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RESEARCH ARTICLE

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Type 2 diabetes mellitus stimulated pulmonary vascular inflammation and exosome biogenesis in rats

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Abstract

It has been shown that type 2 Diabetes Mellitus (T2DM) changes the paracrine activity of several cell types. Whether the biogenesis of exosomes is changed during diabetic conditions is the subject of debate. Here, we investigated the effect of T2M on exosome biogenesis in rat pulmonary tissue. Rats received a high-fat diet regime and a single low dose of Streptozocin to mimic the T2DM-like condition. A total of 8 weeks after induction of T2DM, rats were subjected to several analyses. Besides histological examination, vascular cell adhesion molecule 1 (VCAM-1) levels were detected using immunohistochemistry (IHC) staining. Transcription of several genes such as IL-1β, Alix, and Rab27b was calculated by real-time polymerase chain reaction assay. Using western blot analysis, intracellular CD63 levels were measured. The morphology and exosome secretion activity were assessed using acetylcholinesterase (AChE) assay and scanning electron microscopy, respectively. Histological results exhibited a moderate-to-high rate of interstitial pneumonia with emphysematous changes. IHC staining showed an increased VCAM-1 expression in the diabetic lungs compared with the normal conditions ($p < .05$). Likewise, we found the induction of IL-1β, and exosome-related genes Alix and Rab27b under diabetic conditions compared with the control group ($p < .05$). Along with these changes, protein levels of CD63 and AChE activity were induced upon the initiation of T2DM, indicating accelerated exosome biogenesis. Taken together, current data indicated the induction of exosome biogenesis in rat pulmonary tissue affected by T2DM. It seems that the induction of inflammatory niche is touted as a stimulatory factor to accelerate exosome secretion.

KEYWORDS
exosome biogenesis, inflammation, pulmonary tissue, rat, type 2 diabetes

1 | INTRODUCTION

Most eukaryotic cells can release heterogeneous vesicles, commonly recognized as extracellular vesicles (EVs) which participate in intercellular communication.[1,2] It has been indicated that EVs can transfer numerous signaling cargo like nucleic acids, proteins, lipids, and so forth.[1–3] EVs can be uptaken via active or passive mechanisms, leading to stimulating/inhibiting certain signaling pathways under physiological and pathological conditions.[2] EVs may vary generally in terms of their size, composition, and way of generation.[1] Exosomes (Exo), a subclass of EVs, have

30–150 nm diameters and are being released by many cells following the fusion of multivesicular bodies (MVBs) with the plasma membrane tailored by endosomal systems.[1] The endosomal system consists of numerous effectors which can regulate Exo production, transfer, and release.[4,5] These nano-sized vesicles have multiplied important attention as a significant contributor to therapeutic targets and diagnostic biomarkers because of their strong link with the progress of many pathological conditions spanning from cancer to diabetes mellitus.[4,5] Some studies have suggested that Exo could play significant functions in the pathogenesis, complications, and also treatment of type 2 Diabetes Mellitus (T2DM).[5,6]

Considering the released data, T2DM has become a major health concern globally with the greatest influence and usually arises with insulin resistance.[7] T2DM can promote serious complications such as cardiovascular diseases, permanent wounds, kidney failure, nerve impairment, and vision loss.[8,9] The earliest experiment reported that the amount of platelet-derived EVs was increased in T2DM associated with the development of atherosclerosis.[10] These features indicated that EVs can induce/mediate the process of inflammation in almost cell types during pathological conditions. Based on data, the promotion of metabolic diseases mainly T2DM is related to the alteration of exosomal cargo originating from several cell types.[11] In EVs collected from rat diabetic cardiomyocytes, the increased levels of miR-320 can blunt angiogenesis capacity in cardiac tissue.[12] Likewise, Exo-derived adipocytes transfer miR-155 and induce polarization of macrophages toward M1, resulting in active inflammatory response and insulin resistance in the mouse model.[13] In line with the detrimental properties of T2DM on cardiovascular tissue, these conditions can exert toxic effects on the lungs.[14] At the tissue level, T2DM causes several pathological alterations like inflammation and fibrosis.[15] Considering the critical role of Exo on the transfer of proinflammatory factors during several pathologies, elucidiation of Exo biogenesis under T2DM can give us invaluable information about the critical role of cell paracrine activity on the pathology of lungs. Besides, understanding the role of Exo in T2DM is not only important for addressing cell biological questions in diabetic conditions but also vital to open novel avenues for their clinical application. Here, we designed a rat model of the T2DM model using a high-fat diet (HFD) followed by streptozotocin (STZ) treatment, and the status of Exo biogenesis was investigated in pulmonary tissue.

Significance statement

Type 2 Diabetes Mellitus (T2DM) modify the paracrine activity of cells. We explored the effect of T2M on exosome biogenesis in rat pulmonary tissue using an in vivo rat T2DM model. Findings showed an increase in exosome biogenesis in pulmonary tissue of diabetic rats. It appears that the induction of inflammatory responses is touted as a stimulatory factor to up-regulate exosome secretion.

2 | MATERIAL AND METHODS

2.1 | Animals

In this study, 10-week-old male Wistar rats weighing 210–230 g were used. Rats were supplied with food and water and kept on a 12-h light/dark cycle with the standard heat (24°C–26°C) and standard humidity before experiments. After 2 weeks, 16 animals were randomly allocated into the following two groups ($n = 8$); control diabetic groups. The control rats were fed a normal chow diet. In the diabetic group, rats received HFD (48% carbohydrate, 32% fat, and 20% protein) for 4 weeks and then a single dose of STZ (35 mg/kbw) intraperitoneally.[16] Rats were fed for another 8 weeks (Figure 1A). After the completion of the experimental procedure, animals were anesthetized using an overdose of Ketamin and Xylazine and euthanized. Lungs were taken for histopathological examination and analysis of Exo biogenesis (Figure 1C). All experimental procedures followed the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the ethics committee of Tabriz University of Medical Sciences (No: IR.TBZMED.VCR.REC.1399.369).

2.2 | Histopathological analysis

Lungs were submerged in 10% formalin solution and paraffin-embedded blocks were cut into 5-μm-thick sections. After that, the slides were stained with hematoxylin-eosin solution and imaged using an Olympus microscope (Model: BX41).

2.3 | Monitoring vascular cell adhesion molecule 1 content using immunohistochemistry

To this end, 5-μm-thick sections were incubated with 3% H_2O_2 for 10 min to neutralize endogenous peroxidase activity. Samples were put in citrate buffer (pH = 6.0) and autoclaved at 15 psi for antigen retrieval. The procedure was continued by the incubation of samples with antivascular cell adhesion molecule 1 (VCAM-1) antibody for 30–60 min. After three washes with TBST, slides were incubated with secondary horseradish peroxidase (HRP)-conjugated antibody for 30 min. A 3, 3′-Diaminobenzidine was used as a chromogen to detect VCAM-1 positive cells. Nuclei were stained with Mayer's hematoxylin solution.

2.4 | Quantitative real-time polymerase chain reaction analysis

To monitor the transcription of genes involved in Exo biogenesis (Rab27 and Alix) and inflammation (IL-1β) were measured using real-time polymerase chain reaction (PCR) analysis (Table 1). About 50 mg of lung tissues were collected and total RNA content was extracted using RNA Extraction Kit (YTA). Using the cDNA Synthesis Kit (YTA), 1 μg RNA in both groups was reverse-transcribed into cDNA. The PCR reaction was

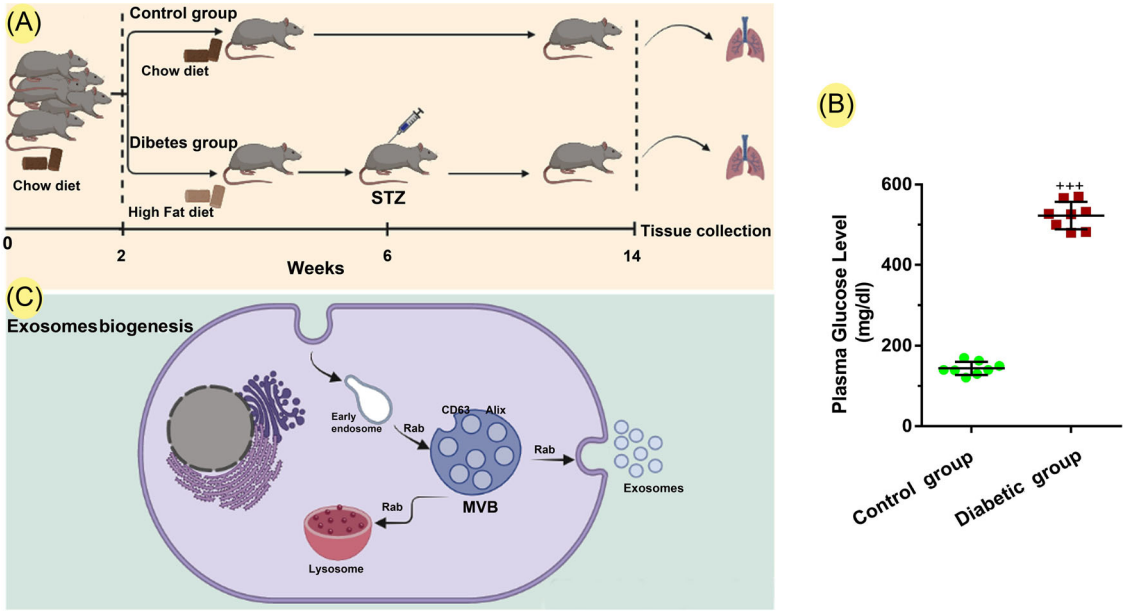

FIGURE 1 (A) Experimental design. (B) Plasma glucose levels increased in the diabetic group. (C) The exosomes biogenesis signaling pathway was measured in lung tissues. ***$p < .001$ diabetic group versus control group. Statistical analysis was done using GraphPad Prism using a *t*-test. Data are means ± SEM ($n = 8$). Exosomes, exo; MVB, multivesicular bodies; SEM, scanning electron microscopy; STZ, streptozotocin.

TABLE 1 Primer list

Gene/NCBI ACC. #	Product length	Annealing temperature	Forward	Reverse
β-actin/NM_031144.3	104	60	TGACAGGATGCAGAAGGAGA	TAGAGCCACCAATCCACACA
IL-1β/NM_031512.2	109	59	AGCTACCTATGTCTTGCCCG	TCGTCATCATCCCACGAGTC
Rab27b/NM_053459.1	86	58	GCTGGACCAAAGGGAAGTCA	TCGCCGCACTTGTTTCAAAG
Alix/NM_001029910.3	80	59	AGTCGAGAGCCTACTGTGGA	ACTTCTTGAGCTTGGGCCAG

Abbreviations: ACC, Acetyl-CoA carboxylase; NCBI, National Center for Biotechnology Information.

done using Rotor-Gene 6000 instrument (Corbett Life Science) and SYBR Green method.[17] The expression of each gene was normalized to an internal housekeeping gene (β-actin) using the $2^{-\Delta\Delta Ct}$ formula.

2.5 | Western blot analysis

To this end, we extracted total protein content using radio-immunoprecipitation assay buffer containing an antiprotease cocktail. Samples were kept in lysis buffer for 30 min and vigorously centrifuged at 4°C for 20 min at 13,000 to achieve supernatant with total protein contents. Protein concentration was measured using bicinchoninic acid BCA and about 40 µg of lysates were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After transferring into the polyvinylidene fluoride membranes and blocking in 1% Bovine serum albumin (BSA) solution (Sigma-Aldrich; 1 h), the samples were exposed to anti-CD63 antibody (Cat no: MCA4754GA) in 5% BSA at 4°C overnight. Following three washes with TBST, secondary HRP-conjugated goat antimouse IgG secondary antibody (DB9571) was added to membranes for 1 h at room temperature. Subsequently, membranes were washed three times and exposed to enhanced chemiluminescence substrate (Roche). For normalization, β-actin (Cat no: sc-47778) was used. The band intensity was measured by ImageJ software (NIH).

2.6 | Bronchoalveolar lavage collection

Bronchoalveolar lavage (BAL) was collected using tubes connected to the trachea via the injection of phosphate-buffered saline (PBS) by five consecutive 1 ml into the tube and aspiration of content.[18]

2.7 | Isolation of Exo

For the isolation of Exo, BAL samples were centrifuged at 2000g for 20 min to remove debris. Then, the medium was filtered through a 0.22 µm filter. Finally, the medium was mixed with reagent A of Exo Isolation Kit (1:5 ratio; EXOCIB) and kept at 4°C overnight. The next day, samples were centrifuged at 2000 *g* for 1 h. Exo were collected and either lysed in protein lysis buffer or resuspended in PBS for further analysis.[18]

2.8 | Characterization of Exo

For morphological characterization, scanning electron microscopy (SEM) was used. Briefly, grids were loaded with a 50 µl Exo suspension and frozen-dried for 40 min. Then, a thin layer of gold was used to coat grids and electron photomicrographs were captured via SEM (MIRA3 FEG-SEM; Tescan) at 40 kV. An average size of isolated Exo was measured using ImageJ software (ver.1.4; NIH).

2.9 | Quantification of Exo-related markers

Exo quantitation was performed by an enzymatic, the-colorimetric acetylcholinesterase (AChE) assay (Cat no. BXC080).[19] In brief, reagent A containing potassium hexacyanoferrate (2 mm) and pyrophosphate (75 mm) was added to 50 µl BAL samples and kept at room temperature for 5 min. Next, the suspension was mixed with reagent B (s-butyryl-thiocholine iodide) and absorbance was obtained at 405 nm at three intervals using a microplate reader system (BioTek). Finally, AChE was expressed as IU per mg of protein.

2.10 | Statistical analysis

Statistical differences between groups were determined with a *t*-test via the GraphPad Prism 7 software. The results are presented as the mean ± SEM. *p* Values less than .05 (*), .01 (**), and .001 (***) were considered significant. Experiments were performed in triplicate.

3 | RESULTS

3.1 | Induction of T2DM

To confirm the induction and stability of diabetic conditions in rats, nonfasting blood glucose levels were measured using a glucometer one day before sacrifice. Results indicated that diabetic rats exhibited increased nonfasting blood glucose levels compared with the control rats ($p < .01$), indicating the promotion of diabetic condition using the current protocol (Figure 1B).

3.2 | Pathological changes

Bright-field imaging revealed that the thickness of interalveolar septa was increased after the induction of T2DM (Black arrows; Figure 2A). Interstitial pneumonitis was detected with massive immune cell recruitment into the interalveolar space. Along with these changes, the promotion of T2DM caused prominent emphysematous foci within the pulmonary tissue. These features showed that the induction of T2DM can contribute to pathological conditions in rat lungs.

3.3 | T2DM increased pulmonary tissue VCAM-1 content

VCAM-1 is an endothelial-associated surface molecule that can be increased in response to the production of proinflammatory cytokines such as IL-β.[20] Here, we monitored the protein content of

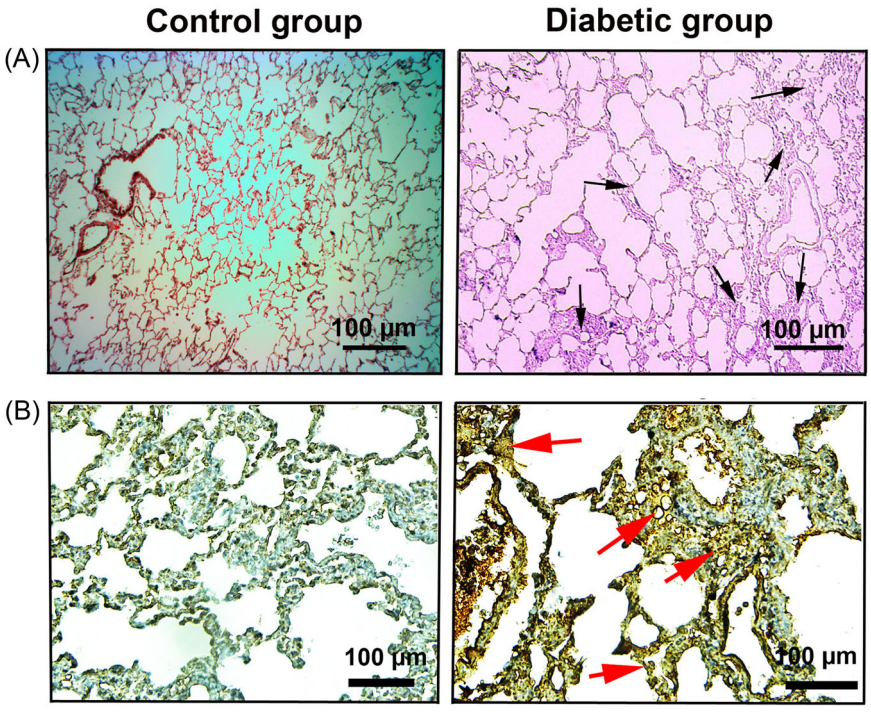

FIGURE 2 (A) Histopathological changes in rat lung tissue sections were monitored by H&E sating. (B) Immunohistochemical staining of rat lung tissue sections using VCAM-1 antibody. Scale bar: 100 µm. H&E, hematoxylin-eosin; VCAM-1, vascular cell adhesion molecule 1.

VCAM-1 and its relationship with IL-β during T2DM. immunohistochemistry (IHC) staining revealed a prominent increase of VCAM-1 positive cells within the diabetic pulmonary niche compared with the control lung samples (Figure 2B). These data showed that the promotion of T2DM causes the promotion of endothelial VCAM-1 within the pulmonary niche.

3.4 | T2DM upregulated the expression of levels of Alix, Rab27b, and IL-1β

Here, we also monitored the expression of genes involved in Exo biogenesis and inflammation. Data showed that the expression of both Alix and Rab27b was increased under diabetic conditions compared with the control samples ($p < .001$; Figure 3A). Likewise, the expression of IL-β was also induced, resulting in a proinflammatory condition ($p < .01$; Figure 3A). Taken together, one can assume that the promotion of T2DM can lead to inflammatory conditions and accelerated Exo biogenesis.

3.5 | T2DM increased the synthesis of endosomal tetraspanin CD63

Western blot analysis was used to monitor the protein content of CD63, a panendosomal tetraspanin. Data indicated a significant increase in CD63 levels under diabetic conditions compared with the normal status ($p < .01$; Figure 3B). These data showed that the induction of T2DM can alter intracellular levels of the endosomal system such as CD63 in the lungs, indicating the stimulation of Exo biogenesis.

3.6 | Exosome characterization

To this end, we monitored the morphology and AChE activity of Exo isolated from BAL using SEM imaging and AChE assay (Figure 4A–C). Ultrastructural imaging revealed the existence of a heterogeneous Exo population with different diameter sizes in the BAL (Figure 4A,B).

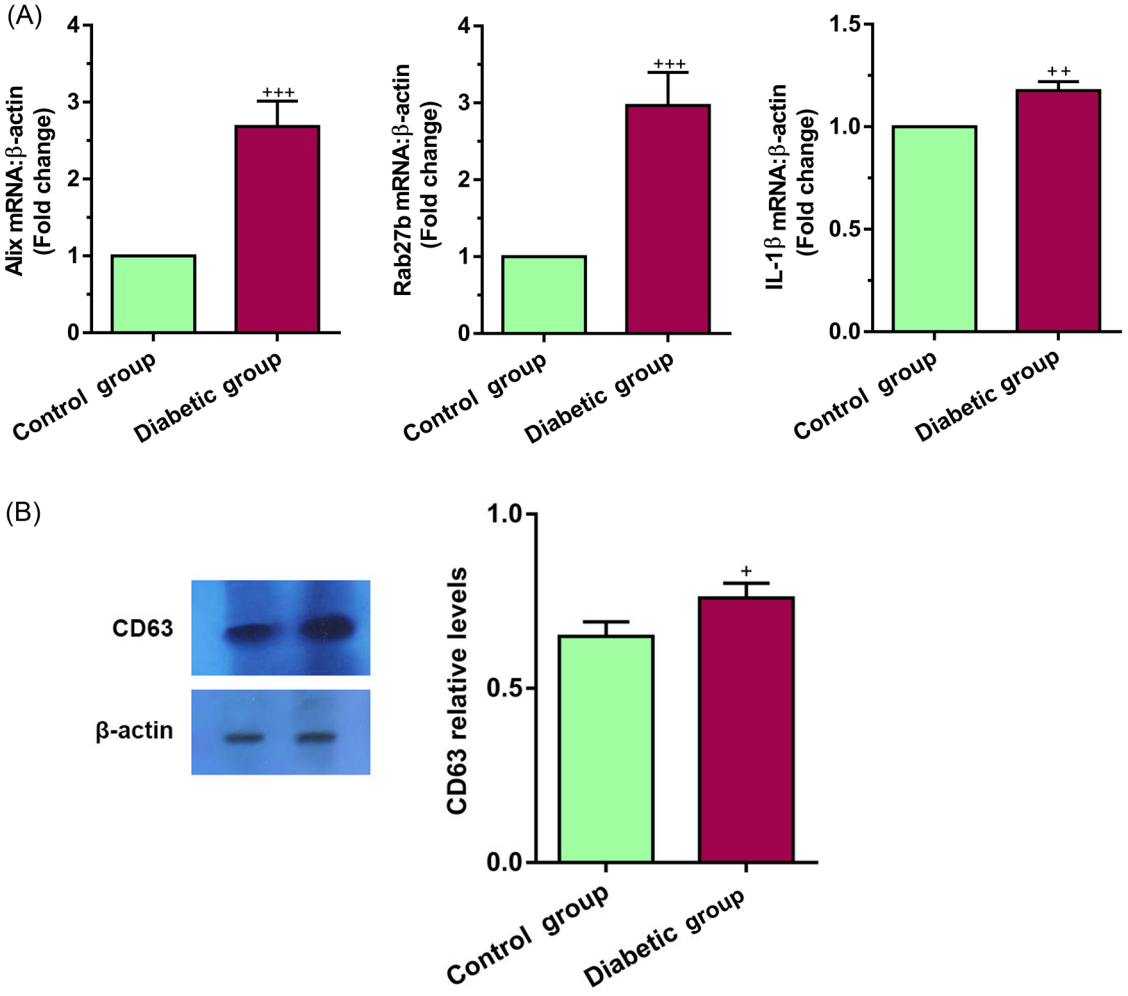

FIGURE 3 (A) Quantitative real-time PCR assay for expression of IL-1β, Alix, and Rab27b in rat lung tissues. (B) Representative western blot analysis for exosomal protein CD63 Rab27b in rat lung tissues. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ diabetic group versus control group. Statistical analysis was done using GraphPad Prism using a *t*-test. Data are means ± SEM, $n = 3$. PCR, polymerase chain reaction; SEM, scanning electron microscopy.

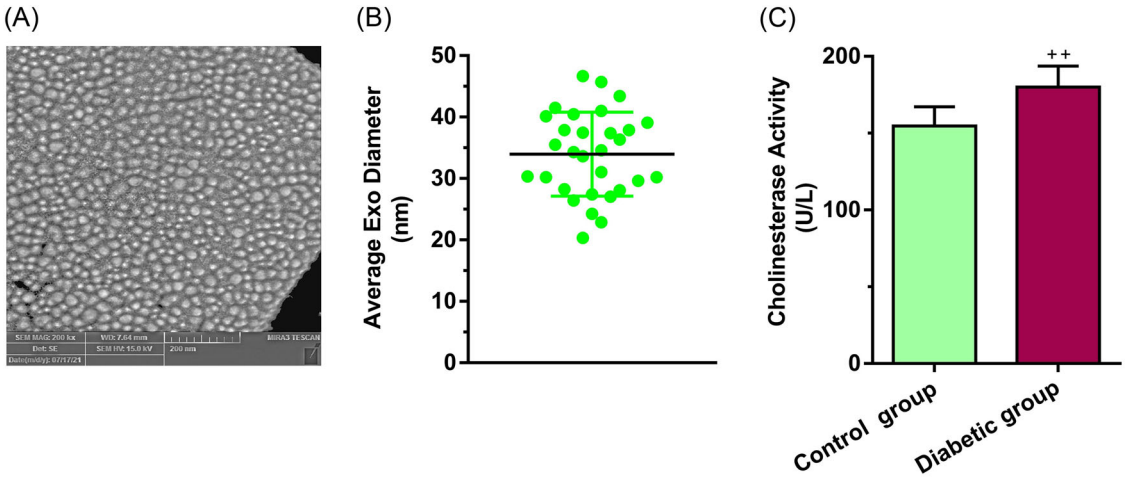

FIGURE 4 (A) Scanning electron microscopy photomicrograph of exosomes. (B) The exosome production was measured by acetylcholinesterase assay. *$p < 0.05$ diabetic group versus control group. Statistical analysis was done using GraphPad Prism using a *t*-test. Data are means ± SEM, $n = 3$. SEM, scanning electron microscopy.

Results showed that Exo secreted into BAL are heterogeneous with varied diameter sizes. We found no differences in the morphology and diameter of isolated Exo from control and T2DM BAL samples. AChE is closely associated with released Exo activity outside the host cells.[19] AChE assay revealed increased enzymatic activity in T2DM rats compared with the control group ($p < .001$; Figure 4C). These features demonstrated that T2DM causes Exo secretion into BAL indicated by higher AChE activity compared with the normal conditions.

4 | DISCUSSION

The possible impact of T2DM on the close relationship between vascular inflammation and Exo biogenesis remains to be elucidated. Here, we exploited a rat model of T2DM to investigate Exo biogenesis in lung tissues and its correlation with vascular inflammation. Our data indicated diabetes-related pathologies within the lungs indicated by interstitial pneumonitis and massive emphysematous changes. Besides, the expression of IL-1β and endothelial VCAM-1 was upregulated in T2DM lungs, indicating vascular inflammation. Like previous studies, we showed that T2DM promoted vascular inflammation and pulmonary tissue remodeling in a rat model.[21–23] It was suggested that several cytokines and proinflammatory factors released by immune cells like IL-1β can contribute to T2DM-related pathologies.[24,25] It is thought that IL-1β, a pyrogenic factor, facilitates inflammation at the cellular and tissue levels.[26] Our IHC data confirmed the pulmonary vessel inflammation, which correlated with induced endothelial VCAM-1.[27,28] VCAM-1 can be expressed via endothelial cells and acts as adhesion molecules. The induction of this factor under proinflammatory conditions (IL-1β) causes inflammation and the recruitment of immune cells into the extracellular matrix.[29–31] Therefore, it could be said that the increase in proinflammatory cytokines causes vascular inflammation and stimulated immune cell recruitment into the interalveolar space.

Exo is key factors involved in both physiological and pathological conditions.[32] In response to T2DM, we found that Exo biogenesis was induced in pulmonary tissue indicated by the expression of Alix and Rab27b and increased AChE activity. Alix is a vital molecule for many biological processes like Exo biogenesis, cargo loading, and intracellular trafficking. These activities are promoted via the close interaction of Alix with several effectors located on the MVBs surface.[33,34] Rab proteins belong to the GTPase proteins family involved in intracellular MVBs trafficking. Among different Rab proteins, Rab27b orient newly generated Exo toward the cytoplasm cortex and the plasma membrane.[35] Another key protein that we found increased in diabetic lung tissues was CD63. This factor participates in Exo biogenesis and cargo loading.[36] To further confirm Exo biogenesis and alteration under T2DM, we performed an AChE assay for BAL samples. Higher AChE activities in diabetic BAL suggest an increase in the amount of secreted Exo.[37] Based on previous data, the changes in the levels of glucose can alter Exo biogenesis. For example, under hypoglycemic conditions cardiomyocytes release high levels of Exo enriched with specific cargo.[38,39] In a study conducted by Almohammai et al., activated Exo biogenesis and secretion were shown in asthmatic rat lungs and BAL.[40] They concluded that increased Exo can be associated with inflammatory responses. More recently, our team found that the inflammatory responses were increased after immune cell recruitment into the lung tissues in a rat model of T2DM.[41] A growing body of evidence indicates that T2DM is associated with hypoxia, proinflammation, oxidative stress, and immune cell infiltration, which may modulate Exo biogenesis in several cell types.[42,43] It seems that the promotion of vascular inflammation can stimulate subsequent immune cell recruitment into the lung parenchyma, leading to immune cell chemotaxis and secretion of inflammatory cytokines under pathological conditions. The production of Exo by immune cells and inured cells within the lung parenchyma under T2DM conditions can reflect the metabolic status and changes in parent cells.[32] A reason for an

increased Exo biogenesis would be that the secretion of Exo is a compensatory mechanism to affluence inflammation and avoid tissue impairment. In immune cells, this is can be done via the production of inflammasomes.[43,44] Nevertheless, in our study, it is obscure whether increased exosomal pathway activity concurred with inflammation responses may facilitate referring particular signals to other cells or that is adaptive machinery for cells to overwhelm stress environments by eliminating impaired biomolecules. This research could serve as a base for future studies, addressing the molecular mechanisms underlying Exo biogenesis/inflammation axis. Additional data collection is essential to determine exactly how inflammation affects Exo signaling in lung tissues. Another possible area of future research would be to investigate BAL exosome cargo and its impact on other cells. The present study faces some limitations that need more consideration. For example, we did not measure protein levels of Alix and Rab27b.

5 | CONCLUSION

This study revealed histological changes and inflammation in lung tissue in the rat model of T2DM. Further, T2DM-induced inflammation might be associated with increased Exo biogenesis. The present study may make an important contribution to the discovery of Exo's role in the pathogenesis of T2DM and possible therapeutic targets.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data are available upon request to the corresponding author.

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