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Characterization of blood-derived exosomal proteins after exercise

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Abstract

Objective: To assess changes in plasma exosome levels and protein content in mice after long-term exercise.

Methods: We subjected 9-month-old adult C57BL/6J mice to daily treadmill running exercise for 4 weeks prior to the isolation of blood-derived exosomes. Exosomal proteins were identified using mass spectrometry.

Results: Extracellular bodies were successfully isolated from mouse blood. Protein levels were altered in blood-derived exosomes after chronic treadmill exercise. Levels of the secretagogue secretogranin 2 were markedly elevated in exercise-induced exosomes.

Conclusion: Our data suggest that levels of secretogranin 2 were increased in mouse exosomes following chronic treadmill exercise. We conclude that exercise increases exocrine secretion of secretogranin 2.

Keywords

Blood, exercise, exosome, secretogranin 2, treadmill, mouse model

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Introduction

Cells can secrete bioactive vesicles,[1] and exosomes are among the most important types of vesicles in the extracellular fluid. Exosomes are found in the nucleoli of most cell types during endosomal maturation and through inverted budding can

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form multivesicular bodies (MVBs), small vesicles 40 to 100 nm in diameter[2–4] that are widely found in various organs.

Exosome biofilms fuse with the plasma membrane for secretion, entering the microenvironment by exocytosis where they can be transferred from one cell to another for communication purposes or undergo long-distance transport.[2,5] Exosomes contain a variety of protein markers, such as CD9, CD63, CD81, HSP70, TSG101, and ALIX,[4,6] which can be used to identify them. Exosomes also contain a variety of lipids, proteins and nucleic acids (such as mRNA and miRNA) and thus can have effects no multiple target cells within a signaling pathway.[5,7,8] Therefore, exosomes play very important roles in biological processes, especially signal conduction. Peripheral blood contains red blood cells, granulocytes, monocytes, lymphocytes, and platelets; these cells can secrete large amounts of exosomes, mainly during red blood cell maturation and platelet activation.[9–11]

Studies have shown that exosomes from multiple cell types occur in peripheral blood at dynamically varying levels.[12] Most plasma exosomes carry large numbers of proteins, lipids, and RNA from their mother cells. The molecular form, number and composition of proteins in exosomes can be used as indicators of the physical and pathological conditions of the cells that produced them and the body in general.[13] Thus, exosomes in peripheral blood are considered biomarkers for the diagnosis and prognosis of disease.[14] In addition, the analysis of exosomes may be important for diagnosis and in treatment monitoring, allowing patients to receive timely and optimal treatments.

Movement is a basic component of human life. There is a general understanding of the various mechanisms through which movement affects quality of life by influencing overall body condition and the level of metabolism. Previous studies have shown that regular exercise reduces the incidence of metabolic syndrome, type 2 diabetes mellitus, cardiovascular disease, stroke, hypertension, colorectal cancer, breast cancer, and depression.[15–19] Studies have also shown that exercise reduces pain-induced and hyperalgesic neuropathic pain through reduction of anti-inflammatory and proinflammatory cytokines.[20] Exercise also increases the release of exosomes from myocardial cells and other cells into the bloodstream.[13] The study of exosomes can provide insights into the mechanisms of exercise-based interventions and guide future research. The present study focused on changes in plasma exosome levels and protein content in mice after long-term exercise. Our findings have theoretical and practical significance.

Materials and methods

Mice, cells, and reagents

Male 9-month-old C57BL/6J mice were given *ad libitum* access to food and water and maintained under a 12-hour/12-hour light/dark cycle. The exercise protocol was approved by the Laboratory Animal Ethics Committee of Jinan University (Institutional Animal Care and Use Committee approval protocol number: 20160302004, Guangzhou, China, 2 March 2016). The treadmill exercise was performed as previously described.[21] Briefly, experimental mice were subjected to treadmill running exercise for 90 minutes/day, 7 days/week for 4 weeks. Control mice could freely move within their cages. The running speed of experimental mice was 9 m/minute on day 1; the speed was incrementally increased each day by 1 m/minute up to 14 m/minute on day 6, where it was maintained for 4 weeks.

The mouse endothelial cell line bEND.3 (CRL-2299) was obtained from American

Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum and antibiotics (Life Technologies, Grand Island, NY, USA). Cells were grown at 37°C under a humidified atmosphere containing 5% CO_2.

All reagents were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated. ExoQuick solution (EXOQ5-1) was obtained from System Biosciences (Palo Alto, CA, USA). Rabbit anti-CD63, rabbit anti-TSG101 and rabbit anti-HSP70 antibodies were obtained from System Biosciences. Rabbit anti-Scg2 antibody was purchased from Gene Tex (GTX116446, Irvine, CA, USA).

Ethics statement

All experiments and methods were conducted strictly in compliance with the relevant guidelines and regulations of Jinan University. All animal procedures were performed according to China's animal welfare legislation for the protection of animals used for scientific purposes and were approved by the Committee on the Ethics of Animal Experiments of Jinan University. All efforts were made to minimize the number of animals used and to decrease their suffering.

Isolation of blood-derived exosomes

Isolation of blood-derived exosomes was performed as described previously with some modifications.[22] Blood from individual mice (approximately 1 mL) was collected from the orbital sinus after anesthetizing the animal with 1.25% tribromoethanol and 2.5% 2-methyl-2-butanol (20 mL/kg body weight). Blood samples from four randomly selected mice in the same group were pooled and centrifuged (3000 ×g, 15 minutes) to remove blood cells and debris. Thrombin (5 units/mL; T6634, Sigma) was then added to the supernatant for 5 minutes at room temperature. The mixture was subjected to intermittent vortexing to remove fibrinogen. After centrifugation (2800 ×g, 5 minutes), four volumes of supernatant were mixed with one volume of ExoQuick solution. The mixture was allowed to rest for 30 minutes at 4°C to precipitate exosomes. After another centrifugation (1500 ×g, 30 minutes), the pellet was resuspended in sterile phosphate-buffered saline (PBS) and subjected to ultracentrifugation (100,000 ×g, 90 minutes). The pellet was washed in sterile PBS once followed by another ultracentrifugation step (100,000 ×g, 90 minutes). The exosome pellet was then resuspended in 200 μL of sterile PBS and stored at −80°C.

Transmission electron microscopy

Fifty microliters of exosomes in PBS were adsorbed on 200-mesh copper grids and stained with 3% phosphotungstic acid for 1 minute. Exosomes were then imaged using a TECNAI 12 transmission electron microscope (FEI, Hillsboro, OR, USA).

Western blotting

One volume of exosomes was lysed with one volume of radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail. Denatured exosomal proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA). bEND.3 cells were treated with exosomes, lysed with RIPA buffer and subjected to SDS-PAGE. Following transfer, exosomal proteins were probed with primary antibodies (overnight, 4°C) followed by the corresponding horseradish peroxidase-conjugated secondary antibodies (1 hour, room temperature). Protein bands were detected using Pierce ECL Western

Blotting Substrate (Life Technologies) and imaged using the ChemiDoc Touch Imaging System (Bio-Rad). The relative levels of proteins of interest were analyzed with ImageJ software using CD63 as a loading control.

Particle size analysis

The particle sizes of exosomes in 100-μL volumes were analyzed using a Zetasizer Nano ZS (Malvern, Worcestershire, UK) according to the manufacturer's protocol.

Bicinchoninic acid (BCA) protein assay

Following lysis of exosomes with RIPA buffer, total exosomal proteins were quantitated using a Pierce BCA protein assay kit (Life Technologies) according to the manufacturer's protocol.

Silver staining

Exosomal proteins were subjected to SDS-PAGE and stained using a ProteoSilver Silver Stain Kit (PROT-SIL1, Sigma) according to the manufacturer's protocol.

Mass spectrometry

Twenty micrograms of exosomal proteins were digested with trypsin and analyzed using a Synapt G1 High-Definition Mass Spectrometer (Waters Corporation, Milford, MA, USA) according to the manufacturer's protocol. The mass spectrometry data were acquired using MassLynx 4.1 SCN639 software (Waters Corporation) and the exosomal proteins were analyzed using a Global Proteome Machine. Gene ontology (GO) analyses of exosomal proteins were performed using the online Princeton GO term finder (http://go.princeton.edu/cgi-bin/GOTermFinder) with the functional annotation category molecular function.

Cell viability assay

bEND.3 cells were plated in a 96-well plate (1000 cells/well) for 24 hours and then treated as indicated for another 72 hours. bEND.3 cells were treated with control or exercise-induced blood-derived exosomes (20 μg of exosomal protein/mL) in the indicated medium. Cell viability was assessed using the Cell Counting Kit-8 (CCK8) assay (Sigma).

Statistical analysis

Data were presented as means ± standard errors of the means. The student's t-test or one-way analysis of variance with Dunnett's post hoc test were used to assess differences between and among groups, as appropriate. Values of $P < 0.05$ were considered statistically significant.

Results

Characterization of exosomes isolated from mouse blood

To investigate blood-derived exosomes in mice, blood samples from four C57BL/6J mice per treatment group were pooled and subjected to exosome isolation using polymer-mediated precipitation and ultracentrifugation (Figure 1a). Exosomes are nanosized extracellular vesicles. The presence of blood-derived exosomes was confirmed by transmission electron microscopy, which revealed vesicles with diameters of 20 to 80 nm (Figure 1b). Exosomes originate from endosomes/MVBs; therefore, endosome-associated proteins, such as tetraspanin proteins (CD9, CD63 and CD81), Rab GTPase and flotillin, and proteins involved in MVB biogenesis such as TSG101 and ALIX, are commonly used as exosomal markers. Using western blotting, we confirmed the expression of CD63 and

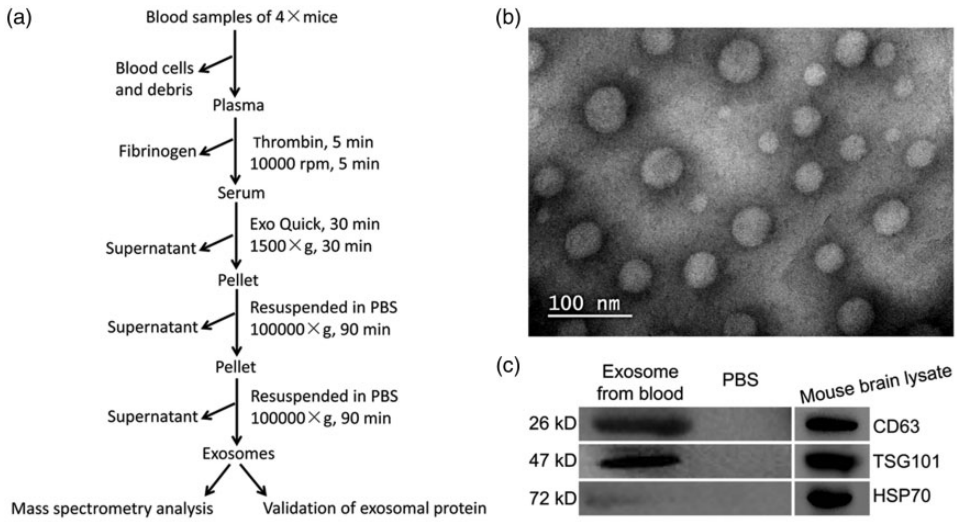

Figure 1. Characterization of exosomes isolated from mouse blood. (a) Schematic of protocol used to isolate blood-derived exosomes. (b) Representative image of blood-derived exosomes by transmission electron microscopy. Scale bar, 100 nm. (c) Expression of exosomal markers (CD63, TSG101 and HSP70) in blood-derived exosomes as assessed by western blotting.

TSG101 in blood-derived exosomes (Figure 1c). Chaperones, such as HSP70 and HSP90, are also thought to be enriched in exosomes. However, we did not detect the expression of HSP70 in exosomal proteins samples, reflecting differential expression of exosomal content in mouse blood-derived exosomes (Figure 1c). Taken together, these data indicated the successful isolation of exosomes from mouse blood.

Characterization of blood-derived exosomes from control and exercised mice

Chronic exercise is beneficial to human health, especially for middle-aged individuals. In this study, we aimed to characterize exosomal proteins in the blood of mice subjected to chronic exercise and compare them with those of control mice. We subjected 9-month-old adult C57BL/6J mice to daily treadmill running exercise for 4 weeks prior to the isolation of blood-derived exosomes (Figure 2a). No significant change in body weight was found between the control and exercised mice (Fig. S1), indicating that the exercise protocol did not cause adverse effects on body health and was well tolerated. Following the isolation of blood-derived exosomes, we analyzed the particle size distributions of exosomes from the control and exercised groups. Both groups had similar distributions of particle size, suggesting that chronic exercise did not affect the vesicular size of exosomes (Figure 2b). The exosomal proteins from the control and exercised groups were further examined by silver staining, which indicated similar enrichment of exosomal proteins of approximately 55 kDa and 70 to 100 kDa (Figure 2c). To quantify the total amount of exosomes after chronic exercise, exosomal proteins were quantitated using a BCA assay. Both control and exercise-associated exosomes had similar protein concentrations (Figure 2d). These results suggested that

chronic exercise did not substantially affect the sizes or total concentrations of exosomes in blood.

Mass spectrometry analysis of blood-derived exosomal proteins

To further assess the expression of proteins in blood-derived exosomes, we performed mass spectrometry to identify exosomal proteins. Following quality control of the exosomal proteins by silver staining (Figure 2c), mass spectrometry was used to generate a full list of exosomal proteins present in the blood samples of control and exercised mice (Appendix). A Venn diagram was constructed and showed that only a few exosomal proteins were found in both groups, while most exosomal proteins were specific to control or exercised mice (Figure 3a). The GO analysis of molecular function suggested an enrichment of control group exosomal proteins (72 of 141 genes) involved in protein binding, while poor enrichment of exercise-induced exosomal proteins was observed (3 of 109 genes enriched involved in platelet-derived growth factor binding, 3 of 109 genes enriched involved in DNA polymerase binding, and 4 of 109 genes enriched were extracellular matrix structural constituents) (Figure 3b).

Exosomal proteins with both ≥ 2 total peptides and ≥ 2 unique peptides identified by mass spectrometry are summarized in Table 1. Among these proteins, Cdk13, Pou3f1, Scg2, Acd, Golim4 and Col25a1 were exclusively present in exercise-associated exosomes. By contrast, Nek9, Wiz, Prep, Syne2, Ppfia4, Stmn1, Cbll1 and Stard9 were control exosome-specific proteins. Taken together, these data suggest a specific expression pattern of blood-derived exosomal proteins after chronic exercise.

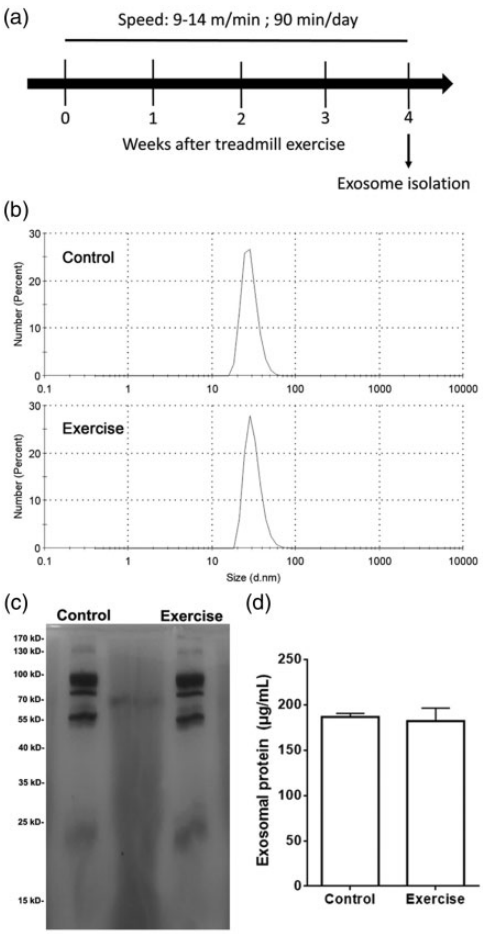

Figure 2. Characterization of blood-derived exosomes from control and exercised mice. (a) Schematic of treadmill running exercise. (b) Analysis of blood-derived exosome particle sizes in the control and exercised groups. (c) Silver staining of exosomal proteins in the control and exercised groups. (d) Concentrations of exosomal proteins in the control and exercised groups. $n = 3$.

Validation of secretogranin-2 expression in exercise-associated blood-derived exosomes

The mass spectrometry data indicated that levels of Scg2 may be upregulated in blood-derived exosomes after chronic exercise. Therefore, we performed western blotting

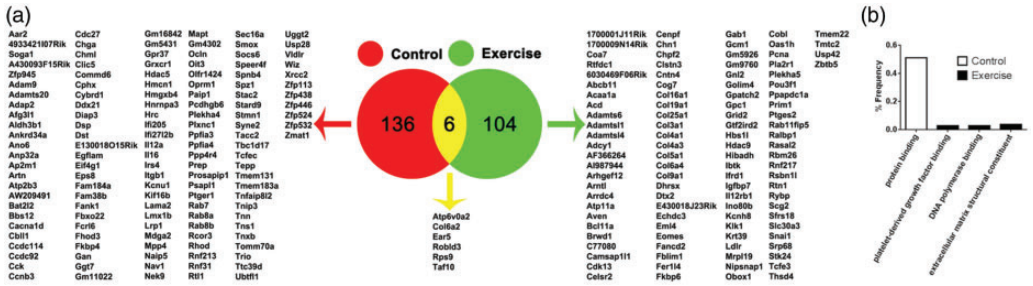

Figure 3. Mass spectrometry analysis of blood-derived exosomal proteins. (a) Venn diagram showing control group-specific, exercised group-specific and common exosomal proteins identified by mass spectrometry. (b) GO analysis of control group-specific and exercised group-specific exosomal proteins categorized by molecular function.

to validate Scg2 expression in exosomal lysates. Using CD63 as a loading control, an ~3-fold increase in Scg2 levels was consistently observed in exercise-associated exosomes (Figure 4).

Secretoneurin increases endothelial cell proliferation

A variety of studies have shown that exosomes mediate the intercellular transfer of intravesicular cargos such as proteins and RNAs.[23,24] Thus, we investigated whether exosomal Scg2, a protein highly expressed in neuroendocrine cells, can be transferred to other recipient cells by exercise-associated blood-derived exosomes. Endothelial cells, which form the inner lining of blood vessels, are in direct contact with blood-derived exosomes. Therefore, we treated bEND.3 cells with control or exercise-induced blood-derived exosomes (20 μg of exosomal protein/mL) (Fig. S2).

Discussion

In this study, we found that exercise can cause changes in the levels and types of expressed proteins in exosomes secreted into the blood of mice. Using mass spectrometry, we identified the expression of exosomal proteins. Earlier studies found that extracellular vesicles in plasma increased after acute exercise,[13,25] a finding that differed from our results. Differences in types of exercise and sampling time points may explain this discrepancy. Our study used long duration, low intensity exercise while previous ones used short duration and high intensity exercise. Exosomes are dynamically changing in the body, and more research is needed to understand the effects of different types of exercise on their levels and contents.

Exosomes are found in almost all bodily fluids including blood plasma, urine, saliva, milk, bronchoalveolar lavage fluid, cerebrospinal fluid, amniotic fluid, and malignant ascites.[26] Exosomes have been suggested to have protective effects on organisms, including protecting the myocardium from ischemia-reperfusion injury.[27] Exosomes are also considered liquid-biopsy markers of cancer. They contain microRNAs that allow early assessment of cancer risk.[28] Exosomes in blood play important roles in body function, metabolism, and self-protection. The role of exosomes in exercise is gradually being uncovered Increases in exosome microRNA levels after exercise can cause changes in protein expression and enhance protection of myocardial cells to prevent fibrosis.[29]

Table 1. Selected proteins identified by mass spectrometry in mouse serum-derived exosomes.

	Control		Exercised				
Gene name	Total peptides	Unique peptides	Total peptides	Unique peptides	Function	Accession number	MW (kDa)
Cdk13	N.D.	N.D.	110	13	Alternative mRNA splicing via spliceosome; hemopoiesis; phosphorylation of RNA polymerase II C-terminal domain	Q69ZA1	165
Pou3fl	N.D.	N.D.	22	3	Positive regulation of gene expression; myelination	P21952	50
Scg2	N.D.	N.D.	20	6	Angiogenesis; intracellular signal transduction; positive chemotaxis	Q03517	68
Acd	N.D.	N.D.	11	3	Intracellular protein transport; positive regulation of telomerase activity	Q5EE38	45
Golim4	N.D.	N.D.	4	2	Plays a role in endosome to Golgi protein trafficking	Q8BXA1	87
Col25al	N.D.	N.D.	3	2	Axonogenesis involved in innervation	Q99M5	65
Nek9	81	12	N.D.	N.D.	Cell division; chromosome segregation; mitotic nuclear division	Q8K1R7	107
Wiz	47	11	N.D.	N.D.	DNA methylation; long-term memory; organ growth	Q9Z148	179
Prep	14	2	N.D.	N.D.	Protein metabolic process; proteolysis	Q9QUR6	85
Syne2	14	5	N.D.	N.D.	Positive regulation of cell migration; centrosome localization	Q6ZWQ0	37
Ppfia4	13	2	N.D.	N.D.	Glutamate neurotransmitter release cycle; norepinephrine neurotransmitter release cycle; acetylcholine neurotransmitter release cycle	A0A087WPJ3	78
Stmn1	9	2	N.D.	N.D.	Brain development; axonogenesis	P54227	18
Cbll1	8	5	N.D.	N.D.	Single organismal cell-cell adhesion; positive regulation of cell migration	Q9JIY2	55
Stard9	4	2	N.D.	N.D.	Cytoskeleton-dependent intracellular transport; metabolic process	Q80TF6	45

N.D., not detected.

Proteins with both $\geq$2 total peptides and $\geq$2 unique peptides identified by MS are shown.

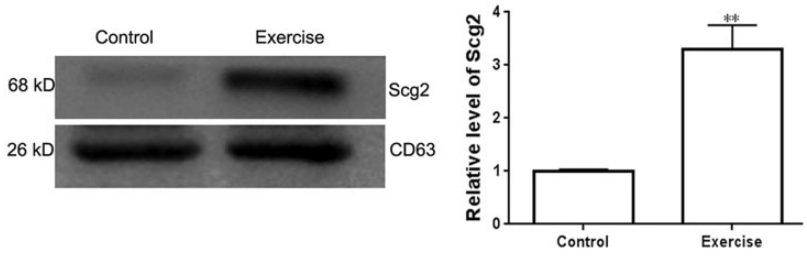

Figure 4. Validation of Scg2 expression in blood-derived exosomes. Expression of Scg2 in exosomes was assessed by western blotting. $n = 3$, Student's t-test $^{**}P < 0.01$.

In proteomic and transcriptomic studies, myocardial and skeletal muscle mitochondria show similar carbonylation levels. However, by increasing skeletal muscle mitochondrial protein carbonylation and thereby increasing the body's carbonylation level,[30] exercise also changes oxidative phosphorylation pathways. Furthermore, exercise improves mitochondrial biology, synthesis of proteins (such as NADH dehydrogenase, ATP synthase, cytochrome c oxidase, and alcohol dehydrogenase[31]), and promotes growth of capillaries and upregulation of genes involved in muscle hypertrophy.[32,33] In contrast, exercise reduces the expression of genes involved in inflammatory/immune responses, endoplasmic reticulum stress genes[34,35] and type 1 voltage-dependent anion channels.[31]

In exosome samples from the experimental group, levels of Scg2 protein was significantly higher than those in samples from the control group. Scg2 modulates inflammatory reactions and neurotransmission, and may participate in neuronal differentiation.[36] Furthermore, Scg2 stimulates migration and vascular smooth muscle cell proliferation, and promotes angiogenesis as an endothelial cell proliferation factor.[36–38] Studies have shown that exercise can also promote angiogenesis; therefore, increasing the amount of Scg2 protein in exosomes may be a mechanism of promoting blood vessel formation. This potential mechanism of exercise-induced Scg2 protein promotion of angiogenesis is worth exploring in future studies.

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Author contributions

Hongkai Xiang performed the treadmill exercise experiments, characterized exosomes, and contributed to data analysis. Shisheng Chen contributed reagents and contributed to writing the paper. Junhan Zhou performed the *in vitro* study and contributed to data analysis. Junxiu Guo performed validation of exosomal proteins and contributed to data analysis. Qingfeng Zhou contributed to the interpretation of the data. Qishuang Zhou conceived and designed the experiments and contributed to writing the paper.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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Appendix.

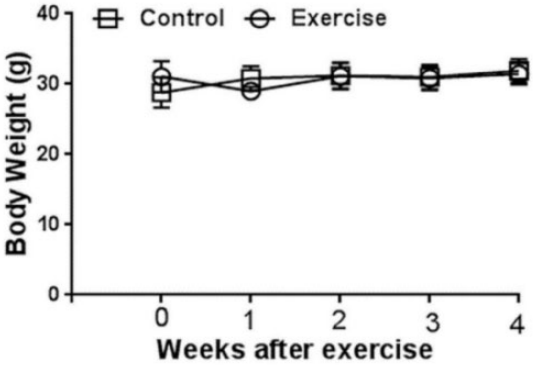

Figure A1. Body weights of control and exercised mice. No significant difference in body weight was observed between the control and exercised groups. n = 26 for the control group, n = 16 for the exercised group.

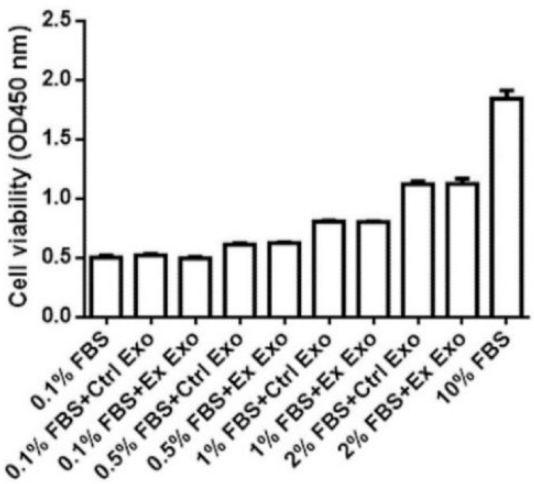

Figure A2. Treatment of bEND.3 cells with blood-derived exosomes did not increase cell viability. No significant increase in cell number or viability was found after treatment of bEND.3 cells with exercise-associated exosomes. n = 5.