Research Article

BIOMEDICAL Journal of Scientific & Technical Research

ISSN: 2574 -1241

DOI: 10.26717/BJSTR.2023.53.008336

MicroRNA Profiling of Plasma Exosomes in Type 2 Diabetes Following Exercises

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ARTICLE INFO

Received: September 12, 2023

Published: September 26, 2023

Citation: Xinwen Cui, Peng Zhao, Rongxin Zhu, Xingya Yang, Yirui Wang and Yimin Zhang. MicroRNA Profiling of Plasma Exosomes in Type 2 Diabetes Following Exercises. Biomed J Sci & Tech Res 53(1)-2023. BJSTR. MS.ID.008336.

ABSTRACT

Background: The study explored postprandial exercise's impact on exosomal miRNAs in men with Type 2 diabetes (T2D).

Methods: A total of 12 male participants, including 7 patients and 5 healthy individuals, were recruited. T2D patients underwent a randomized three-period crossover 1-hour postprandial intervention: high-intensity interval exercise (T2D-HIIE), moderate-intensity continuous exercise (T2D-MICE) with cycling and sedentary control (T2D-CON) for 30 min, while healthy participants served as controls (HP-CON) without exercise intervention. Exosomes were extracted from plasma using differential ultracentrifugation at 1.5 hours postprandial, which equates to immediate post-exercise for T2D patients. Exosomal miRNA sequencing was conducted. KEGG analysis was performed to identify pathways linked with altered expression of miRNAs.

Results: Based on sequencing, we identified 152 differentially expressed exosomal miRNAs following exercises (fold change > 1, $p < 0.05$). Among these, 116 were upregulated in T2D patients and downregulated immediately after exercises, while 36 were downregulated in T2D patients and upregulated immediately after exercises. The top 10 significantly differentially expressed exosomal miRNAs included hsa-miR-96-5p, hsa-miR-126-5p, hsa-miR-142-3p, hsa-miR-144-3p, hsa-miR-30d-5p, hsa-miR-7704, novel-hsa-miR184-3p, novel-hsa-miR286-3p, novel-hsa-miR293-5p, and novel-hsa-miR80-5p. The MAPK signaling pathway showed the highest enrichment ($p < 0.001$, Rich Ratio = 1).

Conclusions: Postprandial exercise can regulate a unique profile of circulating exosomal miRNAs in T2D patients.

Keywords: Type 2 Diabetes; Microrna; Exosome; Postprandial Exercise; Next-Generation Sequencing

Introduction

Type 2 diabetes (T2D), characterized by elevated blood sugar levels, is a prevalent metabolic disorder that poses a significant global health threat. Exercise has long been acknowledged as a crucial element in managing T2D. Despite impaired insulin sensitivity in individuals with T2D, exercise-induced muscle contractions can enhance glucose uptake independently of insulin [1]. Specifically, post-meal exercise has been shown to boost glucose absorption [2]. While moderate-intensity continuous exercise (MICE) is the most commonly recommended exercise for individuals with T2D, recent research has revealed that high-intensity interval exercise (HIIE), differing from MICE in intensity and exercise pattern, can be equally or even more effective in glycemic control [3-5]. This suggests that various aerobic exercise patterns may influence blood glucose levels to varying degrees. To develop improved or novel intervention strategies, researchers must delve into the molecular mechanisms underlying T2D. While altered miRNAs have been implicated in the beneficial effects of physical exercise [6,7], understanding the

involvement of exosomes, the regulation of exosomal miRNAs, and the function of miRNAs› corresponding target genes in T2D patients is still a subject requiring further investigation.

Exosomes, with diameters ranging from 40 nm to 120 nm, are a type of extracellular vesicle (EVs) originating from various cells and can be detected in most biofluids [8,9]. Exosomes regulate cellular functions by transporting proteins, lipids, and nucleic acids [10]. Growing evidence suggests their involvement in various pathological processes like chronic inflammation, endocrine disorders, insulin resistance, and the modulation of biochemical signaling through specific molecule transfer, including miRNAs [11]. Exosomal miRNAs, protected from degradation by enzymes while in circulation, are delivered to target cells. There, they influence gene expression, serving as mediators of intercellular communication [12]. This impact extends to insulin secretion, adipose tissue metabolism, and diabetes development [13,14]. While biomarkers of exosome-carried miRNAs in body fluids for diabetes and its complications have been explored [15-18], the effects of exercise in this context require further investigation. Exercise serves as a trigger, prompting cellular responses like gene expression and exosome release [19-21]. Exosomes from various sources can convey beneficial effects of exercise on crucial metabolic tissues through their cargo. Pioneering studies [22,23] have highlighted unique exosomal miRNA patterns, emphasizing their role as a distinct factor enabling inter-tissue communication throughout the body in response to a single exercise session. Recent reviews have delved into the evolving significance of exosomal miRNAs in exercise physiology and their potential involvement in the novel mechanisms underlying exercise›s positive effects on T2D patients [17,24].

Yet, the role of exosomal miRNAs induced by acute postprandial exercise in T2D, their impact on glucose regulation, and the underlying signaling pathways remain largely uncharted territory. This study, involving HIIE and MICE interventions in middle-aged men with T2D, aims to uncover differentially expressed exosomal miRNAs in response to exercise and explore the pathways involved in postprandial exercise regulation in T2D patients. Our hypothesis posits that acute exercise can modulate a distinct exosomal miRNA pattern in the circulation of middle-aged men with T2D.

Methods

Study Design and Ethical Approval

This study was a randomized three-period crossover intervention conducted solely for T2D patients. Ethical approval was obtained from the CISS Ethics Board in accordance with the principles of the Declaration of Helsinki. All participants provided written consent prior to participation.

Participants

Twelve male participants between 30 and 60 years were enrolled. Among them, 7 participants had T2D, and 5 were age and BMI-matched healthy volunteers. T2D patients eligible if diagnosed for ≥ 6 months and no structured physical activity for ≥ 6 months (>150 min/week). Those with T2D-related complications, blood pressure ≥140/90 mmHg; Body Mass Index (BMI) >28 kg/m2 or<18.5 kg/m2; contraindications to exercise were excluded. Healthy participants met specific criteria, including fasting blood glucose (FBG) < 6:1 mmol/L, oral glucose tolerance test within two hours blood glucose < 7:8 mmol/L; triglyceride <2.25 mmol/L; cholesterol <5.18 mmol/L; free of any chronic diseases as well as any injuries; absence of a family history of diabetes. Insulin users and smokers were also not included in the present study.

Procedures

The procedures for individuals with T2D have been detailed in our previous study [4]. During the first visit, participants underwent fasting blood sampling, resting blood pressure measurement, and anthropometric assessment. They also completed the international physical activity questionnaire (IPAQ) [25], the physical activity readiness questionnaire (PAR-Q), and a checklist of contraindications for the graded exercise test [26]. Healthy individuals had a second visit on a separate day to undergo blood sampling 1.5 hours after breakfast. For T2D patients, a randomized crossover trial encompassed three experimental days: a control day (CON) without exercise and two exercise days-HIIE and MICE. These exercises were performed 1 hour after breakfast and included:

1. HIIE (7 × 1 min, 90% VO2max + 2 min, 30% VO2max) involving cycling.
2. MICE (30 min, 50% VO2max) also using cycling; or
3. A sedentary control (CON) lasting 30 min.

A 10-day washout period separated each intervention. The energy expended in the MICE and HIIE protocols was approximately equivalent. Random allocation was achieved through sealed envelopes, each specifying the type of intervention. Throughout the study, participants were advised to maintain their regular daily routines, including diet, exercise, and medication. They consumed consistent standard breakfast and water intake on each intervention day, along with an identical dinner before the interventions. Alcohol, caffeine, and physical activity were avoided 48 hours before each intervention. Researchers provided breakfast during the study, adjusted to 30% of daily energy intake based on individual body weight.

Venous Blood Sampling and Biochemical Analyses

Blood samples were taken from antecubital vein. During the initial visit, the following biochemical markers were measured following a 12-hour fast: glucose, insulin, HbA1c, free fatty acids (FFA), high-density lipoprotein (HDL-cholesterol), low-density lipoprotein (LDL-cholesterol), triglycerides and total cholesterol. Throughout the three

interventions for T2D patients, exosomes were extracted immediately after exercise, which was performed 1.5 hours after breakfast. During the second visit of healthy individuals, exosomes were extracted from at 1.5 hours after breakfast.

Graded Exercise Test

Graded exercise test was conducted using a stationary cycle ergometer (Ergoselect 100, Ergoline, Bitz, Germany) to measure the maximal heart rate (HRmax) and maximal oxygen uptake (VO2max), as detailed in our previous study [4].

Exosome Isolation from Plasma

Blood samples were first centrifuged at 1900g for 10 minutes at 4°C, and the collected supernatant was further centrifuged at 3000g for 15 minutes at 4°C. The resulting plasma was immediately stored at -80°C. Exosomes were isolated using differential ultra-centrifugation (Beckman, Optima L-100XP), involving centrifugation of plasma at 2000 x g in 4°C for 30 minutes, followed by centrifugation at 12000 x g in 4°C for 45 minutes. The supernatant was then filtered through 0.45 µm disposable filter units (Merck KGaA, Darmstadt, Germany) and centrifuged at 110000 x g in 4°C for 70 minutes. After resuspending with 10 ml nuclease-free water (Qiagen, Germany), the exosomes underwent another round of centrifugation at 110000 x g in 4°C for 70 minutes. Finally, the exosomes were suspended in 0.1 ml nuclease-free water then stored at -80°C until further use.

Characterization of Isolated Exosomes

To assess the purity of the isolated exosomes, rigorous optimization was performed using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and Western blotting analysis. For TEM, 10 µl of resuspended exosomes were fixed in 1% glutaraldehyde and stained with 2% aqueous uranyl acetate. The stained exosomes were air-dried and observed using HT-7800 TEM (Hitachi, Tokyo, Japan) at 80 kV. NTA was conducted using Flow NanoAnalyzer (N30E, China) to measure the size and concentration of particles. Western blotting of exosomes involved examining proteins with primary antibodies against CD63 (1:1000, ab59479, Abcam), CD81 (1:1000, ab109201, Abcam), TSG101 (1:1000, ab125011, Abcam), and Calnexin (1:1000, ab22595, Abcam). Secondary antibodies (1:10,000; Novus Biologicals, Littleton, CO, USA) were then used, and detection was performed using Western Blotting Luminol Reagent (Bio-Rad, Hercules, CA, USA).

Next-Generation Sequencing

Exosomal RNA was isolated using the miRNeasy Serum/Plasma Advanced Kit (217204, TIANGEN, Germany) following the instructions. High-quality sequence reads were obtained by sequencing the qualified RNA library using the BGISEQ-500 platform from BGI-Shenzhen, China. Low-quality reads with adapters were removed to ensure data quality. Clean reads were aligned to the reference genome using Bowtie2, and Rfam mapping was performed using cmsearch. Novel miRNAs were predicted using miRDeep2 based on secondary structure exploration. Differential expression analysis was conducted with DEGseq, applying a threshold of a Q value <0.05 and |log2 fold change (FC)| ≥ 1.

Prediction of Target Genes of miRNAs

Three target prediction algorithms (RNAhybrid, miRanda, and TargetScan) were utilized to predict miRNA target genes. The function annotation of differentially expressed miRNA targets was performed via Kyoto Encyclopedia of Genes (KEGG, http://www.genome.jp/kegg/). Functions with Q values ≤ 0.05 were considered significantly enriched.

Statistical Analysis

Statistical analysis was performed with SPSS 19.0 (IBM Corp., Armonk, NY, USA). Data normality was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Non-normally distributed data were transformed using logarithm or square root methods.

Results

Participant' characteristics are showed in Table 1. All participants were male. Blood samples from 5 healthy individuals and 7 patients with T2D were obtained.

Table 1: Participants' characteristics and pharmacological regimen (n =12). Mean with SD.

Characteristics	Healthy individuals (n = 5)	Type 2 diabetes (n = 7)	p-value
Age (years)	41.4 (1.9)	45.7 (6.0)	P = 0.158
Diabetes duration (years)		3	
Body mass (kg)	72.2 (7.8)	70.0 (8.5)	P = 0.395
BMI (kg/m^2)	26.0 (0.8)	25.5 (1.8)	P = 0.481
Glycemic control			
Fasting glucose (mmol/L)	4.3 (0.4)	7.7 (1.6)	P < 0.001
HbA1c (%)	5.0 (0.3)	7.6 (1.2)	P < 0.001
FFA (mmol/L)	0.6 (0.1)	0.6 (0.1)	P = 0.841
Total cholesterol (mmol/L)	4.6 (0.5)	5.0 (0.9)	P = 0.367

Triglycerides (mmol/L)	1.5 (0.4)	1.7 (1.2)	P = 0.694
HDL-cholesterol (mmol/L)*	1.3 (0.4)	1.1 (0.3)	P = 0.330
LDL-cholesterol (mmol/L)*	2.7 (0.3)	2.9 (0.8)	P = 0.760
Fitness variables			
Maximal oxygen consumption (VO_{2max}) (ml/min/kg)	27.8 (6.4)	28.0 (4.6)	P = 0.853
Maximal heart rate (HR_{max})(bpm)	175.0 (19.7)	160.8 (7.3)	P = 0.108
Oral hypoglycemic agents		n =6	
Metformin only		n = 4	
Metformin + acarbose		n =2	
No medication used		n =1	

Note: *HDL-cholesterol, high-density lipoprotein cholesterol; LDL-cholesterol, low-density lipoprotein cholesterol.

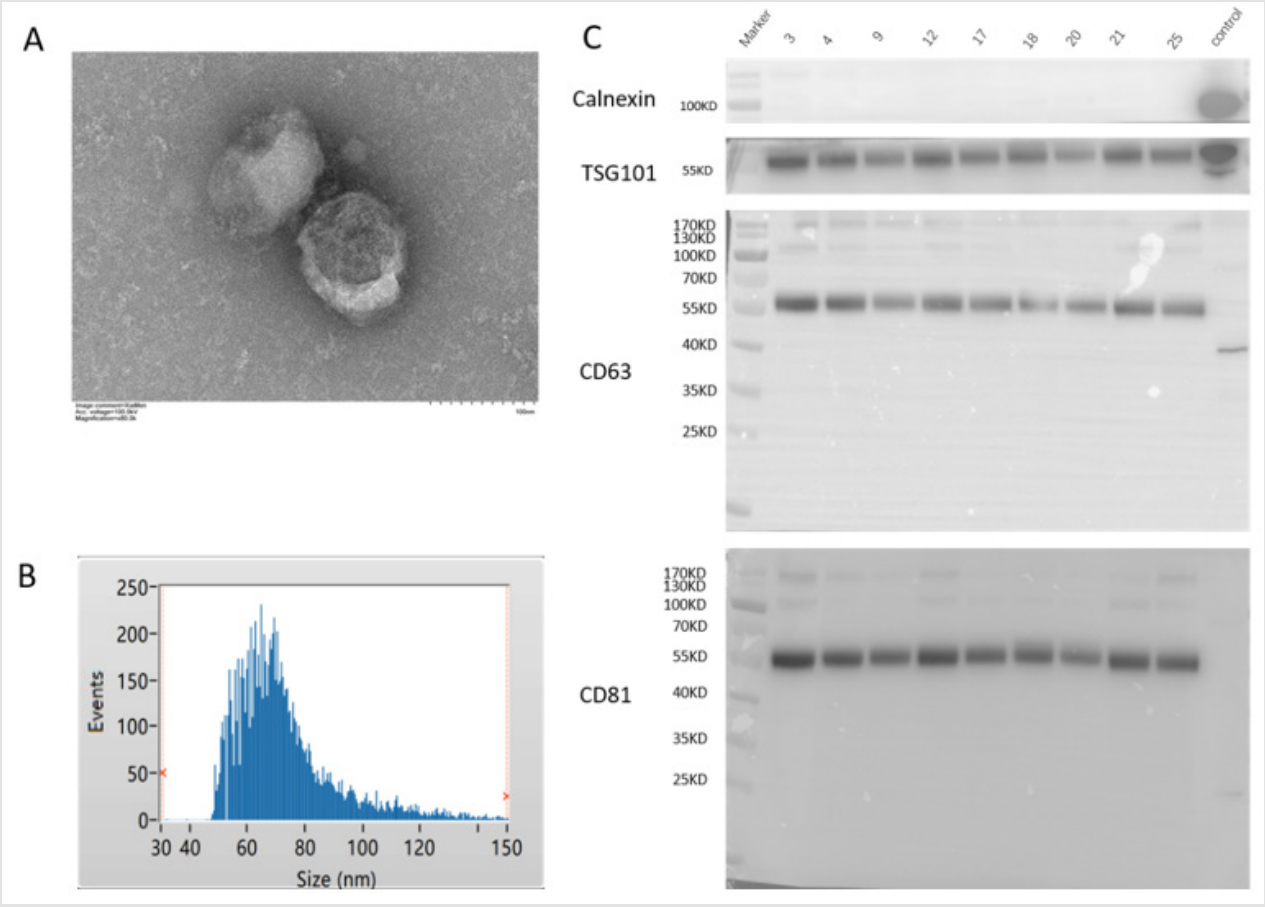

Figure 1: Identification of plasma exosomes.
(A) Transmission electron microscopy (scale bar 100 nm).
(B) Analysis of exosomes isolated from plasma by Nanoparticle Tracking Analysis (size range: 50 nm to 150 nm; concentration: 1.0 E+8 ~ 1.0 E+10 particles/mL).
(C) Confirmation of the exosomes markers with Western blotting indicated the presence of CD63, CD81 and TSG101, but the absence of calnexin in exosomes. 3-12: 4 healthy individuals; 17-25: 4 T2D patients; Numbers are the identification numbers for each individual.

Isolation and Characterization of EVs

The identification of plasma exosomes was illustrated in Figure 1.

Sequencing Analysis of Exosomes miRNA Profiles

In the miRNA sequencing analysis, we identified 204 upregulated miRNAs and 73 downregulated miRNAs in T2D patients compared to healthy individuals. Furthermore, after HIIE, 172 miRNAs were upregulated and 93 miRNAs were downregulated compared to the control (CON) group, while after MICE, 109 miRNAs were upregulated and 158 miRNAs were downregulated compared to the CON group in T2D patients. Among the identified miRNAs, 116 were upregulated in T2D patients compared to healthy individuals and downregulated immediately after either HIIE or MICE compared to CON in T2D patients (Figures 2A & 3A). On the other hand, 36 miRNAs were downregulated in T2D patients compared to healthy individuals and upregulated immediately after either HIIE or MICE compared to CON in T2D patients (Figures 2B & 3B). The top 10 significantly differentially expressed miRNAs were shown in Table 2. MiR-96-5p and miR-30d-5p were upregulated in T2D patients compared to healthy individuals and downregulated immediately after HIIE compared to CON in T2D patients. MiR-126-5p and miR-142-3p were upregulated in T2D patients and downregulated immediately after either MCIE. MiR-144-3p was upregulated in T2D patients and downregulated immediately after both HIIE and MCIE. MiR-7704, novel- miR184-3p, novel-miR286-3p and novel-miR293-5p were downregulated in T2D patients and upregulated immediately after MCIE. Novel-hsa-miR80-5p was downregulated in T2D patients and upregulated immediately after both HIIE and MCIE.

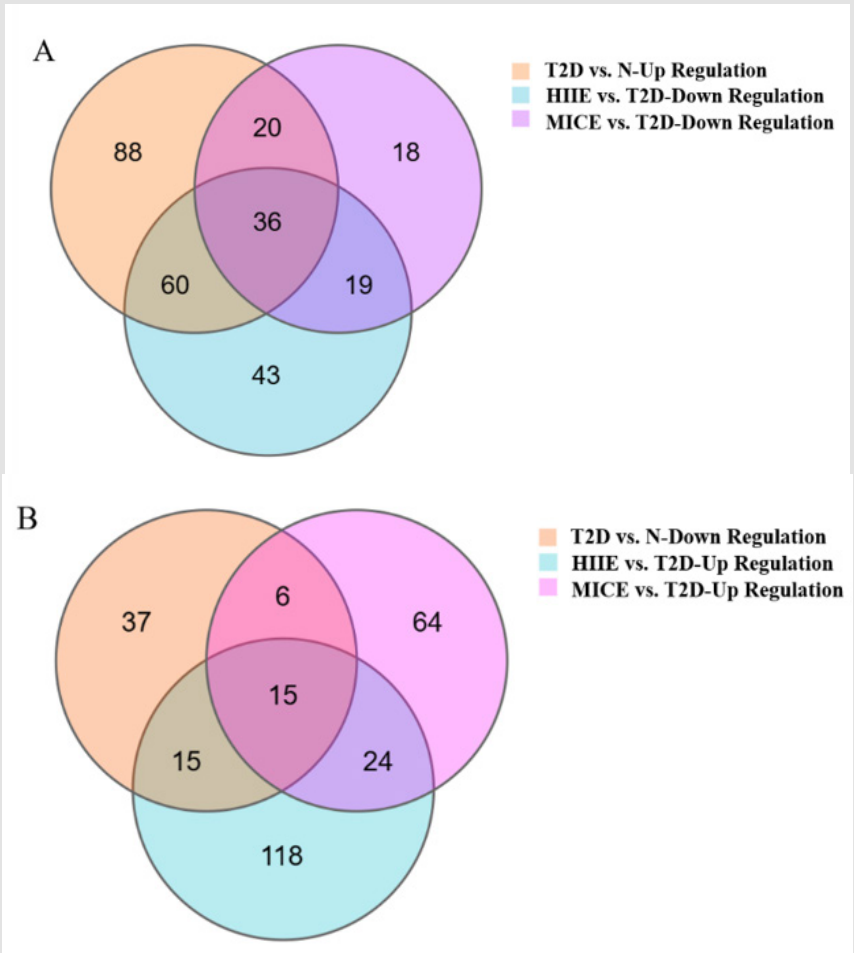

Figure 2: Venn diagrams showing the number of differentially expressed exosomal miRNAs overlapped between T2D patients (T2D, n=7) vs. normal individuals (N, n=5) and T2D patients in CON vs. immediately post-HIIE or immediately post-MICE.

DOI: 10.26717/BJSTR.2023.53.0083336

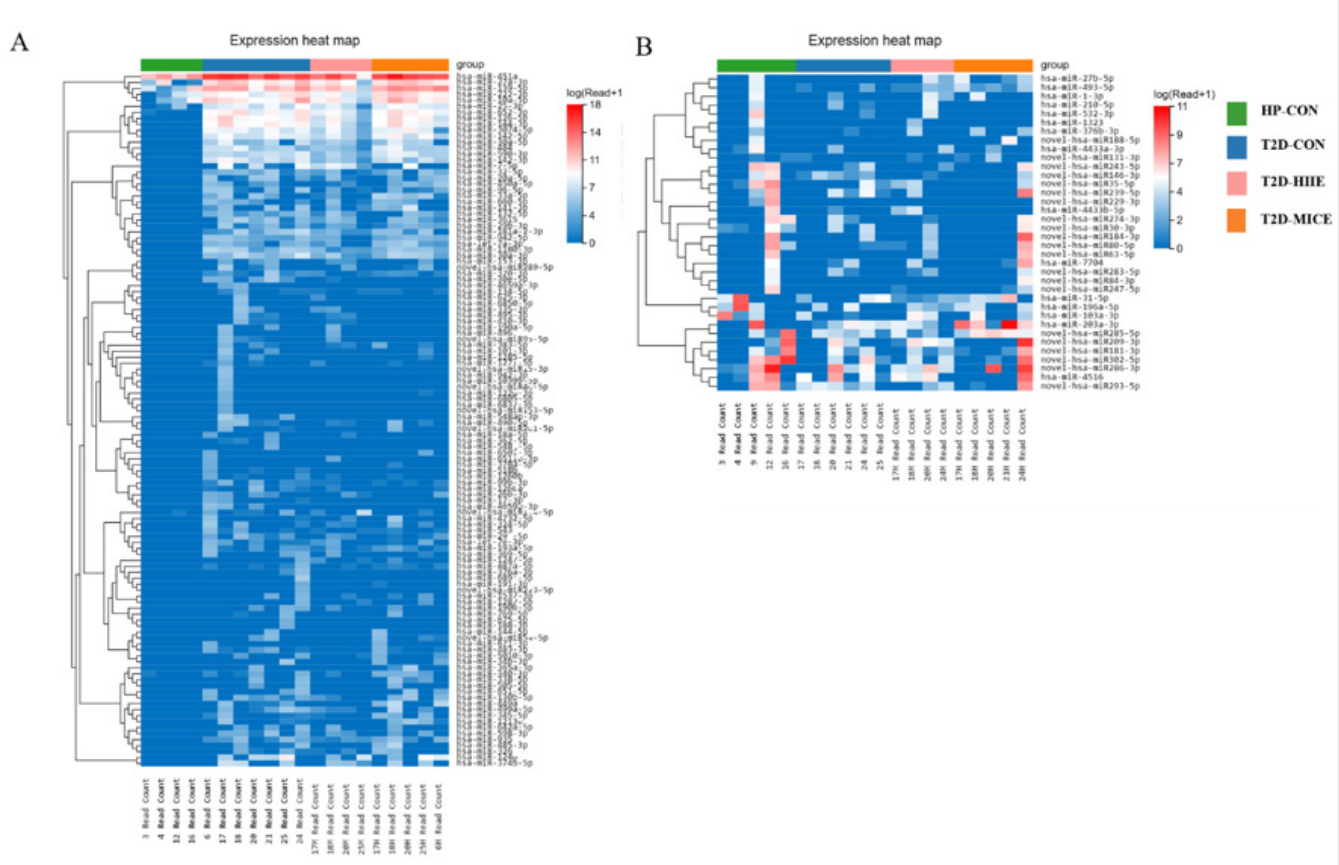

Figure 3: Heat maps of 116 (A) and 36 (B) selected exosomal miRNAs depicted a sample cluster according to the levels of plasm exosomal miRNAs.

Table 2: Top 10 differentially expressed miRNAs.

miRNA	log2 (FC)			p value			Regulation*
	T2D-CON vs HP-CON	T2D-HIIE vs T2D-CON	T2D-MICE vs T2D-CON	T2D-CON vs HP-CON	T2D-HIIE vs T2D-CON	T2D-MICE vs T2D-CON	
hsa-miR-96-5p	9.908392621	-1.6046119	-2.568543	0.0584448	0.05246513	0.192301	Up
hsa-miR-126-5p	3.481311906	-0.0974404	-1.184947	0.0060827	0.28202717	0.008113	Up
hsa-miR-142-3p	2.468128192	-0.8969385	-2.30852	0.0463527	0.14574161	0.002088	Up
hsa-miR-144-3p	1.73399435	-0.2655162	-1.861131	0.037945	0.04687044	0.002048	Up
hsa-miR-30d-5p	1.715934932	-0.440674	-0.307712	0.0507482	0.02633509	0.988321	Up
hsa-miR-7704	-2.30581	3.712718	3.440362	0.04142	0.147399	0.006661	Down
novel-hsa-miR184-3p	-4.24473	4.972693	3.439973	0.018509	0.149466	1.41E-18	Down
novel-hsa-miR286-3p	-2.98334	2.202467	3.793718	9.26E-04	0.519962	3.98E-06	Down
novel-hsa-miR293-5p	-2.5113	1.76986	1.371783	0.008699	0.376296	0.002696	Down
novel-hsa-miR80-5p	-5.73697	4.774055	5.395177	6.60E-05	0.048885	3.67E-05	Down

Note: *T2D-CON vs HP-CON

Enriched Biological Functions of miRNA Targeted Genes

We examined the mRNA targets of 152 differentially expressed miRNAs, where 116 were upregulated in T2D patients and downregulated immediately after exercises, and 36 were downregulated in T2D patients and upregulated immediately after exercises. KEGG pathway analysis revealed the enriched pathways, including ‹MAPK signaling pathway›, ‹Endocytosis›, ‹Axon guidance›, ‹Wnt signaling pathway›, ‹Oxytocin signaling pathway›, ‹mTOR signaling pathway›, ‹Rap1 signaling pathway›, ‹Adrenergic signaling in cardiomyocytes›, ‹Signaling pathways regulating pluripotency of stem cells›, ‹Autophagy - animal›, and ‹Insulin signaling pathway› ($p < 0.01$, Rich Ratio = 1; Figure 4).

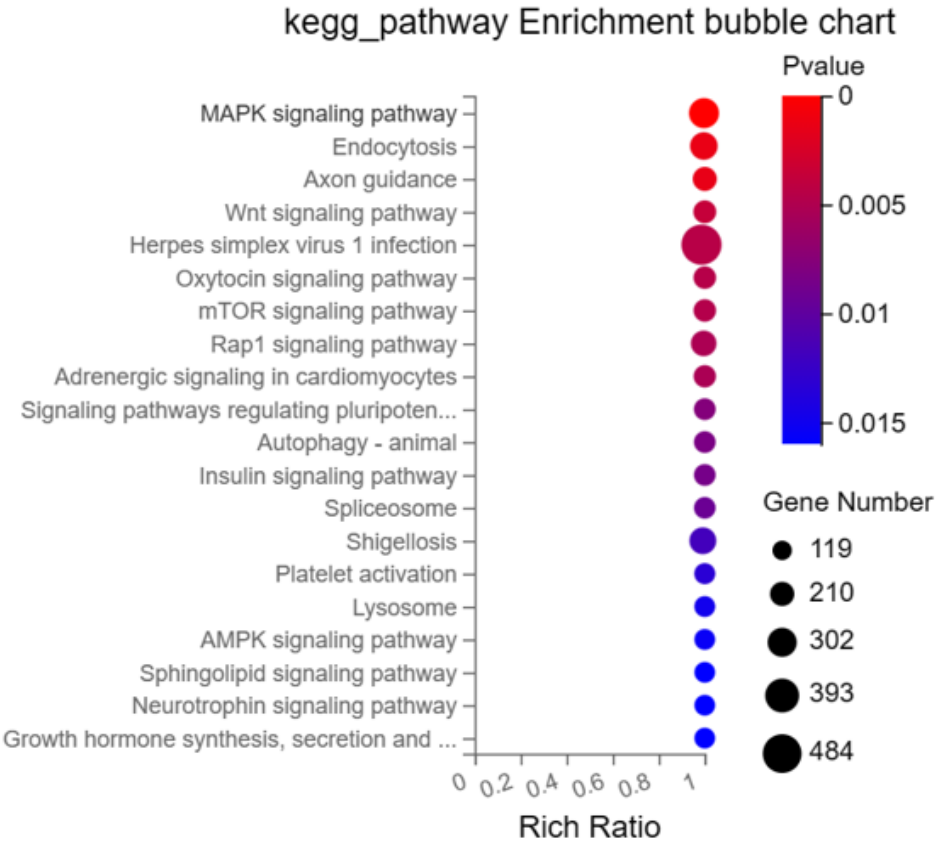

Figure 4: Top Kyoto Encyclopedia of Genes and Genomes pathways regulated by predicted target genes of miRNAs altered by T2D following exercise.

Discussion

To the best of our knowledge, this study represents the first attempt to characterize exosomal microRNA profiles following postprandial exercises in patients with Type 2 Diabetes (T2D). The KEGG functional analysis revealed that the target genes of differentially expressed exosomal microRNAs were significantly enriched in several pathways, including MAPK, Endocytosis, Axon guidance, Wnt, Oxytocin, mTOR, Rap1, Autophagy - animal, Insulin signaling pathway, among others. Exercise acts as a stressor, inducing molecular responses like gene expression and exosome release in different cell types. Exosomal miRNAs transported during exercise undergo modifications, revealing exercise›s biological effects. However, the precise regulation of exosomal miRNAs in post-exercise T2D remains incompletely elucidated. Research findings [17] have shown that diet significantly affects the release and content of exosomes. In this study, we standardized participants› breakfast to minimize diet-related interference and examine the effects of postprandial exercise on exosomal miRNAs. Functional enrichment analysis was performed on 152 differentially expressed exosomal miRNAs identified through RNA sequencing. The results revealed enrichment in pathways associated with the pathogenesis of T2D, suggesting exercise intervention could improve glucose control in T2D patients.

The most enriched KEGG pathway is the mitogen-activated protein kinase (MAPK) signaling pathway, which is related to inflammatory, oxidative, apoptotic processes, glucose homeostasis, cell proliferation,

and survival [27]. Evidence supports its association with T2D development [28]. MAPK pathway was shown to inhibit obesity and the associated T2D and was involved in glucose metabolism, exercise, meanwhile, could lead to favorable changes through the activation of MAPK signaling pathway [29,30]. Besides the MAPK signaling pathway, our identified exsosmal miRNAs revealed a participation of endocytosis, axon guidance, wnt signaling pathway, oxytocin signaling pathway, mTOR signaling pathway, Rap 1 signaling pathway and so on, which could be the potential mechanisms underpinning the intervention of T2D by acute exercises. The pathways mentioned above have previously implicated in treating diabetic complications as well as in various diabetes models [31-35]. Further research is needed to investigate the association between the identified miRNAs and the enriched biological pathways, which may help elucidate the hidden mechanisms of exercise effects.

In this study, we selected top 10 significantly differentially expressed miRNAs based on sequencing results. Previous studies have also reported increased circulating blood levels of miR-96-5p, miR-142-3p and miR-144-3p in T2DM patients, suggesting their potential as biomarkers for T2D [36-41]. MiR-126, primarily expressed in endothelial cells, plays a crucial role in regulating endothelial function, angiogenesis, and vascular integrity [42,43]. Past research has indicated that plasma miR-126 shows promise as an early predictive biomarker for T2D in susceptible individuals [44]. MiRNA-144-3p potentially modulates glucose metabolism and energy homeostasis by directly targeting insulin receptor substrate 1, a key component of the insulin signaling pathway, and glucose transporter 1 [45]. However, physical exercise can influence miRNA levels in T2DM patients by rapidly altering exosome release and circulating miRNA levels, including exosomal miRNA levels [7,22,46]. Studies exploring changes of exosomal miRNAs profiles have been relatively limited. Guay, et al. [47] T lymphocyte-specific exosomal miR-142-3p/-5p could induce chemokine expression and apoptosis in pancreatic β cells in type 1 diabetes (T1D), and inhibiting miR-142-3p/-5p in β cells improved insulin levels, reduced inflammation, and prevented T1D development. MiR30d-5p may be involved in glucose metabolism, insulin signaling, blood coagulation, platelet activation, and inflammation. Dysregulation of miR-30d has been observed in circulating blood and urinary exosomes [48,49].

None of the other differentially expressed miRNAs (miR-7704, novel-miR184-3p, novel-miR286-3p, novel-miR293-5p, novel-miR80-5p) identified in this study have been previously associated with T2DM. It is worth mentioning that this study has identified novel dysregulated miRNAs in patients with T2DM, at least in the Chinese cohort. Our present study identified differentially expressed miRNAs following both HIIE and MICE. It is worth noting that distinct patterns of postprandial exercise may result in varying profiles of exosomal miRNAs, potentially contributing to differences in the degree of glucose-lowering effects. Future research should include qPCR validation of the identified exosomal miRNAs to further explore these findings. This study underscores the potential impact of exercise-induced mechanisms on miRNAs, but further research is necessary to thoroughly elucidate their roles. Meanwhile, we must acknowledge several limitations. Firstly, it was an exploratory study with a relatively small cohort of T2D patients and healthy participants. Secondly, medication was not standardized in this study. Future research will aim to control patients› medication to minimize the potential impact of medications on exosomes. Additionally, the differentially expressed exosomal miRNAs identified by sequencing were not verified by qPCR. Therefore, qPCR will be conducted to validate the currently identified exosomal miRNAs. Finally, circulating exosomes likely originate from various cell types, such as skeletal muscle, adipose, and islet cells. It is possible that exosomes released by distinct cells may possess varying miRNA profiles. Further experimental investigations are needed to clarify the origin of these miRNAs.

Despite its limitations, this study offers valuable insights into potential contributors to the benefits of exercise for T2D. The use of high-throughput sequencing is a strength, allowing for the identification of candidate miRNAs with high power. Our results reinforce the role of postprandial exercise-induced exosomal miRNA in the regulation of glucose and may potentially provide new targets for the intervention of T2D.

Conclusion

Postprandial exercise can regulate a unique profile of circulating exosomal miRNAs in T2D patients. These miRNAs may be promising contributor for exercise-induced benefits in patients with T2D.

Data Availability

The miRNA sequence data generated during the current study are available at NCBI under the accession number PRJNA1007966.

Conflicts of Interest

The authors confirm no financial conflicts of interest related to the manuscript›s content.

Funding

This study was funded by the Fundamental Research Funds for the China Institute of Sport Science (2021-28).

Author's Contributions

Xinwen Cui and Peng Zhao significantly contributed to the study design. Xinwen Cui, Rongxin Zhu, Xingya Yang, and Yirui Wang were involved in conducting the experiments. Peng Zhao conducted data analysis and interpretation. Xinwen Cui drafted the manuscript. All authors, including Rongxin Zhu, Xingya Yang, Yirui Wang, and Yimin Zhang, critically reviewed and revised the manuscript. The final version was approved by all authors.

Acknowledgements

The authors wish to thank Tao Yuan from Peking Union Medical College Hospital for her theoretical and technical assistance and the volunteers who took part in the study.

Ethical Approval and Consent to Participate

This study was approved by the Ethics Committee of the China Institute of Sport Science (CISSLA-20210909). All participants provided written informed consent prior to enrollment.

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ISSN: 2574-1241

DOI: 10.26717/BJSTR.2023.53.008336

Xinwen Cui. Biomed J Sci & Tech Res