pulmonary lesions found in the control rats. The STZ group had a higher lung histopathologic damage score than the control group (Figure 2). Lung injury was characterized by vascular congestion, thickened alveolar wall, and inflammatory cell infiltration. This destruction was manifested as a significant increase in alveolar wall thickening and higher volume fractions of the alveolar wall.

To determine the collagen content of lung tissues, sections were analyzed by Masson's trichrome staining. Collagen accumulation in the lung tissues of diabetic rats was increased compared to the control group. We examined the relationship of STZ-induced diabetic lung tissue with fibrosis. Accordingly, connective tissue fibers were irregularly deposited in the alveolar walls of diabetic animals and were more prominent than the control group (Figure 2).

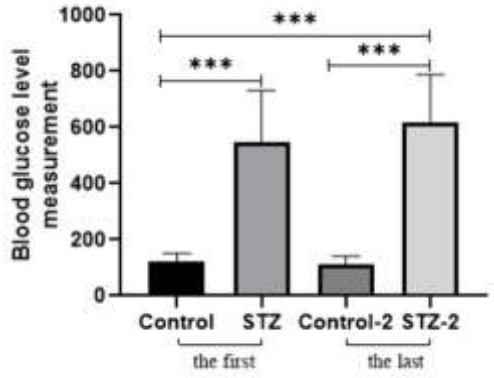

Figure 1. Measurement results of blood glucose levels of rats. (*P<0.05;P<0.01;***P<0.001).**

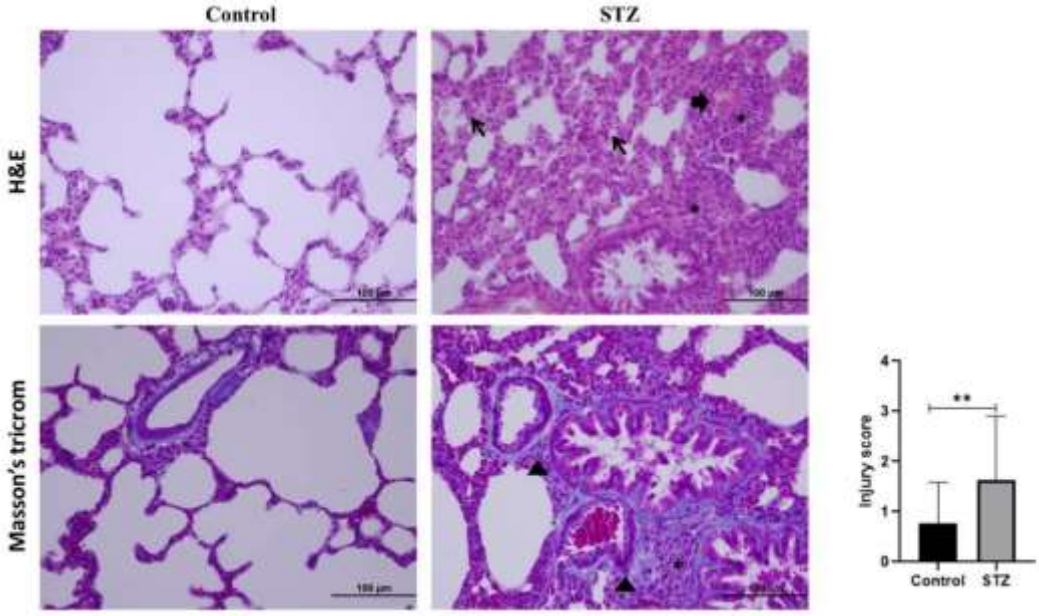

Figure 2. Representative images of H&E staining of STZ-induced DM and control lung tissues.

While the lung tissues of the control group showed normal alveolar structure and thin interalveolar septa. The STZ group exhibited significantly alveolar wall thickness, lower alveolar airspace volume than the control group. The representative graph showed the injury score. Connective tissue fibers accumulated in the alveolar wall of diabetic rats were demonstrated by Masson's trichrome staining. Arrow; thickened alveolar wall, star; inflammatory cell infiltration, bold arrow; vascular congestion, arrowhead; collagen fiber accumulation. (H&E staining, 40×) (*P<0.05;**P<0.01;***P<0.001).

To demonstrate apoptosis in lung tissues, apoptosis-positive cell marking was performed with caspase-3 immunohistochemical staining. Caspase-3 immunoreactive cells were observed as brown in the control and STZ groups (Figure 3). In the caspase-3 immunohistochemistry staining, the density of positive immunoreactive cells in the lung tissues of the STZ group was significantly higher than the control group (P<0.01).

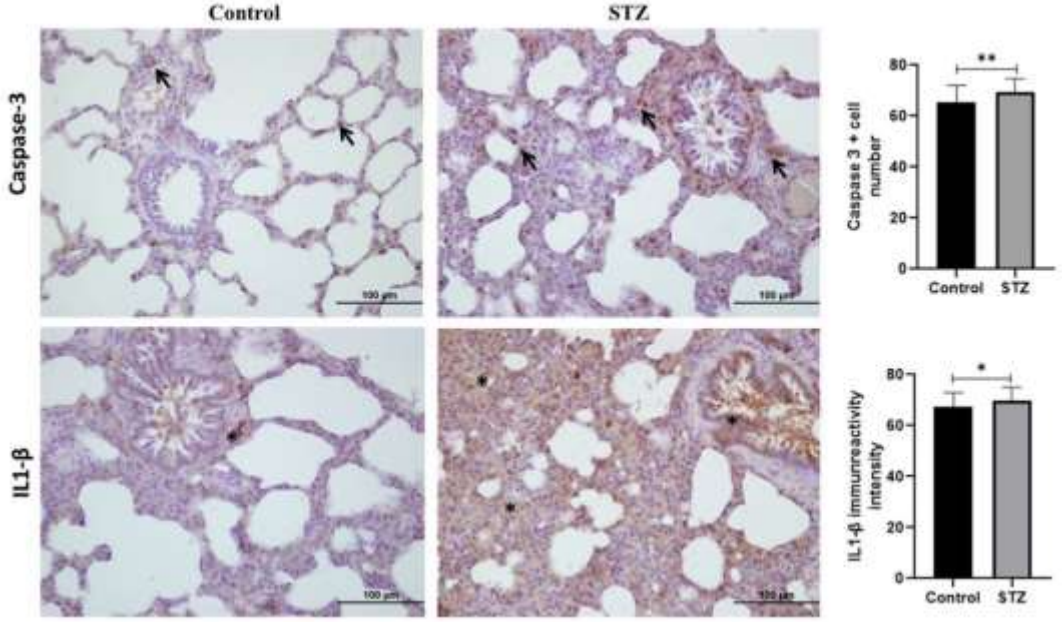

Figure 3. Caspase-3 and IL-1β immunohistochemistry light microscopic images of the lung tissues in the experimental groups. arrow; Caspase 3 immunoreactive positive cell, star; IL-1β immunoreactivity (Caspase-3 and IL-1β IHC, 40×) (*P<0.05;P<0.01;***P<0.001).**

To show the severity of inflammation, immunohistochemical staining was performed by the IL-1β immunohistochemical method in both the control and STZ groups. Brown-stained cells showing expression were displayed under a light microscope. Expression intensity was higher in the STZ group according to the control group. It was determined that this difference was statistically significant with the density measurement we made with the image j software program (P<0.05).

DISCUSSION

DM is a common metabolic disease with high mortality and morbidity. Complications are common in many organs in the body. We created an experimental DM model on rats to show the complications that may occur in lung tissue. STZ is a commonly used molecule in experimental DM models and destroys β-cells of islets of Langerhans in pancreatic tissue, resulting in elevation of blood glucose level, and thus, inhibition of insulin secretion[13]. In this context, the lung tissues of the DM group and the control group were examined by histopathological and immunohistochemical methods, and lung damage due to DM was confirmed. An increase in blood glucose levels due to the complications of DM is expected. An increase in blood glucose levels after DM occurs in the STZ group in our study was consistent with the literature[14]. We used the histopathological damage scoring used in the literature to present our findings more objectively with HE staining. In a study in which no pulmonary lesions were observed in the control group, the histopathological findings in which edematous changes in the lungs of STZ-induced mice, leukocyte infiltration in the alveolar interstitium, and mild decrease in the alveolar air space were similar to our study[6]. In another study, STZ-induced diabetic mice were shown to have

higher lung damage scores than non-diabetic mice[15]. In the study showing findings such as bleeding in the lung tissues induced by STZ, thickened alveolar wall, and inflammatory cell infiltration, the lung damage score was higher than the control group[14]. In the study of Onk et al., while the histological findings of control rats were normal, it was reported that there was a thickened interalveolar septum, peribronchial cell infiltration, desquamation in the bronchial epithelium, and hyperemia in the peribronchial vessels in the DM group[16]. In our study, similar to the studies in the literature, there was no lesion in the control group with HE staining; in the STZ group, lung scoring was significantly increased in the presence of vascular congestion, thickened alveolar wall, and inflammatory cell infiltration findings. Therefore, histopathological results were supported by experimental data.

Pulmonary fibrosis, the transformation of lung fibroblasts into myofibroblasts, is characterized by the excessive accumulation of extracellular matrix proteins such as collagen. This may result in decreased gas exchange and impaired lung function[17]. Therefore, we examined the relationship between diabetes and fibrosis in lung tissues. In this study, lung tissues were analyzed by Masson's trichrome staining to determine collagen content. Accordingly, collagen accumulation was clearly increased in the STZ group compared to the control group. In particular, connective tissue fibers were irregularly accumulated in the alveolar walls of diabetic rats and were more prominent than the control group in the STZ group. As is known, in pathological conditions, epithelial cells can also transform into fibroblasts that cause organ fibrosis[18]. Collagen produced by fibroblasts increases in the early stages of lung injury and affects lung functions. The increase in collagen accumulation in the lung tissues of diabetic rats compared to the control group showed us that fibrosis was induced. Consistent with the results we obtained with Masson's trichrome staining, it was shown that irregularity and collagen accumulation in the lung tissue was increased in the STZ group[14].

Apoptosis plays an important role in the pathophysiology of DM and also is involved in the pathogenesis of lung fibrosis. The expression of caspase-3, a key apoptosis executioner, can induce apoptosis. In cells receiving apoptosis signal, caspase-3 activate CAD (caspase-activated deoxyribonuclease), and this enzyme rapidly breaks the DNA into nucleosomal fragments. The apoptosis pathway is regulated by caspases, and caspase-3 activation is induced by high glucose in the lungs of diabetic rats[19]. In our study, caspase-3 expression was relatively low in the control rats. However, there was a significant increase in the STZ group. Thus, we found that DM induces apoptosis in lung tissue. In parallel with our findings, it was observed in the literature that the amount of caspase-3 increased in the lungs of rats with DM[20]. Again, in another study, it was observed that the expression of caspase-3 was strongly increased in the DM group compared to the control group[16]. In agreement with this the present study shows increased caspase-3+ in the pulmonary epithelium and interstitium which are likely signs of diabetes-mediated tissue damage. We postulate that diabetes induces cell apoptosis and proliferation in both pulmonary epithelium and interstitium in rat lungs as a response to tissue damage. On the other hand, the pulmonary epithelium is close to pulmonary capillaries. Cell junctions between pulmonary epithelial cells prevent accumulation of fluids in alveolar areas. Therefore, diabetic complications in lung tissue may related to pulmonary epithelial and endothelial dysfunctions.

In pathological conditions where body hemostasis is impaired, such as infections and injuries, the body develops an inflammatory response as a defense mechanism. In the region where the inflammatory response will occur, macrophages, monocytes, leukocytes, fibroblasts, and endothelial cells secrete cytokines such as TNF-α, IL-1, IL-6, and interferons. Although the vast majority of studies have focused on the production of IL-1β by monocytes and macrophages, it is produced by various cell types[21]. It is known that an increase in cytokines such as IL-1β, TNF-α lead to increased cellular stress and consequently, structural damage develops in diabetic lungs. The pro-inflammatory cytokine IL-1β was found to be high in the lung tissues of diabetic rats.[6] In the present study, hyperglycemia led to similar structural damage in diabetic rat lungs. In the study of Xiong et al., DM was shown to increase inflammation in the lungs[15]. The increase in TNF-α in the lungs of diabetic rats, which increased with IL-1β during the inflammation process, was significant[22,23]. Diabetes is a systemic disease and affects many organs in the body, including the lungs. In this context, studies showing that pro-inflammatory cytokine (IL-1β and TNF-α) levels increase in kidney tissue of diabetic mice also support us[24]. Cleavage of pro-IL-1β by caspase-1 can lead to inflammatory activation and active production of

mature IL-1β. Later, active mature IL-1β may mediate lung inflammation and fibrosis. Thus, it can be said that fibrosis increases with collagen accumulation in the lung tissues of rats with STZ-induced DM. In the lungs noted hyperglycemia-induced inflammatory mediators such as IL-1β and TNF-α. An increase in oxidants and a decreased in pulmonary antioxidant enzymes lead to increased cellular stress and consequently, structural damage develops in diabetic lungs. In the present study, hyperglycemia led to similar structural damage and alterations of inflammatory mediators in diabetic rat lungs. Therefore, it is likely that when pro-inflammatory IL-1β family members are suppressed, they might have therapeutic effects on many inflammatory diseases (e.g., viral infections such as covid-19)[25]. There are some limitations in our experimental study that should be mentioned. We did not compare the control group and DM lung weight/body weight ratio exposed to high glucose levels. Although many studies have demonstrated that there is a correlation between the results obtained from the immunohistochemistry quantification and the tissue concentration, another limitation is that we did not measure protein concentrations by western blot analysis. These issues should be addressed in future studies.

As a result of our histopathological studies, it was observed that pulmonary complications due to DM occurred in the lung tissues of rats exposed to DM induced by STZ. Lung histopathologic damage score in STZ group; IL-1β and TNF-α cytokines; It was observed that the caspase-3 level increased significantly. Therefore, these findings shed new light on the histopathology of diabetic lung injury and provide a theoretical base for further research of diabetic lung fibrosis therapeutics.

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