ORIGINAL ARTICLE

EXPLORING THE STREPTOZOTOCIN-NICOTINAMIDE MOUSE MODEL IN BALB/C MICE: GAINING INSIGHTS INTO DIABETIC PNEUMOPATHY

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Background: Diabetic pneumopathy has been a topic of interest in the present era, and it caught the eye during the COVID-19 pandemic. Extensive research provides compelling data supporting the association between diabetes and lung injury, establishing the lung as a principal organ affected by diabetes. However, the understanding of diabetic lung has always remained under shadow. For this purpose, we aimed to search for diabetic lung conditions by using a type 2 diabetic model in BALB/c mice. **Methods:** The study spanned from May to June 2023 at Ziauddin University. Nicotinamide, followed by streptozotocin, was injected into the mice. Mice were confirmed diabetic on the 7th day and housed for the onset of diabetic lung complications. After 28 days, mice underwent an oral glucose tolerance test and were then sacrificed, followed by the collection of bronchoalveolar lavage fluid and the extraction of the lungs. Lungs were fixed for histological analysis. **Results:** The presence of increased glycaemic levels, further verified by abnormal glucose tolerance, confirmed the establishment of the type 2 diabetic model. Additionally, the diabetic group showed lung fibrotic changes. **Conclusion:** The foundation of a type 2 diabetic model with the target of generating pneumopathy was accomplished successfully.

Keywords: Type 2 diabetes, Streptozotocin, Nicotinamide

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INTRODUCTION

Diabetes is a widespread, possibly catastrophic medical disorder that has become a serious public health issue in the 21st century. Diabetic complications include macrovascular disorders like coronary heart disease, stroke, and peripheral artery disease, as well as microvascular conditions like diabetic kidney disease, retinopathy, and peripheral neuropathy.[1]

In 1976, a decrease in lung elastic recoil was reported for the first time among young individuals with type 1 diabetes, suggesting that the lungs might be a target organ affected by the condition.[2] Subsequently, there has been a growing body of research investigations, primarily centred on the adult demographic, with a particular emphasis on individuals suffering from type 2 diabetes.[3] Epidemiological studies and surveys have indicated a potential association between diabetes and impaired respiratory functions particularly in a restrictive pattern.[4–7] Individuals with type 2 diabetes experience a decrease in pulmonary function and strength of the inspiratory muscles due to inadequate glycaemic control.[4]

Diabetic lung, also known as diabetic pneumopathy, is a recently recognized condition characterized by the slow progression of lung disease as a result of the microvascular complications associated with diabetes mellitus.[8] Diabetic pneumopathy gained prominence during the COVID-19 pandemic because the diabetic patients developed respiratory issues, required ICU hospitalization and had a higher mortality rate.[9] It has been emphasized that diabetic lung is neglected, suggesting a deficiency in understanding the complex pathophysiology underlying pulmonary disorders and efforts to resolve this complication.[10] Since type 2 diabetes is more prevalent in adults (90–95%) than type 1[11], our study aimed to examine the pulmonary alterations in type 2 diabetes mellitus using a Streptozotocin-Nicotinamide (STZ-NA) animal model.

METHODOLOGY

This preclinical experimental study was conducted at Ziauddin University from May'2023 to June'2023. The research protocol for this study was approved by the Animal Ethics Committee at Ziauddin University (Protocol number: 2023-01/AM/FHS).

The experiment utilized healthy male BALB/c mice, aged 10 weeks and with an average weight of 25 grams. These mice were kept in the Animal House Facility at Ziauddin University, housed in standard cages under controlled conditions at a temperature of 22–23 °C with a light-dark cycle of 12 hours. Throughout the study, the mice had unrestricted access to rodent chow and water, and underwent a period of acclimatization before the experiment began. The mice were then randomly divided into two groups: diabetic and control, each consisting of six mice.

A Type 2 diabetes model was developed in mice by subjecting them to a 6-hour fasting period in the morning. Nicotinamide was administered intraperitoneally at a dosage of 100 mg/Kg, given 15 minutes before the administration of Streptozotocin (BioShop Canada Inc., Burlington, ON, L7L 6A4).

Freshly prepared Streptozotocin in citrate buffer (*p*H 4.5) was then delivered intraperitoneally at a dose of 200 mg/Kg. On the seventh day, blood glucose levels were measured via the tail vein using a glucometer. Mice with fasting blood glucose levels between 150–300 mg/dL were selected for inclusion in the study. The group of mice induced with diabetes was monitored for 28 days to observe diabetic lung complications development. During this period, weight and fasting blood glucose levels were monitored weekly.

After 28 days, both the diabetic and control groups underwent an oral glucose tolerance test. The mice fasted for 6 hours in the morning before receiving an oral dose of 2 g/Kg of glucose. Blood glucose levels were then measured at various time points; before the administration of glucose, at 15-minute intervals up to 60 minutes, then 90- and 120-minutes post-administration.

The animals were euthanized using methods approved by the Ethics Committee of Ziauddin University. After euthanasia, the mice's lungs were removed. Broncho-alveolar lavage was performed by inserting a 22 G catheter into the trachea and instilling and withdrawing 1 mL of PBS three times. The Broncho-alveolar lavage fluid was collected, centrifuged at 1,500 rpm for 5-minutes, and the supernatant was stored at -80 °C for protein analysis. The left lung tissues were placed in a 10% formaldehyde solution for fixation, dehydrated through successive alcohol solutions of varying concentrations (70%, 80%, 90% and 100%), cleared with xylene, and then underwent paraffin infiltration and tissue embedding. Tissue sections measuring at a thickness of 4µm were obtained using a microtome, and slides were made subsequently for histological evaluation.

The tissue sections embedded in paraffin underwent a deparaffinization and rehydration process using xylene and alcohol, followed by water baths. Then, the slides went through haematoxylin staining for about 10-minutes. After staining, they were rinsed with water and placed in an acid solution for differentiation until the sections displayed a blue coloration. Eosin staining followed for 2-minutes, accompanied by a brief water wash. Subsequently, the slides underwent dehydration using graded alcohols before being cleared in xylene, mounted, and examined under a light microscope (Nikon Eclipse Ts2R-fl) at 10× magnification. The assessment of fibrotic changes utilized the semi quantitative Ashcroft histological index which has a scoring scale ranging from 0 to 8.[12] An Ashcroft score was allotted to each field aiming to allocate odd-numbered grades; intervening even scores (2, 4 or 6) between two odd numbers were assigned where decision-making became challenging. Finally, the mean Ashcroft score was calculated from all fields of view.

Deparaffinied sections were utilized for Masson's Trichrome staining. The sections underwent treatment with Weigert's iron haematoxylin solution for 5-minutes, followed by thorough washing with distilled water. Subsequent incubation involved Biebrich scarlet-acid fuchsin solution for 5-minutes and extensive washing. Differentiation was then performed in a phosphor-tungstic acid solution for 10-minutes, followed by transfer to a light green staining solution for 5 minutes. After a brief rinse in distilled water, differentiation was carried out in a 1% acetic acid solution for 2–5 minutes. Following this step, slides were washed again with distilled water and immediately dehydrated using 95% alcohol, absolute alcohol, and xylene. The finalized process included mounting the slides for subsequent analysis under a light microscope. The images were analysed using the Masson's Trichrome option in Colour Deconvolution 2 plugin in Fiji.[13]

Statistical significance between means in two groups was analysed using independent sample *t*-test and for measures over time was carried out by 2-way ANOVA and Tukey's Multiple Comparison using GraphPad Prism-8.0 for Windows, GraphPad Software, Boston, Massachusetts USA, and $p \leq 0.05$ was considered significant.

RESULTS

First, we assessed the development of the STZ-NA Type 2 Diabetes Model. Following treatment with STZ-NA, the mice showed a significant increase in blood glucose levels (Figure-1A) and decline in weights (Figure-1B) over the entire 28-day period. Furthermore, the OGTT revealed impaired glucose metabolism with delayed and elevated rise in blood glucose levels compared to non-diabetic mice (Figure-1C), confirming our successful establishment of a non-obese type 2 diabetic model for the first time in BALB/c mice.

The lungs were examined for alterations using protein analysis through Broncho-alveolar Lavage and histological examination with the Hematoxylin and Eosin (H&E) and Masson's Trichrome staining method. The BAL fluid exhibited a notable rise in total protein levels in comparison to the control group, as illustrated in Figure-2, indicating exudation and inflammation.

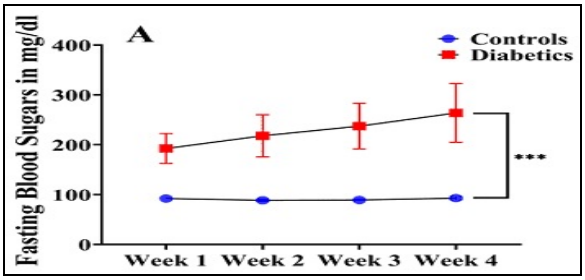


Figure-1A

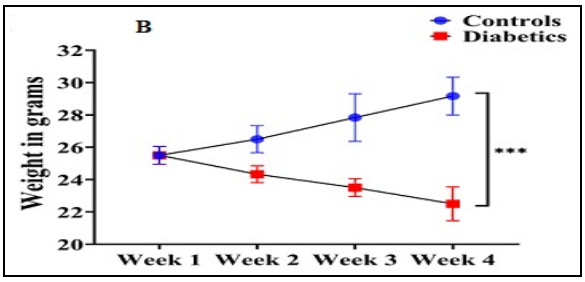


Figure-1B

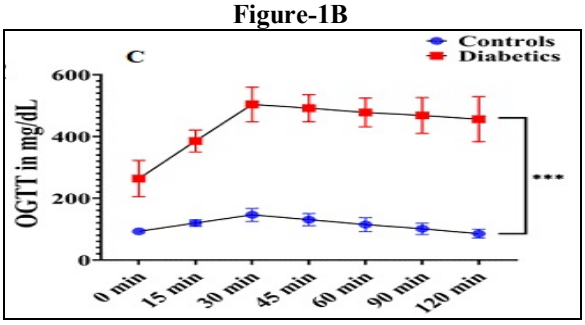


Figure-1C

Figure-1: Assessment of STZ-NA type 2 diabetes Model. A) Fasting Blood Sugar levels of mice, B) Weights in grams of mice over a period of 4 weeks, C) Oral Glucose Tolerance Test of mice over the duration of 120 minutes. ***p<0.001

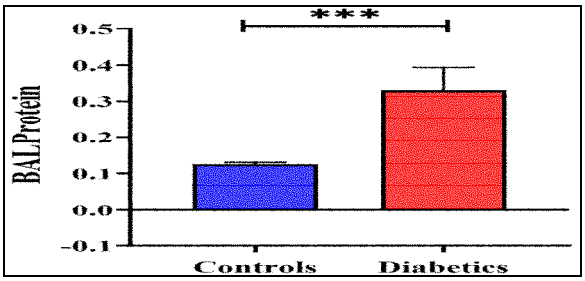


Figure-2: Total Protein levels in BAL fluid of mice with STZ-NA-induced type 2 diabetes were increased compared to controls. ***p<0.001

Histological analysis by H&E of the lung tissue showed significant alterations in the diabetic mice. The image shows areas of diffuse alveolar damage. Most of the alveoli were collapsed with thickened interstitium containing infiltrated inflammatory cells, and some dilated alveoli with damaged walls. Mild alveolar oedema was evident. Additionally, hypertrophy of bronchial epithelial cells, luminal obstruction, inflammatory cell infiltration, and thickening of smooth muscle around bronchioles was observed which are indicative of bronchoconstriction in the viable lung. Furthermore, congested blood vessels with increased wall thickness, encircled by infiltrating inflammatory cells, were noted. Ashcroft Score demonstrating increased lung fibrosis in mice with STZ-NA-induced type 2 diabetes compared to controls as shown in Figure-3.

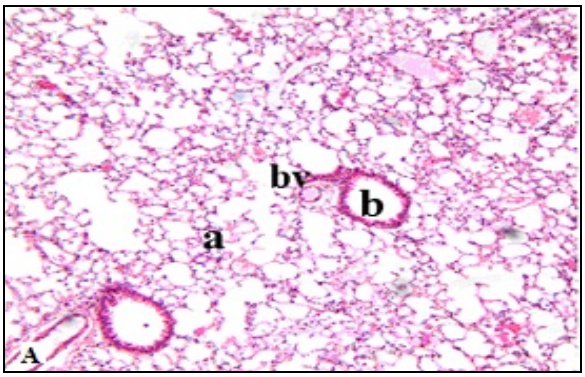


Figure-3A

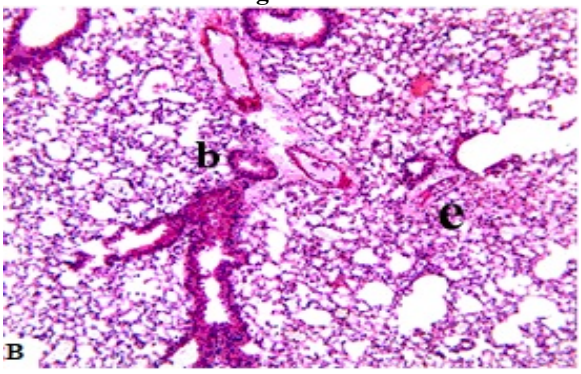


Figure-3B

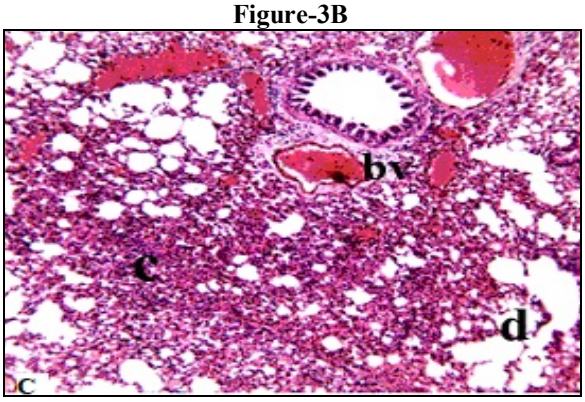


Figure-3C

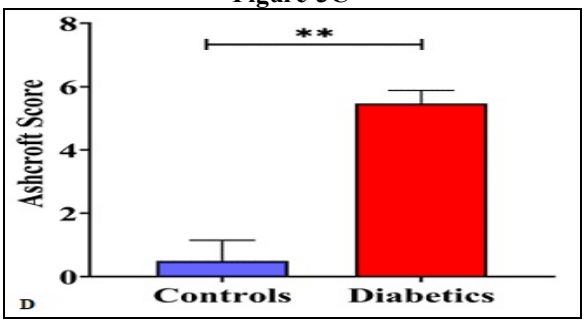


Figure-3D

Figure-3: H&E stained lung from Control vs Diabetic Mice: A) Control B & C) Diabetic Lung showing Bronchiolar, Vascular and Alveolar Changes D) Ashcroft Score. **p<0.01

a: Alveoli of normal lung, bv: Blood vessels, b: Bronchioles, c: Collapsed Alveoli, d: Dilated Alveoli, e: Alveolar oedema

Masson's Trichrome staining of the lung tissue confirmed the presence of collagen deposition in the lung tissue. The diabetic group exhibited a significant rise in the percentage area of collagen accumulation compared to the control group, indicating the development of fibrotic changes as shown in Figure-4.

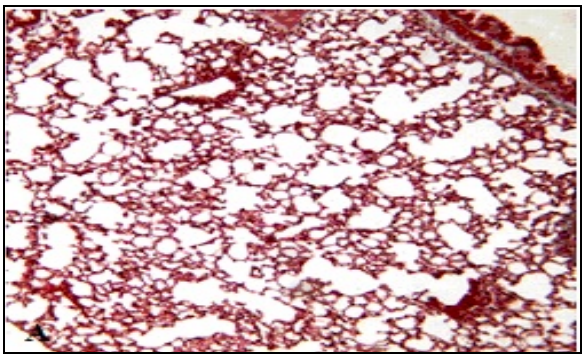


Figure-4A

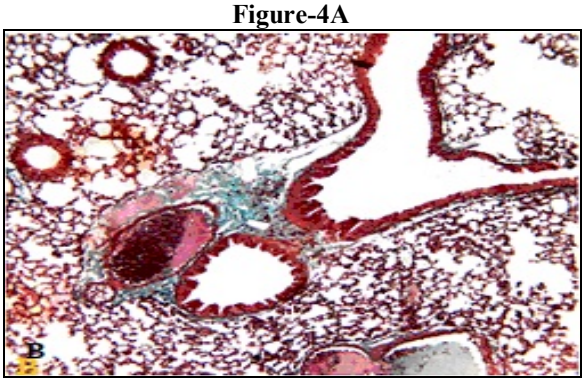


Figure-4B

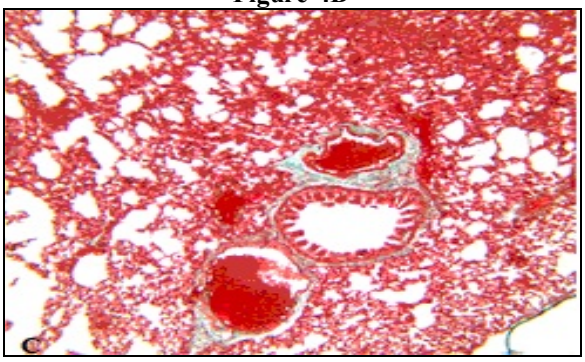


Figure-4C

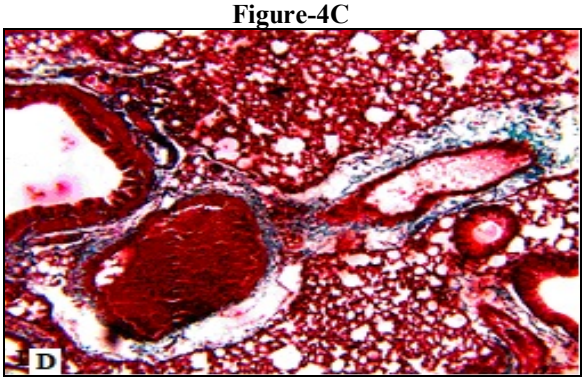
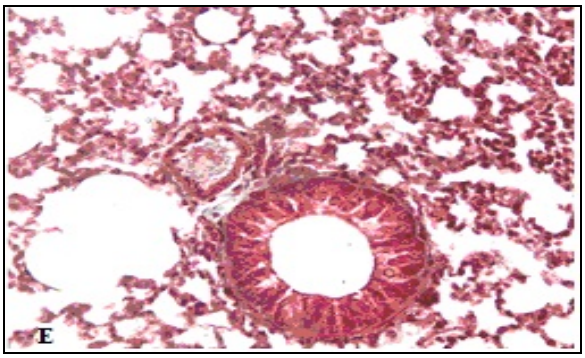


Figure-4D



Figudre-4E

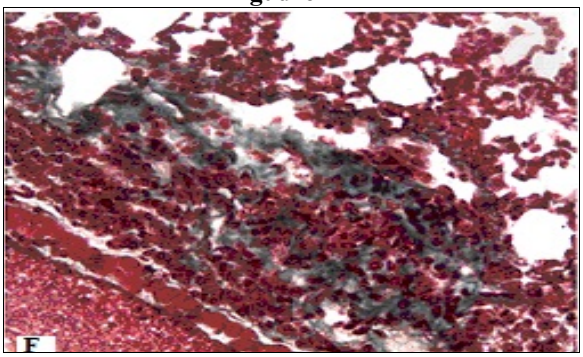


Figure-4F

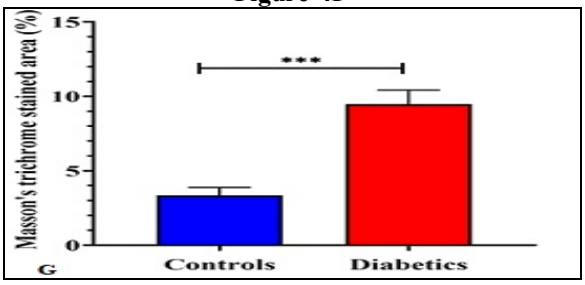


Figure-4G

Figure-4: Masson's Trichrome Staining: A-D images in 10× (A) Control (B, C and D) Diabetic lungs showing increased deposition of collagen around the alveoli, peri-bronchiolar and perivascular regions, E–F images in 40× E) control lung and (F) Diabetic Lung, (G) Percent area of collagen. ***p<0.001

DISCUSSION

Diabetic pneumopathy is an emerging area of research, shedding light on the intricate interplay between diabetes mellitus and pulmonary changes. According to the study, a notable proportion ranging from 10 to 42% of persons diagnosed with idiopathic pulmonary fibrosis exhibit comorbidity with diabetes.[14] As advocated by another study, the term 'Diabetes-induced pulmonary fibrosis' has been suggested as a replacement for idiopathic pulmonary fibrosis in individuals with diabetes[15], thereby reflecting a causal relationship between diabetes and the development of pulmonary fibrosis which needs to be elucidated.

We have used the STZ-NA induced Type 2 Diabetic Model in BALB/c mice to explore diabetic lung changes. Our results confirmed the presence of diabetes-related fibrotic alterations in the lungs. Diabetes is characterized by the existence of high levels of glucose in the blood, which is referred to as hyperglycaemia. In our study, it was shown that the STZ-NA induced diabetic group had a significant elevation in blood glucose levels following the confirmation of diabetes. The findings were in alignment with the existing body of literature.[16] Our study shows lung fibrotic changes consistent with the literature.[17] In another study, STZ-induced type 1 diabetic model exhibited mild oedematous changes, leukocytes infiltration in alveolar interstitium, and slight decrease in alveolar air space.[18] Another study reports similar findings; however, the fibrosis was localized predominantly in the lung interstitium.[19,20] However, our study reports Type 2 diabetes associated lung fibrotic changes, with respect to bronchiolar, vascular and alveolar changes which are consistent with the findings reported in type1 diabetic model of rats.[21] Another study reports findings consistent to our results, the control group showed thin alveolar wall, inter-alveolar septa, and a limited number of immune cell infiltrations. However, the diabetic group's lungs showed large accumulations of mononuclear cells, alveolar collapse, and some areas showed diffuse alveolar damage.[22] We used Ashcroft scoring to present our findings more objectively. Our study indicates higher score in diabetics than controls, indicating hyperglycaemia related lung damage, as supported by literature.[22]

Lung fibrosis involves the build-up of extracellular matrix, which we confirmed by Masson's trichrome stain. The diabetic group exhibited a significant rise in collagen levels in comparison to the control group. These findings align with the previous studies.[23,24]

The relationship between diabetes and lung disease is complex, involving genetic, molecular and physiological factors. The complex relationship between diabetes and pulmonary issues highlights the necessity for further experimental inquiries employing this type 2 diabetic model. Such investigations aim to elucidate the underlying pathophysiological mechanisms and pinpoint potential therapeutic targets for effectively managing diabetic lung complications.

CONCLUSION

The STZ-NA induced type 2 diabetic model serves as a significant framework for examining the impacts of diabetes on lung within various scientific realms. Our study delineated the association between diabetes and lung injury, characterized by immune cell infiltration and heightened collagen and extracellular matrix levels in the lungs, indicative of fibrosis. This model not only facilitates the investigation of the underlying molecular pathways of diabetes but also offers a robust platform for devising therapeutic strategies.

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REFERENCES

1. Tomic D, Shaw JE, Magliano DJ. The burden and risks of emerging complications of diabetes mellitus. Nat Rev Endocrinol 2022;18(9):525–39.
2. Schuyler MR, Niewoehner DE, Inkley SR, Kohn R. Abnormal lung elasticity in juvenile diabetes mellitus. Am Rev Respir Dis 1976;113(1):37–41.
3. Mameli C, Ghezzi M, Mari A, Cammi G, Macedoni M, Redaelli FC, *et al*. The diabetic lung: Insights into pulmonary changes in children and adolescents with type 1 diabetes. Metabolites 2021;11(2):69.
4. Al-Khlaiwi T, Alsabih AO, Khan A, Habib SH, Sultan M, Habib SS. Reduced pulmonary functions and respiratory muscle strength in Type 2 diabetes mellitus and its association with glycemic control. Eur Rev Med Pharmacol Sci 2021;25(23):7363–8.
5. Adeoti AO, Raimi TH, Fadare JO, Ibidapo R. Spirometric Indices in Type 2 Diabetes Mellitus Patients in a Nigerian Tertiary Institution. Eur J Med Health Sci 2021;3(3):10–4.
6. Choi HS, Lee SW, Kim JT, Lee HK. The association between pulmonary functions and incident diabetes: Longitudinal analysis from the ansung cohort in Korea. Diabetes Metab J 2020;44(5):699–710.
7. Rambaran K, Bhagan B, Ali A, Ali F, Toolsie S, Lobin R, *et al*. High prevalence of diabetes mellitus in a cohort of patients with chronic obstructive pulmonary disease in Trinidad, West Indies. Turk Thorac J 2019;20(1):12–7.
8. Rajasurya V, Gunasekaran K, Surani S. Interstitial lung disease and diabetes. World J Diabetes 2020;11(8):351–7.
9. Pugliese G, Vitale M, Resi V, Orsi E. Is diabetes mellitus a risk factor for CoronaVirus Disease 19 (COVID-19)? Acta Diabetol 2020;57(11):1275–85.
10. Kolahian S, Leiss V, Nürnberg B. Diabetic lung disease: fact or fiction? Rev Endocr Metab Disord 2019;20(3):303–19.
11. Rais N, Ved A, Ahmad R, Parveen K, Gautam GK, Bari DG, *et al*. Model of streptozotocin-nicotinamide induced type 2 diabetes: a comparative review. Curr Diabetes Rev 2022;18(8):58–69.
12. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. J Clin Pathol 1988;41(4):467–70.
13. Landini G, Martinelli G, Piccinini F. Colour deconvolution: Stain unmixing in histological imaging. Bioinformatics 2021;37(10):1485–7.
14. Wang D, Ma Y, Tong X, Zhang Y, Fan H. Diabetes mellitus contributes to idiopathic pulmonary fibrosis: A review from clinical appearance to possible pathogenesis. Front Public Health 2020;8:196.
15. Kopf S, Kumar V, Kender Z, Han Z, Fleming T, Herzig S, *et al*. diabetic pneumopathy —a new diabetes-associated complication: mechanisms, consequences and treatment considerations. Front Endocrinol (Lausanne) 2021;12:765201.
16. Birgani GA, Ahangarpour A, Khorsandi L, Moghaddam HF. Anti-diabetic effect of betulinic acid on streptozotocin-nicotinamide induced diabetic male mouse model. Braz J Pharm Sci 2018;54(2):e17171.
17. Jeon HY, Lee AJ, Kim EB, Kim M, Park WS, Ha KS. C-peptide attenuates hyperglycemia-induced pulmonary fibrosis by inhibiting transglutaminase 2. J Mol Endocrinol 2022;68(4):209–23.
18. Wang LM, Zhong NZ, Liu SJ, Zhu XY, Liu YJ. Hypoxia-induced

acute lung injury is aggravated in Streptozotocin diabetic mice. Exp Lung Res 2015;41(3):146–54.
19. Yang J, Tan Y, Zhao F, Ma Z, Wang Y, Zheng S, *et al*. Angiotensin ii plays a critical role in diabetic pulmonary fibrosis most likely via activation of NADPH oxidase-mediated nitrosative damage. Am J Physiol Endocrinol Metab 2011;301(1):E132–44.
20. Liu XY, Wei DG, Li RS. Ghrelin attenuates inflammation in diabetic lung disease by TLR4 pathway *in vivo* and *in vitro*. BMJ Open Diabetes Res Care 2023;11(2):e003027.
21. Onk D, Onk OA, Erol HS, Özkaraca M, Çomaklı S, Ayazoğlu TA, *et al*. Effect of melatonin on antioxidant capacity, ınflammation and apoptotic cell death in lung tissue of diabetic rats. Acta Cir Bras 2018;33(4):375–85.
22. Elewa YHA, Ichii O, Nakamura T, Kon Y. Pathological alternations of mediastinal fat-associated lymphoid cluster and lung in a streptozotocin-induced diabetic mouse model. Microsc Microanal 2021;27(1):187–200.
23. Kumar V, Agrawal R, Pandey A, Kopf S, Hoeffgen M, Kaymak S, *et al*. Compromised DNA repair is responsible for diabetes-associated fibrosis. EMBO J 2020;39(11):103477.
24. Bolat D, Ülger M, Baran M, Turan It, Yay A. Lung injury aggravated in Streptozotocin-induced diabetes: an experimental study. Cukurova Med J 2022;47(1):175–82.

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AM: Data acquisition, drafting, analysis and interpretation of data, critical revision
STA: Concept, design of project, revision, final approval
AH: Concept, design of project, revision
ARB: Concept, design of project, analysis and interpretation of data, revision, final approval
KJJB: Analysis and interpretation of data, revision

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Disclaimer:

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