

Evaluation of VEGF, Cytokeratin-19 and Caspase 3 Immunolocalization in the Lung Tissue of Rat with Experimentally Induced Diabetes ^[1]

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Abstract

Diabetes Mellitus (DM) manifests itself with changes in the functional structure of the lungs and impairments in gas exchange. These changes in diabetic lung tissue may be due to various factors. Our aim in this study is to correlate the damage of diabetes with lung tissue in terms of VEGF, CK19, caspase 3 immunolocalizations. In this study, animals were divided into 4 groups, 60 mg/kg streptozotocin was given to each of the groups with experimental diabetes and the physiological saline solution was given intraperitoneally to the control group. On days 7 and 14 of the experiment, diabetic and control groups were euthanized, and lung tissues were removed. Tissue samples were evaluated histochemically and immunohistochemically by monitoring with standard light microscopy. In the diabetic group, the localization of CK19 and Caspase 3 increased on the 7th and 14th days compared to the control group, but the immunolocalization of VEGF decreased. Based on our findings, it was determined that lung tissue was one of the target organs of diabetes. The increase in pulmonary parenchyma due to hyperglycemia is accepted as a source of fibrosis. We concluded that due to increased CK19 localization of fibrosis source, decreased VEGF localization has increased apoptosis in the pulmonary capillary endothelium, which has a significant role in the blood-air barrier in the lung parenchyma, especially in endothelial cells.

Keywords: Experimental diabetes, Lung, CK19, VEGF, Caspase 3

Deneyel Olarak Diyabet Oluşturulan Ratların Akciğer Dokusunun Caspase 3, Cytokeratin 19 ve VEGF İmmünolokalizasyonunun Değerlendirilmesi

Öz

Diabetes Mellitus (DM), akciğerlerin fonksiyonel yapısındaki değişikliklerle ve gaz değişimindeki bozukluklarla kendini gösterir. Diyabetik akciğer dokusunda bu değişiklikler çeşitli faktörlere bağlı olabilir. Bu çalışmada amacımız VEGF, CK19, kaspaz 3 immunolokalizasyonları ile ilgili olarak diyabetin akciğer dokusuna verdiği zararı ilişkilendirmektir. Çalışmada hayvanlar 4 gruba ayrıldı, deneyel diyabetli gruplara 60 mg/kg streptozotocin verildi ve kontrol grubuna serum fizyolojik solüsyonu intraperitoneal olarak verildi. Deneyin 7 ve 14. günlerinde diyabetik ve kontrol grupları sakrifiye edildi ve akciğer dokuları çıkarıldı. Doku örnekleri, standart ışık mikroskobu ile izlenerek histokimyasal ve immünohistokimyasal olarak değerlendirildi. Diyabetik grupta 7. ve 14. günlerde kontrol grubu ile kıyaslandığında CK19 ve Kaspaz 3 lokalizasyonu artmış, ancak VEGF immunolokalizasyonu azalmıştır. Bulgularımıza göre, akciğer dokusu diyabetin hedef organlarından biri olduğunu göstermektedir. Hiperglisemiye bağlı pulmoner parankimde artış fibrozis kaynağı olarak kabul edildi. Sonuç olarak, pulmoner fibrozis kaynağının CK19 immünolokalizasyonunun artmasına bağlı olarak, azalmış VEGF lokalizasyonunun, özellikle endotelial hücrelerde, akciğer parankimindeki kan-hava bariyerinde önemli bir rolü olan, pulmoner kapiler endoteliumda apoptosisi arttırdığı sonucuna varıldı.

Anahtar sözcükler: Deneyel diyabet, Akciğer, CK19, VEGF, Caspase 3

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease with an increase

in blood glucose levels, and its prevalence has increased rapidly in the last ten years. Some of the causes of this disease can be expressed as a lack of insulin secretion,



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decreased insulin action or decreased insulin receptor sensitivity [1]. Hyperglycemia and lack of insulin cause various organ dysfunction in patients with DM. DM has negative effects on the lung as well as in many organs. Some of the complications with DM in the lungs can be infections, pulmonary function abnormalities, pleural effusion, and obstructive sleep apnea. DM can also cause lung cancer [2]. Biochemical changes in the lungs in diabetics include decreased glutathione peroxidase activity, NO-induced endothelial dysfunction, and increased heparan sulfate level of the vascular endothelial basement membrane. Biochemical changes in the diabetic lung cause structural changes in the lung parenchyma. Some of these may be expressed as narrowing and interstitial involvement in alveolar areas. Pulmonary vessels, alveolar epithelial basement membrane, bronchial epithelium, and pulmonary capillaries are also affected by diabetes. The most common pathology of this disease is the deterioration in the vascular structure [3,4]. It is very difficult to examine the damages on diabetic lung tissue on the human. Therefore, experimental diabetes models have been developed. Streptozotocin (STZ) is used as an agent to induce experimental hyperglycemia in rats [5]. STZ reduces insulin biosynthesis and secretion and induces hyperglycemia as β -cells cause excessive free radical production [6]. Therefore, STZ is a widely used model to investigate the effects of DM on cells and tissues in experimental studies. High blood glucose levels can damage the blood vessels and cause endothelial dysfunction. Therefore, DM is a risk factor for cardiovascular diseases [7]. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is an angiogenic factor that causes the proliferation and permeability of vascular wall endothelial cells [8,9].

Oxidative stress plays a major role in the development of micro- and macrovascular complications. Accumulation of free radicals in the vasculature of diabetic patients is responsible for the activation of detrimental biochemical pathways, miRs deregulation, disruption of apoptosis mechanisms, and epigenetic changes contributing to vascular inflammation and reactive oxygen species (ROS) generation [10]. So oxidative stress induced by hyperglycemia in lung endothelial cells due to DM induces apoptosis [11]. Apoptosis is regulated by specific functional genes and their protein products. Caspases are vital mediators of programmed cell death (apoptosis) in lung tissue as well as in many cells. Among these, caspase-3 is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types and helps in determining the initial stage of apoptosis. Hyperglycemia induces apoptosis and causes damage to many organs and systems including the reproductive system [12]. Diabetes mellitus induces apoptosis by regulating signal molecules such as Bcl-2/Bax/Caspase-3-9 in the apoptosis pathway [13]. Diabetes mellitus causes pulmonary fibrosis by increasing collagen fibers in the lung parenchyma [14,15]. Cytokeratin 19

(CK19) is a kind of cytoskeleton element for many epithelia including bronchial epithelium [14]. In addition, it has been reported that CK19 which is a kind of cytoskeleton element for many epithelia including bronchial epithelium is expressed due to lung cancer, especially from type 2 pneumocytes [16,17]. However, no studies are reporting the localization of CK19 in lung tissue. In this study, we hope to provide the first understanding of the mechanism of damage in lung tissue due to diabetes and the expressions of Caspase3, CK19, and VEGF in the literature and we hope to benefit from the development of treatment methods with the studies which are planned to be carried out.

MATERIAL and METHODS

Animals

The animals used in the study were obtained from the Experimental Animal Research Laboratory of Cumhuriyet University Faculty of Medicine. The rats were housed at 26-28°C with a 12 h light:12 h dark cycle and free access to standard diet and water stainless steel cages. All procedures were approved by the Ethical Committee (Cumhuriyet University, 65202830-050.04.04-225)

Twenty Wistar albino male rats were used in this study. The control group was divided into 4 equal groups on the 7th day (n = 5), the control group on the 14th day (n = 5) and the DM group on the 7th day (n = 5) and the DM on the 14th day (n = 5). One dose of STZ (60 mg/kg) dissolved in citrate buffer was given i.p. to an animal in DM group. Citrate buffer (vehicle) was given i.p. to the animals of the control group. Blood samples were collected after 48 h in the diabetic group, and those with glucose levels higher than 140 mg/dL were included in the study. On days 7 and 14 of the experiment, rats were anesthetized with a cocktail of ketaminehydrochloride (50 mg/kg) and xylazine (5 mg/kg) which were administered i.p. before the animals were killed [18].

Histology

Lung tissues were fixed in 10% buffered neutral formalin for 24-48 h at room temperature, then washed with tap water, dehydrated through 70, 80, 95 and 100% alcohol, cleared in two baths of xylene, embedded in paraffin and sectioned at 4-6 μ m. Paraffin sections were stained with Hematoxylin-Eosin (H&E), Van Gieson, Silver precipitation methods. Lung sectioning and staining are essential methods for studying lung development or lung pathology. H&E staining is most widely used in histology studies [19,20] and medical diagnosis Verhoess's Van Gieson staining detects elastic fibers and collagen deposition in tissues [21]. Silver impregnation lends itself especially well to the demonstration of the reticular connective tissue [22].

The stained sections were evaluated according to Zhou and Moore [23] methods under the microscope.

Immunohistochemistry

Briefly, after deparaffinization in xylene and rehydration, antigen retrieval was performed by microwaving sections in Citrate Buffer, pH 6.0 for 3x5 min. After cooling at room temperature, the sections were washed with phosphate buffer solution then they were treated with 3% hydrogen peroxide (Thermo, Rockford, USA) 10 min. The sections were washed three times with phosphate-buffered saline (PBS) (pH 7.6) (Sigma, Darmstadt, Germany). The sections were treated with blocking reagent for 20 min and incubated (90 min) at (37°C) with VEGF Ab1 (RB-222-R7; Neomarkers, Fremont, California), Caspase 3 Ab4 (RB-1197-R7, Neomarkers, Fremont, USA), Cytokeratin 19 (A53-B/A2.26 (Ks 19.1), ScyTec, Logan, USA)

Sections then were washed three times in PBS and incubated with biotinylated Goat anti-mouse secondary antibody for 10 min at room temperature. Sections were washed three times in PBS and incubated in streptavidin-HRP conjugate (TP-125-HL, Lab Vision, Fremont, USA) for 10 min at room temperature. After rinsing in PBS, the sections were incubated in DAB (3,3 'diaminobenzidine, TA-XXX-QHCX, Lab Vision, Fremont, USA) for 5 min for visualization. Sections were washed with distilled water and observed under the light microscope (BX51, Olympus, Japan) and photographed. The specificity of the antibody was previously confirmed. Negative control experiments were performed by omitting primary antibodies and were also used for comparison in case of residual expression.

RESULTS

In the diabetic group, the alveolar epithelium was gradually thinner than the control group, and the basal lamina of the pulmonary capillaries increased, and the alveolar parenchyma was steadily increased in the HE staining. Van Gieson staining was performed to see the density of collagen fiber, and it was determined that diabetic groups had more intense collagen life than the control group. In the case of silver precipitated preparations regarding reticular fiber density, the amount of reticular fiber is much higher in diabetic groups than in control (Fig. 1). In immunohistochemical studies, the localization of VEGF was gradually decreased in DM groups at 7 and 14 days compared to the control group, but the immunolocalization of CK19 and Caspase 3 increased on DM 7th and 14th days compared to the control group (Fig. 2).

DISCUSSION

Diabetes develops some chronic complications, including pulmonary dysfunction. Little is known about the effects of pulmonary dysfunction on diabetes. Findings in human diabetic subjects and experiments with diabetic rats thickened alveolar epithelium, pulmonary capillary basal lamina, centrilobular emphysema, and pulmonary microangiopathy. Other authors describe ultrastructural changes in pneumocytes, bronchiolar epithelium and connective tissue proteins in streptozotocin-induced diabetic rats. These anatomical changes may result from

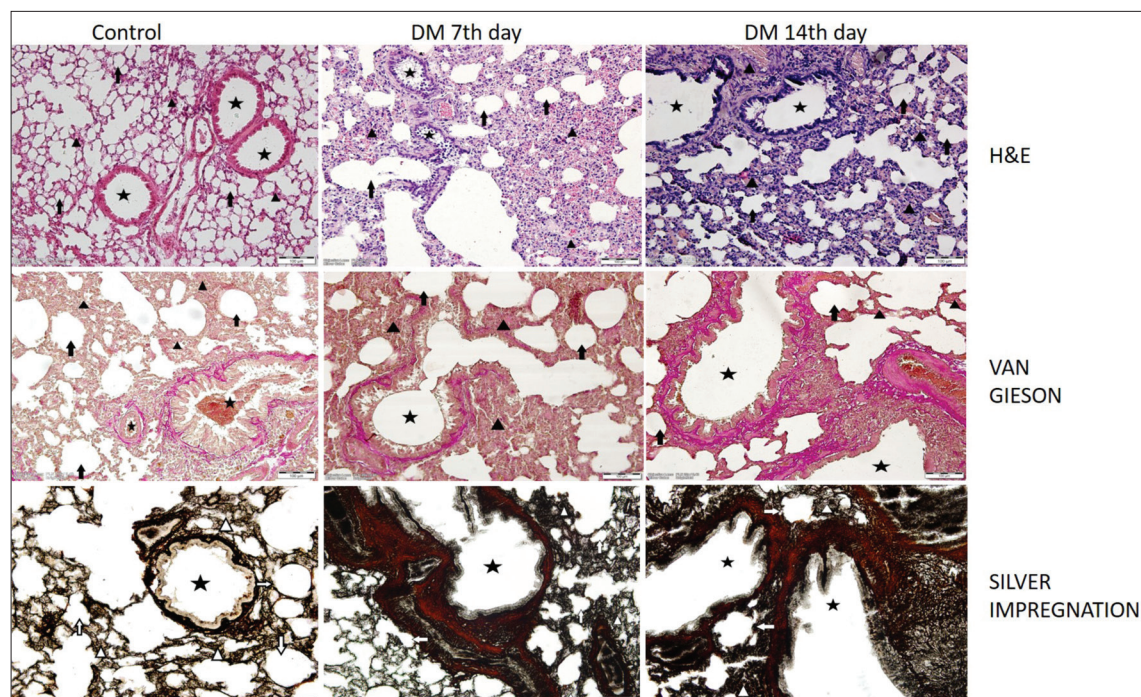


Fig 1. Comparison between H&E, Van Gieson and Silver impregnation staining between the control group, the DM 7th-day group, and the DM 14th-day group (20X Magnification). In the control and experimental groups, alveoli (→), bronchioles (*), and interalveolar septum (▲) are shown

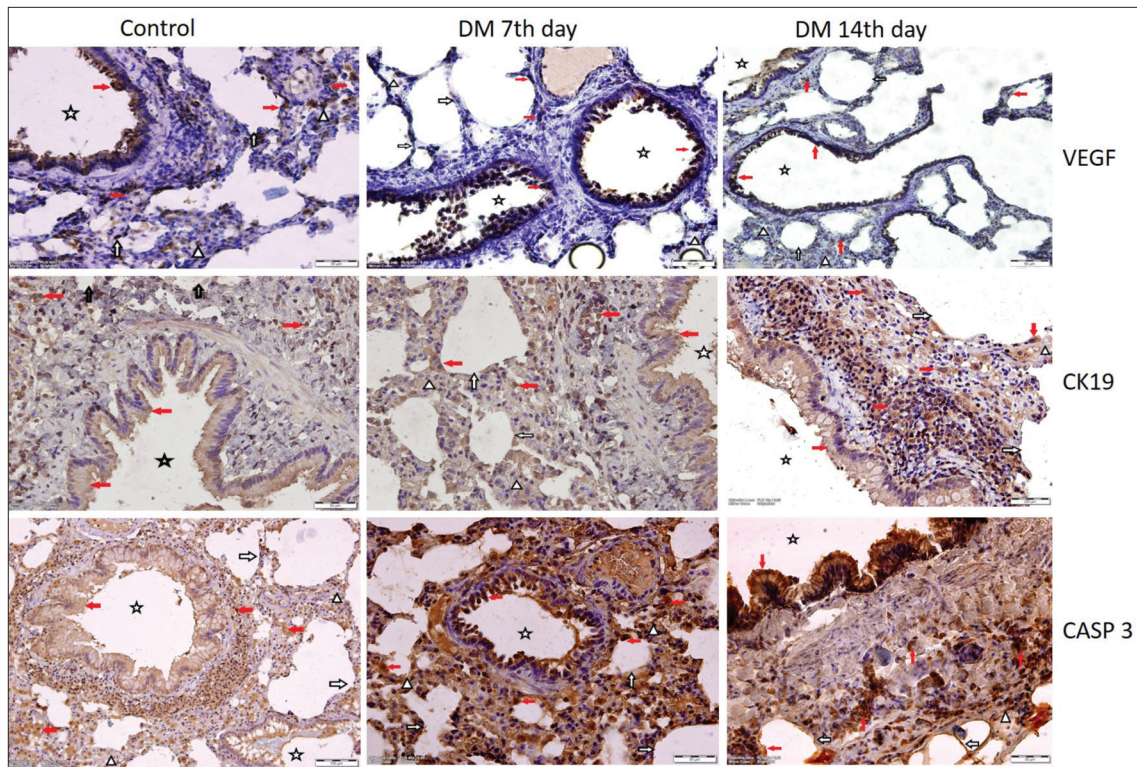


Fig 2. Control and DM groups immunostaining of VEGF, CK19 and Caspase 3 in the lung tissue bronchiolar, parenchymal and alveolar areas of DM groups (40X magnification). In the control and experimental groups, alveoli (→), bronchioles (*), interalveolar septum (▲) and VEGF, CK 19, CASP3 immunopositive cells (red →) in alveoli, bronchioles, interalveolar septum are shown

biochemical changes of the connective tissue components caused by proteins and non-enzymatic glycosylation of peptides caused by chronic high circulating glucose [24-26]. Histological evaluations were done on slides stained with H&E, Von Gieson, and silver impregnation. In the lung tissue of the diabetic groups, it was determined that the alveolar epithelium was progressively thinner than the control group, the basal lamina of the pulmonary capillaries decreased, and the alveolar parenchyma was gradually increased. However, it was seen that the amount of collagen fiber and reticular fiber in the diabetic group showed much more accumulation on day 14 than on day 7 (Fig. 1). Researchers have shown that [24,25], diabetes causes lung fibrosis by increasing the amount of collagen fiber in the lung tissue parenchyma. In this study, an increase in collagen accumulation was observed in the lung tissue of hyperglycemic rats treated with STZ. In previous studies, it is stated that collagen accumulation increases as a result of high glucose, high fat and high oxidative stress caused by diabetes [26].

According to the immunohistochemical findings in our study, CK19 localization was observed in 7 and 14 days in the diabetic group (Fig. 2). Any study that determines the immunolocalization of CK19 in diabetic lung tissue, is not found. CK19 is expressed in epithelial cells [16]. In some studies, however, CK19 has been reported to be profoundly expressed in lung injury [15] or lung fibrosis [15]

by hyperblastic type II cells. It was emphasized that excessive collagen accumulation occurred in the lung parenchyma and this resulted in fibrosis. In our study, CK19 immunostaining of the bronchiolar epithelium and alveolar type II cells were found to be more than 7 days on the 14th day with diabetes (Fig. 2).

However, immunostaining of Caspase 3 significantly increased in diabetic group compared to that of control groups. In particular, diabetes was observed to be more intense localization than 14 days at 7 days (Fig. 2). Hyperglycemia induces apoptosis and causes damage to many organs and systems [11,13]. Oxidative stress resulting from hyperglycemia has been reported to play a major role in the initiation of apoptosis [27,28].

However, when compared to the control group, VEGF immunolocalization was observed to be decreased gradually on the 7th and 14th days (Fig. 2). In diabetics, reduced glutathione peroxidase activity due to biochemical changes in lungs, NO-induced endothelial dysfunction, increased heparan sulfate level of the vascular endothelial basement membrane, structural changes in lung parenchyma, contraction of alveolar areas, and interstitial involvement. Pulmonary vessels, alveolar epithelial basement membrane, bronchial epithelium, and pulmonary capillaries are affected by diabetes. The pathology that is always associated with the disease is microangio-

pathy^[3,4,29]. Microangiopathy is the cause of the multiorgan complication of diabetes^[29].

In the pathogenesis, elevated glucose levels in the serum and extra enzymatic glycosylation of proteins and peptides in the extracellular matrix play a major role. As a result of nonenzymatic glycosylation occurring in the extracellular area of all organs, end products (adversely glycation end products, AGEs) are formed. These end products are highly concentrated in the vessel walls due to high blood pressure. By immunohistochemical methods, these end products can be shown in vascular tissue. Microangiopathy has been shown in renal, retinal and many other organs^[4]. Studies on diabetic rats and hamsters indicated that the target organ in the lung, thickening of the alveolar walls, increased collagen and elastin fiber in the basal lamina^[29]. It has a dense and extensive capillary system network in the lung. However, in the literature, there are very few studies investigating the effect of diabetes on lung capillaries. In particular, there are few studies that express VEGF expression in the diabetic lung^[30]. However, there is no study on immunolocalization of it. In a few studies, it was reported that testicular VEGF decreased in diabetic rats and the decrease in VEGF was associated with increased apoptosis and testicular damage^[9,31]. According to the studies, it is known that the pathogenesis of the complex biological processes involved in diabetic pulmonary dysfunction can make lung tissue one of the target organs of diabetes. An increase in lung parenchyma due to hyperglycemia is accepted as the source of fibrosis. Although this relationship has not been fully elucidated, our findings suggest that CK19 immunolocalization increases and VEGF immunolocalization decreases hyperglycemia-induced fibrosis. The increase in the number of apoptotic cells may be due to oxidative stress associated with hyperglycemia. In this study, it was determined that fibrosis increased with the change of reticular structure and collagen accumulation in diabetic lung parenchyma. However, the localization of VEGF in the endothelium of lung capillaries decreased, and vascular pathology developed. Diabetes-induced vascular pathology is caused by an increase in apoptosis of the vascular endothelium. Thus, immunolocalization of CK19 in diabetic lung tissue also increased and triggered lung fibrosis, leading to diabetic lung pathology. In conclusion, we believe that this study will be useful in understanding the mechanism of damage in the lung tissue due to diabetes and the development of treatment methods.

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