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ORIGINAL ARTICLE

High-fat diet-induced biogenesis of pulmonary exosomes in an experimental rat model

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Abstract

Background High-fat diets (HFD) have recently become a public health concern. We hypothesize that HFD induces exosomes biogenesis in the lung tissue of rat model.

Methods and results Sixteen adult male Wistar rats were fed with HFD or a regular chow diet for 3 months. The histopathological changes in lung tissues were measured by hematoxylin and eosin (H&E) staining. Bronchoalveolar lavage (BAL) was performed to assay exosomes by acetylcholinesterase enzyme (AhCE) activity. Real-time PCR (qPCR) was used to evaluate Rab27-b, Alix, and IL-1β expression, while the immunohistochemical examination was performed for CD81 expression in lung tissues. In addition, expression of IL-1β was detected by ELISA. We found pathological alterations in the lung tissue of HFD animals. AhCE activity along with the expression level of Rab27-b, Alix, and IL-1β was increased in HFD animals ($p < 0.05$). Immunohistochemical staining showed that expression of CD81 was increased in lung tissues of HFD animals compared with the control group ($p < 0.05$).

Conclusion Hence, HFD induced exosomes biogenesis and histopathological changes with IL-1β expression in rats' lung tissues.

Keywords Exosomes · AhCE activity · HFD · Obesity · Inflammation

Introduction

HFD have become a public health concern due to their role in prompting individuals to obesity and also diabetes [1, 2] as well as inducing overproduction of phosphatidylcholine and insulin resistance [3]. Unusually high levels of cellular phosphatidylcholine lipid impact energy metabolism, which is associated with insulin resistance and obesity [4, 5]. During obesity, oxidative stress and chronic systemic inflammation may affect the function of several organs [6]. In addition, obesity has been considered as important risk factor for several diseases including, asthma, chronic obstructive pulmonary disease (COPD), hypertension, liver and heart diseases, reproductive disorders, and several forms of cancers [7, 8]. An abundant vascular bed in the lungs may make it susceptible to inflammation in an obesity condition, resulting in lung dysfunction. In addition, the accumulation of excess fat tissue in the chest and abdomen regions causes disturbances in the movement of respiratory muscles, especially the diaphragm and intercostal muscles, which causes changes in the functional parameters of the lungs following

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breathing tests [8, 9]. Despite much research, the detailed mechanism behind lung tissue damage in obesity has not been fully determined. Many obesity-related diseases are associated with the hypertrophic and hyperplastic growth of adipocytes which results in adipocyte dysfunction [7, 8]. It is well well-known that the oxidative stress and pro-inflammatory conditions of the adipocytes may interrupt the secretions of cytokines, adipokines, and other metabolic regulators [10]. Furthermore, under obese conditions, exosomes play a vital role in facilitating the pathogenesis of many diseases [11]. The key roles of adipocyte-released exosomes linking obesity and its comorbidities have been reported in several studies [12, 13]. Exosomes are 30–150 nm membrane-bound vesicles producing from many cells like lung cells, and are present in most body fluids such as bronchoalveolar fluid (BAL) [14]. These vesicles contain many types of proteins, lipids, and RNAs that circulate in almost body fluids, participating in cell-to-cellcommunication by transferring their contents to nearby or distant cells [15]. The inflammatory condition may cause an alteration in exosomes secretion and cargo [16]. Moreover, there is an association between exosomes secretion and an increase in the level of inflammatory and pro-inflammatory interleukins in patients with COPD and asthma [17]. Therefore, it seems that lung inflammation in obesity may impact exosomes signaling in the lung tissue. Regarding the role of exosomes in obese conditions, in this study, we were looking at whether HFD would impact the secretion and biogenesis of exosomes in lung tissue of the rat HFD model. Understanding exosomes kinetics in lung tissue under HFD conditions may be useful for uncovering the physiological and pathological roles of these exosomes and may offer hints for the progress of exosome-based therapy.

Materials methods

Animals

In this study, we included 16 male Wistar rats weighing between 190 and 10 g, purchased from the animal laboratory unit of Urmia University of Medical Sciences, Urmia, Iran and kept under a 12-h light–dark cycle, temperature control (22 ± 1 °C), with free access to food and water.

Experimental groups

After 1 week of adaptation, rats were randomly allocated into 2 experimental groups, with 8 rats for each group: Control: fed a standard diet; HFD: fed a high fatty pellet diet for three months (Fig. 1).

Diet composition

The essential and standard diet in animal laboratories comprised 11% fat, 22% protein, and 67% carbohydrate. The HFD used in this research contained 32% fat, 20% protein, and 48% carbohydrates for 3 months [18].

Obtaining lung tissues

At the end of the third month, animals were euthanized by anesthetization with xylazine (10 mg/kg) and ketamine (100 mg/kg). The lungs were removed by surgery and immediately, after washing with physiological serum, they were placed in nitrogen and then saved at − 80 °C for downstream experimental panels.

Histopathological study

H&E staining was used to investigate the pathological changes caused by HFD in lung tissue. For this purpose, the lung tissues of animals were placed in a 10% formalin solution for fixation. Then, the fixed tissues were placed in different grades of ethanol from 70 to 100% each for 45 min to one hour for dehydration. Next, to prepare tissue blocks, tissue samples were placed in paraffin. Thin sections with a thickness of 4 to 5 µm were prepared and stained with H&E dye. In this method, hematoxylin turns the nucleus of the cells into purple blue and eosin turns the cytoplasm into pink. Finally, using an optical microscope, pathologic photographs were prepared.

Preparation of BAL

The lungs along with the lower part of the trachea (trachea) were removed surgically. For collecting the BAL, about 1 to 1.5 ml of physiological serum was injected into the lung through a blue branol inserted in the trachea, and then the fluid was instantly collected. This procedure was repeated for each animal a total of 6 times to collect about 5 ml of BAL from each animal.

AhCE activity

AhCE, associated with extracellular vesicles like exosomes, is an important qualitative tool for measuring the exosomes. For this purpose, according to a commercial kit recommendation (BXC080; Iran), 100 µl BAL samples were incubated with reagent A for 10 min at 37 °C. Then, reagent B was added and after 5 min incubation the absorbance, at five intervals, was calculated at 412 nm

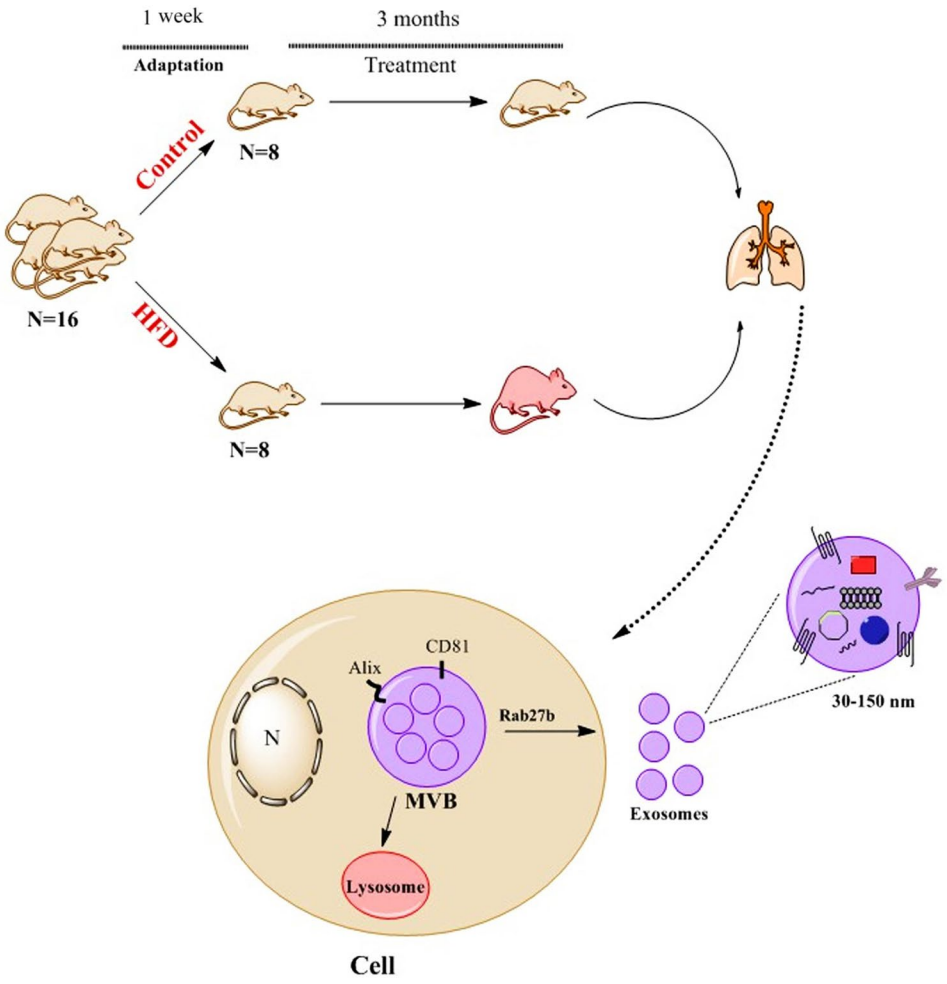

Fig. 1 Timetable and design of the current study

using the recommended formula of a commercial kit as the AhCE activity.

RNA extraction and cDNA synthesis

Total RNA was extracted from the equally homogenized lung tissue using a commercial total RNA extraction mini kit (YektaTajhiz; Iran). UV spectrophotometry technique was used to measure the RNA concentration by NanoDrop 1000 Spectrophotometer (BioTek). The 260 nm/280 nm ratio was also considered a qualitative factor. For reverse transcription, according to the cDNA synthesis kit's recommendation, 1 µg total RNA was denatured at 70 °C for 5 min, chilled on ice for 5 min and then Transcription Oligo (dT) was added. The samples were incubated with cDNA Synthesis components Mixed at 42 °C for 1 h. The reaction was stopped at 70 °C for 5 min. cDNA samples were stored at − 80 °C for additional amplification.

Real-time PCR (qPCR)

The expression level of Rab27-b, Alix, and IL-1β was determined by *q*PCR analysis. GAPDH was used as the housekeeping gene. The primers were designed using Oligo-7

Table 1 List of primers

Gene	Primer sequence (5′-3′)	
	Forward	Reverse
Rab27-b	GCTGGACCAAAGGGAAGTCA	TCGCCGCACTTGTTTCAAAG
Alix	AGTCGAGAGCCTACTGTGGA	ACTTCTTGAGCTTGGGCCAG
IL-1β	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGTG
GAPDH	TTGCCATCAACGACCCCTTCA	AGCACCAGCATCACCCCATTT

software and validated by NCBI database (Table 1). *q*PCR with the cDNA samples was then performed with SYBR green Kit as recommended by the manufacturer (YektaTajhiz; Iran). The PCR reactions were accomplished in a final reaction volume of 14 µl containing the final concentration of 0.5 µM of forward and reverse primers in MIC48 instrument (Swiss). Relative expression of genes was measured from Cq values by ΔΔCq method.

Immunohistochemistry

An immunohistochemical examination was used to evaluate the expression of CD81 in lung tissue samples. In brief, after fixation, tissue samples were embedded in paraffin blocks and mounted on glass slides. Samples were deparaffinized in xylene and then rehydrated in graded alcohol to prepare 5 µm thick sections. Then, to inhibit endogenous peroxidase activity, sections were treated with hydrogen peroxide (3%) for 10 min and washed with PBS. The slides were then treated with CD81 primary antibody overnight. Next, the slides were incubated with a secondary antibody and then with diaminobenzidine chromogen to visualize the marker. After adding Mayer's hematoxylin and washing with PBS, slides were evaluated under a light microscope and photographed using 5 fields from 3 sections from each rat.

ELISA

To investigate the expression of IL-1β, we used ELISA according to the manufacturer's recommendations (Rat IL-1β ELISA kit (ab100768, Abcam)) recommendations. Briefly, Samples were added into the wells for 60 min at room temperature (RT). Then, the wells were washed with PBST solution, and a biotinylated antibody was added. After washing with PBST solution, HRP-conjugated streptavidin was added to the wells. The wells are again washed, and a chromogenic 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added to the wells and kept for 15min for color change in proportion to the amount of IL-1 beta bound. The reaction was stopped by adding Stop Solution and the color was changed from blue to yellow, and the intensity of the color was measured at 450 nm.

Statistical analysis

The statistical analyses were performed using the program GraphPad Prism 8.0 (California, USA) by T-test. Data were considered statistically significant when p value was <0.05. Results obtained from three separate experiments are expressed as the mean ± SEM.

Results

Histopathological results

As shown in Fig. 2, we observed interstitial pneumonia (black arrow sign) in the lung tissue of the HFD group. No pathological lesion was seen in the control group.

Expression of the IL-1β gene was up-regulated in HFD rats

The results of both gene expression analysis and ELISA showed that the level of IL-1β gene expression in the lung tissue of the high-fat diet (HFD) group was significantly increased compared to the control group ($p<0.001$ and $p<0.01$; respectively) (Fig. 3A, B), which indicates the inflammatory induction in the lung tissue.

AChE activity increased in exosomes of HFD rats

The AChE activity test showed that the activity of this enzyme in exosomes derived from HFD rats was significantly higher than the control group ($p<0.01$) (Fig. 4).

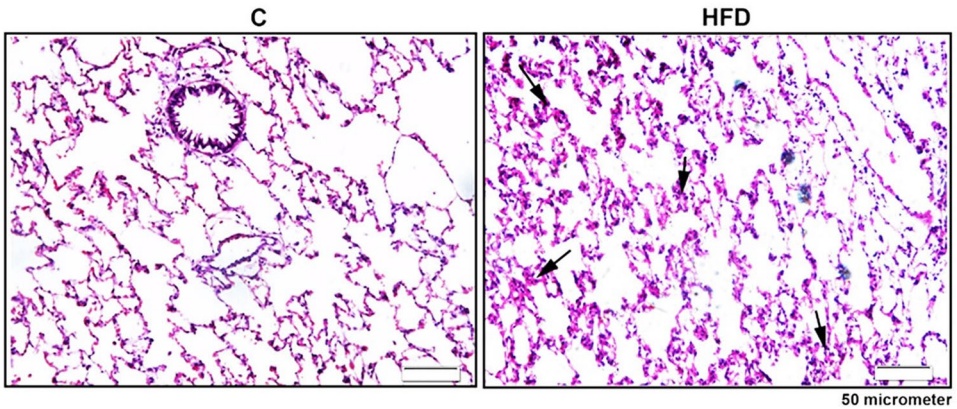

Fig. 2 Lung histology in different groups. Healthy lung tissue in the control group (C) and interstitial pneumonia (black arrow sign) in the HFD group are shown. Scale bar was 50 µm

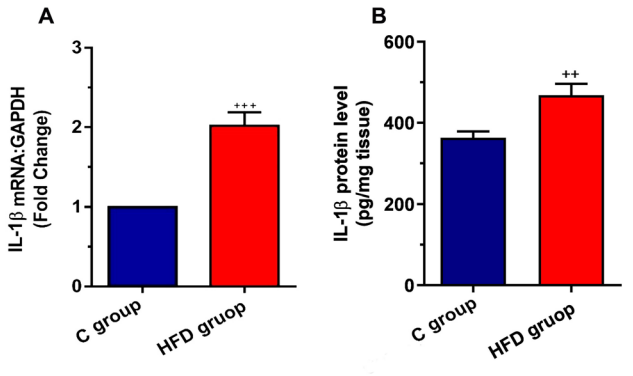

Fig. 3 Expression of IL-1β in rats. IL-1β gene expression in the control group (C) and the high-fat diet group (HFD) (**A**). The protein level of IL-1β by ELISA experiment (**B**). In each group, the results are presented as mean ± SEM for 8 animals. Student's t-test. $^{**}p < 0.01$; $^{***}p < 0.001$ versus the control group

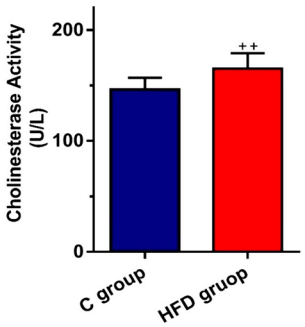

Fig. 4 The level of acetylcholinesterase (AChE) enzyme activity in the lung lavage fluid of the control group (C) and the high-fat diet group (HFD). In each group, the results are presented as mean ± SEM for 8 animals. Student's t-test, $^{**}p < 0.01$ versus the control group

Expression of Alix and Rab27-b was increased in HFD rats

QPCR analysis showed that the mRNA levels of Alix and Rab27-b genes were significantly increased in the lung tissue of the HFD group compared to the control group ($p < 0.001$ and $p < 0.01$; respectively) (Fig. 5A). These genes participate in exosomes biogenesis and secretion.

Expression of CD81 was increased in HFD rats

Immunohistochemical staining showed that the level of CD81protein was increased in the lung tissue of HFD groups (Fig. 5B). This may indicate an increase in the production of exosomes in the lung tissue of the HFD group compared to the control group.

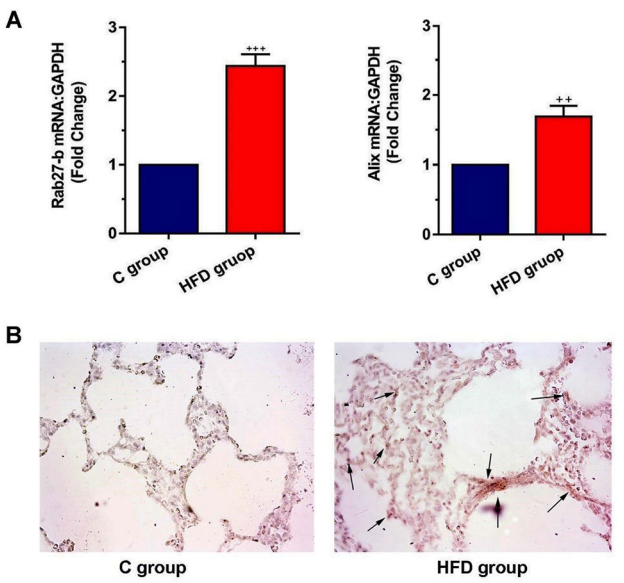

Fig. 5 QPCR analysis for Alix and Rab27b (**A**). Data showed that expression of Alix and Rab27b was increased in the high-fat diet group (HFD). Immunohistochemical examination for CD81 in the lung tissue samples of the control group (left) and the HFD group (right) (**B**). Black arrows show CD81 staining in lung tissue. The scale bar was 50 µm. In each group, the results are presented as mean ± SEM for 8 animals. Student's t-test, $^{**}p<0.01$ and $^{***}p<0.001$ versus the control group

Discussion

Exosomes secreted by lung cells into the BAL may contribute to homeostasis and regulate cellular signaling pathways in the lung tissues. Therefore, investigating the exosomes kinetics in the lung tissue can be useful for the diagnosis, treatment, and follow-up of various lung diseases [19, 20]. Previous studies have shown that lung tissue is vulnerable to chronic inflammatory conditions caused by obesity and overweight, however, the main mechanism of lung tissue damage in HFD/obesity has not yet been fully understood. Therefore, this study was conducted to investigate the biogenesis of exosomes in the lung tissue of HFD male rats. The results showed that using an HFD for three months caused inflammation and pathological changes in the lung tissue of male rats along with an increase in IL-1β expression and the incidence of interstitial pneumonia. These results are consistent with other studies that have shown that obesity and HFD cause inflammation and pathological changes in the lung tissue [21, 22]. Confirmed that obesity and excess adipose tissue contribute to pro-inflammatory conditions [23]. It seems that investigating other lung inflammatory cytokines may uncover the expression level of relevant genes, understanding the connection between HFD pathology and inflammatory cytokines. In the mice model of HFD, it was shown that the level of oxidative stress and inflammatory factors

mediators increased in the lung tissue and alveolar lavage fluid of the animal [21]. We found that rats receiving HFD have a high density of collagen fibers in the lung tissue, possibly due to pulmonary pneumonia and increased collagen expression in the connective tissue, suggesting the incidence of pulmonary fibrosis in these animals [24]. It seems that chronic systemic inflammation in obesity and abundant blood vessels in the lungs are prompting to lung inflammation, pathological changes, and a decrease in its function, however, its exact mechanism has not yet been fully determined. In keeping, we observed that the AChE activity of BAL samples of HFD rats increased, proposing an increase in exosomes/extracellular vesicles production [25]. Then, we were curious if the exosomes biogenesis pathway could be affected by HFD or not. Our finding demonstrated that exosomal markers including Rab27b, Alix, and CD81 were upregulated in tissue samples of HFD group. Alix and CD81 contribute to exosomes biogenesis and loading of biological cargo into exosomes [26], while Rab27b participates in the intracellular trafficking of exosomes [27, 28]. Our findings would seem to imply that exosomes biogenesis increased in the lung tissues of HFD rats [27, 29]. Previous studies have shown that the levels of circulating fat-derived extracellular vesicles were increased in humans and obese animals, however, decreased following energy restriction [30, 31]. In general, the evidence from this study suggests that an increase in exosomes biogenesis and production was associated with histopathological changes and an increase in IL-1β expression within the lung tissue of HFD rats. A growing number of studies have found that such pathological conditions as inflammation can modulate exosomes production and cargo loading [32, 33]. Under inflammation, exosomes from affected cells can facilitate pathological conditions [34]. At the same time, alterations in exosomes biogenesis/cargo may serve as disease biomarkers [35]. These data are preliminary and in our opinion, these results widen our knowledge of exosomes biogenesis in HFD condition, however further studies are essential to determine whether HFD condition can alter the contents of exosomes, which can play roles as disease biomarker. In addition, further studies are needed to investigate other inflammatory cytokines to uncover link between exosomes biogenesis and inflammatory condition. In our study, it was not clear how and from which immune cell the exosomes were secreted. Moreover, as the focus of this study was on exosomes biogenesis, there is a possibility that these exosomes carry copies of IL-1β, which was upregulated in tissue samples.

Conclusions

Taken together, our research has highlighted inflammation and histopathological changes in rat lung tissue along with an increase in exosome biogenesis after HFD induction. Studying the dynamic of exosomes in the lungs of HFD animals can help us to better understand the pathophysiology and, as a result, innovation of new tools for the diagnosis and treatment of lung-related damage.

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Author contributions M-Sh and JR conceived and designed the experiments and analyzed the data. RR prepared the figures. JR and MA wrote the manuscript. R M-H, MA, and MJ-B performed the experiments. All authors read and approved the final manuscript.

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Data availability The data that support the findings of this study are available on request from the corresponding author.

Declarations

Competing interests The authors have no competing interests to declare.

Ethical approval The animal experimental procedures were conducted according to the principles of guidelines for the ethical use of animals in applied studies and approved by the Ethics Committee on Animal Use of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1399.236) in compliance with the ARRIVE guidelines.

Consent for publication Not applicable.

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