

Evaluation of histopathological changes and exosomal biogenesis in pulmonary tissue of diabetic rats

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Abstract

Diabetes mellitus is one of the leading causes of death globally. The development of cellular injuries and impaired energy metabolism are involved in the pathogenesis of diabetes mellitus, leading to severe diabetic complications in different tissues such as the pulmonary tissue. Autophagy is a double-edged sword mechanism required for maintaining cell survival and homeostasis. Any abnormalities in autophagic response can lead to the progression of several diseases. Here, we aimed to assess the effect of diabetic conditions on the autophagic response and exosome secretion in a rat model of type 2 diabetes mellitus. The experimental diabetic group received 45.00 mg kg⁻¹ streptozocin (STZ) dissolved in 0.10 M sodium citrate. After 4 weeks, we monitored autophagic response and exosome biogenesis in the pulmonary tract using immunohistochemistry (IHC) and Real-time polymerase chain reaction analyses, respectively. Histological examination revealed the interstitial bronchopneumonia indicating enhanced immune cell infiltration into the pulmonary parenchyma. Immunohistochemistry staining displayed an enhanced autophagic response through the induction of microtubule-associated protein light chain 3 (LC3) and protein sequestosome 1 (P62) compared to the control rats. These changes coincided with significant induction of tetraspanin CD63 in STZ-induced diabetic rats relative to control rats. In conclusion, a diabetic condition can increase the autophagic response in pulmonary tissue. The accumulation of P62 in the pulmonary niche exhibits an incomplete autophagic response. The abnormal autophagic response can increase pulmonary cell sensitivity against injuries.

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Introduction

Diabetes mellitus (DM) is a common chronic disease and is characterized by hypoglycemic conditions due to insulin dysfunction and/or secretion. According to the recent data, it has been estimated that the prevalence of diabetic patients will have risen to approximately 7.70% of the total human population in 2030.¹ Of these diabetic patients, about 90.00% are suffering from type 2 DM (T2D) and the rest are diagnosed with type 1 DM. In both forms, life-threatening complications arise from the long-lasting hypoglycemia in patients.² Lowering blood sugar and maintaining it in a normal range can reduce the risk of macro- and micro-vascular complications.³⁻⁵ There are several treatments available for this condition; the most

common of which are oral blood sugar-lowering drugs such as sulfonylureas or insulin.^{6,7} However, current drugs are not very effective in the long-term control and maintenance of blood sugar balance, and it has even been shown that long-lasting use of them increases the susceptibility to dangerous and debilitating drug-induced side effects. Most previous studies have aimed to examine the detrimental effects of diabetic conditions on certain organs such as the cardiovascular system organs and kidneys. However, the lack of sufficient evidence about the other tissues such as the pulmonary tissue has led to the promotion of further studies to estimate diabetic condition impacts. It has been shown that DM can increase the possibility of pulmonary diseases such as asthma and chronic obstructive pulmonary disease.⁸

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Autophagy is an intra-cellular mechanism being triggered in response to surrounding insults and plays a key role in the dynamic activity of each cell in the pathological and physiological conditions.⁹ The activation of autophagy occurs in the early stages following injuries and coincides with the sequestration and releases of misfolded proteins and exhausted organelles out of the cells to restore the normal function of host cells.^{9,10} The phenomenon of sequestration facilitates the formation of autophagosomes subsequently fusing with lysosomes and constituting autophagolysosomes, leading to cargo enzymatic degradation and releasing.^{9,10} The procedure of autophagolysosome formation is initiated by engaging several factors such as beclin-1, LC3 and P62, regulating nucleation, elongation and maturation of autophagolysosome.^{9,10} Despite the protective role of autophagy in cell resistance during pathological conditions, the overactivity of autophagy machinery can make the host cells sensitive undergoing apoptotic changes.¹¹

It has been shown that autophagy can regulate the different activities of cells. For instance, previous studies have revealed the close interplay between autophagy and exosome (Exo) biogenesis.¹² The Exos are nano-sized vesicles, ranging from 50.00 - 200 nm, harboring several signaling molecules and participating in paracrine activity between the cells. The Exos are generated inside the cell in endosomes via membrane invagination into the luminal surface inside the multi-vesicular bodies (MVBs). Further fusion of MVBs with plasma membrane releases the Exo into the extra-cellular matrix.¹³ The Exos are identified by the existence of surface molecules on their membrane such as CD63, Tsg101 and Alix.¹⁴ In previously conducted studies, it has been shown that the promotion of diabetic conditions can alter the secretion capacity of Exo in host cells.¹⁵

Whether and how the promotion of diabetic condition can alter the secretion capacity of Exo in pulmonary tissue is the subject of area. In the current experiment, we aimed to examine the effect of diabetic conditions on the autophagic response and Exo secretion in a rat model of T2D induced by streptozocin (STZ).

Materials and Methods

Animals. In this study, 2- to 3-month-old mature Wistar rats were randomly divided into two groups including control and diabetic groups ($n = 10$; weight: 250 ± 25.00 g). All animals were obtained from the Animal Facility of the Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. All animals were kept in a standard condition with 300 - floor lamps and a regular 12 hr cycle at 23.00 ± 2.00 °C and a humidity of 55.00%. The noise level was about 50.00 decibels, and ventilation was set to 10 - 15 air changes per hr. All of the rats had free access to water and food. All experiments were performed two weeks after the

acclimatization of rats to the ambient conditions. All procedures were approved by the Research Deputy of Urmia University, Urmia, Iran (Ethical code: 4553).

Diabetes induction. To induce a diabetic model of rat, a single intra-peritoneal injection of STZ (Sigma-Aldrich; St. Louis, USA) at a dose of 45.00 mg kg^{-1} dissolved in 0.10 M sodium citrate (Merck, Darmstadt, Germany) with a pH value of 4.50 was done. Blood glucose was measured 72 hr after STZ injection. Blood glucose levels being more than 250 mg dL^{-1} were considered as a diabetic condition.

Pathological examination. Four weeks after DM induction, the animals were euthanized humanly using an over-dose of ketamine (100 mg kg^{-1} ; Alfasan, Woerden, Netherlands) and xylazine (10 mg kg^{-1} ; Alfasan). For pathological examination, the right lungs were selected. Samples were fixed using 10.00% buffered formalin solution (Merck). In this study, $5.00 \mu\text{m}$ thick sections were prepared from paraffin-embedded blocks and stained with Hematoxylin and Eosin (H & E; Merck) and Masson's trichrome solutions (Merck) to examine the pathological condition and tissue fibrosis rate. The autophagic response was studied in diabetic lungs using immunohistochemistry (IHC) staining. In short, $5.00 \mu\text{m}$ thick sections were deparaffinized and exposed to a 3.00% H_2O_2 solution to neutralize endogenous peroxidase activity. For antigen retrieval, slides were exposed to citrate buffer (pH = 6.50) for 20 min. Following washes with phosphate-buffered saline (PBS) solution, slides were incubated with anti-rat LC3 (1: 100; Elabscience Biotechnology Inc., Texas, USA) and P62 (1 :100; Elabscience Biotechnology Inc.) antibodies for 1 hr at room temperature. After several PBS washes, slides were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., California, USA) for 1 hr followed by PBS wash and addition of 3,3'-diaminobenzidine as a chromogenic agent. The slides were visualized through light microscopy (BX41, Olympus; Tokyo, Japan) and changes were compared between diabetic and control rats.

Real-time polymerase chain reaction (RT-PCR) analysis. To this end, the left lungs were immediately removed and stored at -80.00 °C until use. Total RNA content was extracted using an RNA extraction kit (Sinaclon, Tehran, Iran). The integrity and levels of extracted RNA were detected using the Nano Drop™ 1000 spectrophotometer (Thermo Fisher Scientific; Carlsbad, USA). For cDNA synthesis, a cDNA synthesis kit was used according to the manufacturer's recommendation (Sinaclon). The RT-PCR reaction was done using a SYBR Green RT-PCR kit (Sinaclon) and LightCycler 96 (Roche, Mannheim, Germany) instrument. The expression of CD63 was studied using an appropriate primer (Table 1). In this study, the fold change of target genes was calculated using $2^{-\Delta\Delta\text{CT}}$ after normalization against GAPDH.

Table 1. Primers used in this study.

Gene	Sequence	Annealing temperature (°C)
CD63	Forward : 5'- TTGGCGTAGCAGTTCAGGTTGT - 3'	60
	Reverse : 5'- ACGAGCATGATAAGAGACAGGAAG - 3'	
β -actin	Forward: 5' - TCCCTGGAGAAGAGCTACG - 3'	60
	Reverse: 5'- GTAGTTTCGTGGATGCCACA - 3'	

Statistical analysis. Descriptive analysis was reported as mean \pm standard deviation. Analysis of all raw data was performed by Student's *t*-test. The SPSS Software (version 20.0; IBM Corp., Armonk, USA) statistics software was used for all analyses. In the current study, three sets of experiments were conducted.

Results

Histopathological evaluation. Histopathological alterations revealed an extensive immune cell infiltration into the pulmonary parenchyma in the diabetic group compared to the control group (Figs. 1A and 2B). Besides, the accumulation of immune cells and the promotion of inflammation led to the occurrence of interstitial pneumonia and emphysematous foci (Fig. 1B). Along with these changes, Masson's trichrome staining exhibited the deposition of blue-colored collagen fibers around the bronchioles within the lung parenchyma in the diabetic group (Fig. 1D) relative to the control group (Fig. 1C). It was notified that these fibers are surrounded by infiltrated cells, indicating active fibrotic changes. Commensurate with these changes, we noted that the promotion of diabetic condition led to pathological changes in the rat model after 4 weeks.

Diabetic condition contributed to abnormal autophagy response. According to the IHC data, the induction of T2D increased protein levels of LC3 and P62 in the lung parenchyma (Figs. 2B and 2D). By contrast, very slight levels of LC3 and P62 were found in control pulmonary tissue (Figs. 2A and 2C). The significant increase of LC3 and P62 inside the lung parenchyma exhibited the stimulation of autophagic response against the hyperglycemic condition. Of note, intra-cellular accumulation of P62 infers that the procedure of loss of ubiquitinated and misfolded protein was not completed and can lead to accumulation of exhausted proteins and injured organelle inside the cells. In line with histological staining and IHC imaging, one can hypothesize that the abortion of autophagic response can lead to pathological changes in pulmonary tissue after the onset of a diabetic condition (Figs. 2A and 2D).

Type 2 DM increased CD63 expression in pulmonary tissue. To monitor the biogenesis of Exo, the RT-PCR analysis of CD63 was performed. This factor belongs to tetraspanin and is thought as a main component of the lysosomal membrane as well as Exo.¹⁶ The RT-PCR analysis showed a significant increase of CD63 in diabetic conditions compared to the control group (Fig. 3; $p < 0.01$). These data demonstrated that diabetic conditions can increase Exo biogenesis via the induction of CD63.

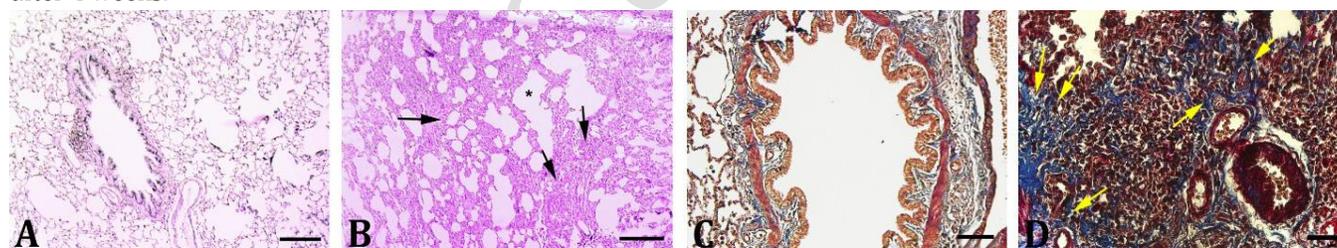


Fig. 1. A) Control group; B) Bright-field staining revealed enhanced immune cell infiltration into the pulmonary niche after the induction of inflammatory cells. These features led to the thickening of alveolar septa (black arrows) and generation of several emphysematous foci (asterisk), (H & E staining; bars = 250 μ m). C) Control group, and D) showed type I collagen deposition (blue-colored areas; yellow arrows) within the pulmonary parenchyma in the diabetic group (Masson's trichrome staining; bars = 50 and 20 μ m, respectively).

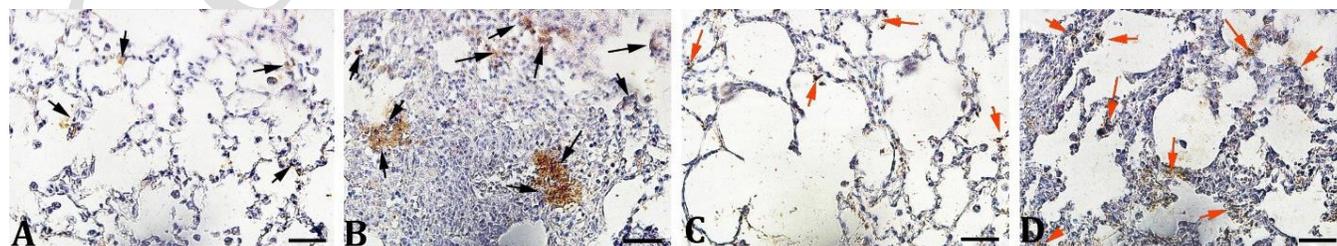


Fig. 2. Immunohistochemical staining of in pulmonary tissue. Black arrows indicate LC3 autophagic marker in A) Control group; B) Diabetic group. Data showed promotion of LC3 factors after the induction of diabetic conditions (bars = 100 μ m). Intra-cellular accumulation of P62 (Red arrows) showed an incomplete autophagy response in C) Control group, and D) Diabetic group (bars = 100 μ m).

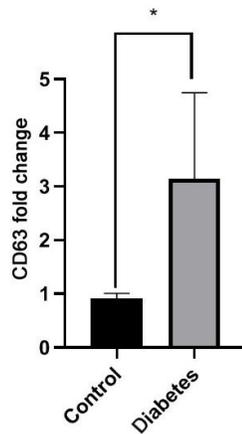


Fig. 3. Real-time polymerase chain reaction analysis of CD63 in pulmonary tissue after diabetes mellitus induction. * indicates statistically significant up-regulation of CD63 after diabetes mellitus induction ($p < 0.01$).

Discussion

The present work studied the detrimental effects of diabetic conditions on autophagic response and Exo biogenesis within the pulmonary tissue and its relationship with pathological conditions. In a recent study being done by Almohammadi *et al.* it was shown that inflammation induction in asthmatic mice can increase lung cells paracrine activation via Exo biogenesis.¹⁷ In another study, Deng *et al.* have proposed that adipose-derived Exos can link communications in macrophages and adipose tissues. They also found that these Exo-like vesicles can induce tumor necrosis factor- α and interleukin 6 production in macrophages and Toll-like receptor 4 (TLR4)/TIR-domain-containing adapter-inducing interferon- β (TRIF) pathway being necessary for insulin resistance.¹⁸ Data showed the induction of autophagy response indicating the high levels of LC3 and P62 within the diabetic lungs compared to the control group. According to our study, the increase of LC3 indicated active autophagosome formation; but, increased intra-cellular P62 levels stood for an incomplete autophagic flux. It is considered that the accumulation of P62 after autophagy machinery induction leads to incomplete autophagy response and reduced autophagic flux, resulting in caspase-mediated apoptosis.^{19,20}

Based on previous data, it has been shown that autophagic response can play a vital regulatory role in different tissues such as cardiac and renal tissues during diabetic conditions.²¹ The normal activity of autophagy can regulate cellular homeostasis and bioactivity; but, the aberrant autophagic response can lead to impaired homeostasis.^{22,23} Of note, the close relationship between the insulin receptor and P62 has been previously found.^{24,25} Indeed, the absence of insulin and insulin receptor activation induces autophagy signaling due to the

energy depletion.^{24,25} Therefore, it is logical to imagine that the occurrence of a diabetic condition can provoke autophagic signaling as a cellular compensatory mechanism that can be activated in the early stages after stressful conditions. Whether and how autophagy can restore cellular function during diabetic conditions is the subject of interest.

We further indicated that an aberrant autophagic response coincided with the increase of Exo biogenesis signaling molecules (CD63). It was suggested that the increase of autophagic response without sufficient flux (an accumulated P62) led to the induction of pathological changes and Exo biogenesis. Evidence showed the close interplay and shared molecular machinery of autophagy and Exo.¹² According to our data, the promotion of autophagic response led to an increased CD63 level.²⁶

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Conflict of interest

Authors declare there is no conflict of interest related to this study.

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