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DYSREGULATION OF CIRCULATING miRNAs IN HIGH-FAT DIET AND STREPTOZOTOCIN-INDUCED TYPE 2 DIABETES MELLITUS IN WISTAR ALBINO RATS

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Keywords:

microRNA, Type 2 diabetes mellitus, Endothelial dysfunction, Platelet reactivity, Biomarkers, High-fat diet, Streptozotocin, miR-126, miR-30a-5p

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ABSTRACT: Background: Endothelial dysfunction is a major contributor to the vascular complications of type 2 diabetes mellitus. Circulating microRNAs (miRNAs) have emerged as promising biomarkers and potential modulators of metabolic and cardiovascular diseases. This study aimed to investigate the expression profiles of selected circulating miRNAs in a rat model of T2DM induced by a high-fat diet (HFD) and low-dose streptozotocin (STZ). **Methods:** Male Wistar albino rats were divided into four groups and subjected to dietary and chemical interventions to induce T2DM. Serum and circulating miRNAs were isolated and quantified using real-time quantitative reverse transcription PCR (qRT-PCR). The expression levels of miR-126, miR-30a-5p were analyzed across control and diabetic groups, with U6 small nuclear RNA as an internal control. **Results:** The diabetic groups exhibited significant downregulation of miR-126, suggesting its protective role in vascular integrity and metabolic regulation. In contrast, miR-30a-5p was markedly upregulated in response to High-Fat Diet + STZ (HFD+STZ) induced metabolic stress, implicating it in diabetes-associated metabolic disturbances. Notably, combined HFD and STZ exposure further amplified these alterations in miRNA expression. **Conclusion:** Our findings reveal distinct alterations in circulating miRNA expression associated with T2DM and its vascular complications. Specifically, reduced miR-126 expression and increased miR-30a-5p expression may contribute to endothelial dysfunction and metabolic dysregulation. These miRNAs may serve as potential non-invasive biomarkers for early diagnosis and disease monitoring in T2DM. Further clinical validation and mechanistic studies are warranted.

INTRODUCTION: The objective of this study is to investigate the role of circulating miRNAs associated with endothelial dysfunction and metabolic disturbances in the context of endothelial dysfunction, using a high-fat diet (HFD) and low-dose streptozotocin (STZ) model in Wistar albino rats.

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Cardiovascular diseases, including hypertension, atherosclerosis, and coronary artery disease, are closely associated with endothelial dysfunction and insulin resistance both hallmark features of metabolic disorders such as type 2 diabetes mellitus (T2DM) and obesity [13].

Previous bioinformatics analyses have identified key platelet-related miRNAs, NotablymiR-126, which are highly abundant in platelets and have been implicated in the modulation of platelet reactivity [15]. miRNAs are endogenous, non-coding RNAs that regulate gene expression post-transcriptionally [1, 2]. Remarkably stable in circulation, these molecules have diverse regulatory

functions and exhibit aberrant expression patterns under pathological conditions [8].

It is well-established that obesity particularly the accumulation of excess adipose tissue leads to insulin resistance and hyperglycemia [9]. The global rise in obesity and T2DM is largely attributed to rapid urbanization, industrialization, dietary changes, and reduced physical activity [3].

Endothelial dysfunction arises from several metabolic insults, including persistent hyperglycemia, protein kinase C (PKC) activation, flux through the polyol and hexosamine pathways, and the formation of advanced glycation end products (AGEs), all of which compromise vascular homeostasis [2]. Obesity-related reduction in nitric oxide (NO) bioavailability further impairs endothelial function, as reduced expression of endothelial nitric oxide synthase (eNOS) leads to endothelial dysfunction [1, 4].

miRNAs, which are highly conserved and typically 21–23 nucleotides in length, predominantly bind to the 3' untranslated regions of target mRNAs, promoting mRNA degradation or translational repression [7, 8]. The discovery of circulating and platelet-derived miRNAs as potential biomarkers has spurred interest in their clinical utility for diagnosing and managing endothelial dysfunction, particularly in T2DM [5]. Notably, platelets play a critical role in the development and progression of atherosclerosis, with their activation contributing to ischemic complications in advanced disease stages [13]. Given their stability and disease-associated expression profiles, circulating and plasma-derived miRNAs are promising candidates for novel biomarkers of prognosis and therapeutic response [16]. Emerging research is also exploring their potential as therapeutic targets, as miRNAs regulate key physiological and pathological processes, including metabolism, immune responses, and cellular proliferation [3]. Platelets, a major source of circulating miRNAs, contribute significantly to the miRNA pool found in plasma and other bodily fluids [7]. The abundance of platelet-derived microvesicles correlates with circulating miRNA levels, underscoring their relevance as diagnostic and prognostic tools [14]. Advances in sequencing technologies and qRT-PCR have enhanced the sensitivity and specificity of miRNA detection, facilitating the discovery of novel miRNAs and elucidating their biological functions.

Among the available methodologies, qRT-PCR and microarrays are considered the gold standards for accurate miRNA quantification [2]. While Northern blotting remains a useful technique for its specificity, it is less suitable for low-abundance targets due to its higher RNA requirement and labor-intensive protocol [6].

This study aims to elucidate the expression patterns of selected miRNAs, including miR-126, miR-30a-5p, to understand their roles in platelet reactivity and endothelial dysfunction in a model of diet- and chemical-induced T2DM. The findings could potentially contribute to the identification of novel biomarkers and therapeutic targets for metabolic and cardiovascular diseases.

Experimental Animals and Study Design: Male Wistar albino rats were housed in polypropylene cages procured from Biogen Laboratory Animal Facility Bangalore, India. After a week of acclimatization, they were divided into four groups and treated with different diets for 42 days. Group III and IV received a 60% HFD table, while groups I and II received a standard diet. The rats were fed the appropriate diets for 42 days, with the HFD purchased from VRK Nutritional Solutions.

Development of Type 2 Diabetes Model Caused by HFD/STZ: The rats received a single intraperitoneal injection of streptozotocin (STZ) (Ref no JPS 22-23 1386 Code S0130 Sigma Aldrich, USA) at a low dose (40 mg/kg body weight, dissolved in 0.05 M citrate buffer, pH 4.5) after 42 days of consuming a high fat diet. The HFD was administered to the rats for 42 days in order to produce type 2 diabetes. The effects of STZ (40mg/kg body weight) and HFD were both studied to find the optimum dose for producing T2DM. One week following the injection of STZ, the plasma glucose levels in the control groups (ND and HFD) were determined, and rats with a glucose concentration we got greater than 250 mg/dl the vehicle (0.9% saline solution). The rats' individual diets were permitted to be consumed until the study's conclusion. Because of their HFD, the diabetic group of rats developed insulin resistance;

as a result, even a little insult from a modest dose of STZ would impair -cell activity and cause hypoinsulinemia. All of the rats were starved for the duration of the experiment, and then they were all given an overdose of the anaesthetic ketamine hydrochloride (Ketalar, Pf) before being euthanized. Microcapillary tubes will be used to retro-orbitally plexus collect blood. For the examination of serum Fasting plasma glucose, circulatory microRNA and platelet microRNA expression was perform in serum was separated by centrifugation (4000 rpm, 10 min), after the blood collection [5].

Real-Time Quantitative RT-PCR (qRTPCR) with RNA Isolation: Blood drawn into sodium EDTA-containing tubes, spin at 1000 g to separate plasma, then transferred to RNase-free tubes and kept at 80°C until needed.

Real-Time qPCR Expression of miR-126:

Procedure: The serum kept in liquid nitrogen used to extract the miRNA, then kept at 80°C until needed. Total RNA extracted using the Trizol® reagent from Invitrogen (USA). The RNA pellet dissolved in 10 ml of distilled water that has undergone DEPC treatment. With RNA samples that had been verified by UV spectrophotometry to be free of phenol and protein, reverse transcription was carried out. Using a specialized resin as a separation matrix for RNA, this kit system isolates RNA from cell components using the spin column technique. Using a nanodrop 1000 spectrophotometer from Thermo scientific, RNA purity and content were assessed between 260 and 280 nm. For CDNA production, a real-time miRNA PCR kit (Qiagen) utilized. A thermal cycler (BIORAD) set to 100 reverse transcribe 20 ng of the extracted RNA into cDNA for 5 min at 95 °C and 60 min at 42 °C.

Real-Time qPCR Expression of miR-30a-5p

Procedure: The serum kept in liquid nitrogen used to extract the miRNA, which then kept at 80°C until needed. Total RNA extracted from 250 l serum the Trizol® reagent from Invitrogen (USA). RNA pellet dissolved in 10 l of distilled water that has undergone DEPC treatment. With RNA samples that had been verified by UV spectrophotometry to be free of phenol and protein, reverse transcription was carried out. Using a nanodrop 1000 spectrophotometer from Thermo scientific, RNA purity and content were assessed between 260 and 280 nm. Total RNA from serum extracted using a miRNeasy Mini Kit (Invitrogen). In brief, 200 L mixed with 1 mL of Qiazol, incubated for 5 min at room temperature, 2 g of RNA carrier, and then mixed with 200 L of chloroform. Next, centrifugation at 12,000 g for 15 min at 4 °C was completed, RNA separated by adding 100% ethanol. Mixture applied after being added to a miRNeasy Mini spin column, the mixture centrifuged at 8000 g for two minutes. 500 liters of buffer and 500 liters of 80% ethanol were used to wash the column's contents. The quality and quantity of the eluted RNA in 14 L of RNase-free water were assessed using a nanodrop 1000 spectrophotometer (Thermo scientific). The wavelength ranged from 260 to 280 nm. A Qiagen TaqMan MicroRNA Reverse Transcription Kit was used to retro transcribe RNA. In a final volume of 7.5 ml, the reverse transcriptase mix 2.5 l included 2 l of RNA and 3 l of primer.

TABLE 2: THE PRIMER SEQUENCES USED FOR THE PCR AMPLIFICATION WERE AS FOLLOWS

Genes	Forward primer	Reverse Primer
miR-30a-5p	5’-GCGTGTAAACATCCTCGAC -3’	5’- GTGCAGGGTCCGAGGT-3’
U6	5’- CTCGCTTCGGCAGCACA-3’	5’-AACGCTTCACGAATTTGCGT-3

The thermocycler used to incubate the plates for 30 minutes at 16 °C, 30 minutes at 42 °C, and 5 minutes at 85 °C. During this stage, the cDNA can be maintained at 20 °C for a week. Then, we prepared a mixture of 20 L of TaqMan PreAmp Master Mix (ThermoFischer Scientific), 7.5 L of reverse transcriptase mix, and 10 L of a miRNA primer to create a final volume of 40 L. The mixture was then placed through 20 cycles of denaturation at 95 °C for 10 min, 55 °C for 2 min, and 72 °C for 2 min, followed by 20 cycles of amplification (15 s at 95 °C, 4 min at 60 °C), before being incubated in a thermocycler (BIORAD) for 10 min at 99.9 °C. The preamplified products were then diluted in real-time RT-PCR experiments at a ratio of 1:40 using RNase-free water.

Finally, the 2Ct technique was utilized to compute the mRNA expression ratio, with U6 serving as the internal reference.

RESULTS:

Expression of miR-126: As illustrated in **Fig. 1**, the normal control group exhibited the highest expression levels of miR-126. In contrast, the STZ-treated group demonstrated a moderate reduction in miR-126 expression, while both the High-Fat Diet (HFD) and High-Fat Diet + STZ (HFD+STZ) groups showed significantly lower levels of miR-126 expression compared to controls. These findings suggest a potential involvement of miR-126 in the pathogenesis of diabetes, warranting further investigation into its mechanistic roles and therapeutic potential. The expression levels were quantified using qRT-PCR (n = 8), and data were expressed as mean ± SEM. Statistical significance was determined using the 2^−ΔΔCt method, with P < 0.05 versus respective controls.

TABLE 1: RELATIVE EXPRESSIONS OF MICRO RNA

Relative Expressions of micro RNA	High-Fat Diet (HFD)	High-Fat Diet + STZ (HFD+STZ)	Normal Diet + STZ	Normal Diet (Control)	P-value
126	0.47±0.02	0.72±0.06	1.09±0.26	1.86±0.05	<0.001**
30a-5p	1.55±0.04	3.28±0.34	2±0.39	0.42±0.07	<0.001**

Expression of miR-30a-5p: Fig. 2 demonstrates that miR-30a-5p expression was significantly upregulated in the STZ group compared to the normal control group. Notably, the HFD+STZ group exhibited further enhancement in miR-30a-5p expression compared to the HFD group alone. These results suggest a synergistic effect of STZ-induced hyperglycemia and HFD on miR-30a-5p upregulation, implicating this miRNA in diabetes-related metabolic disturbances. qRT-PCR analysis (n = 8) showed that these differences were statistically significant (P < 0.01, *P < 0.001 versus control).

Finally, we placed particular emphasis on miR-126 due to their extensive prior investigation and established roles in platelet biology and reactivity. The observed differential expression patterns of these miRNAs across experimental groups highlight their potential involvement in the interplay between metabolic dysfunction and platelet activation.

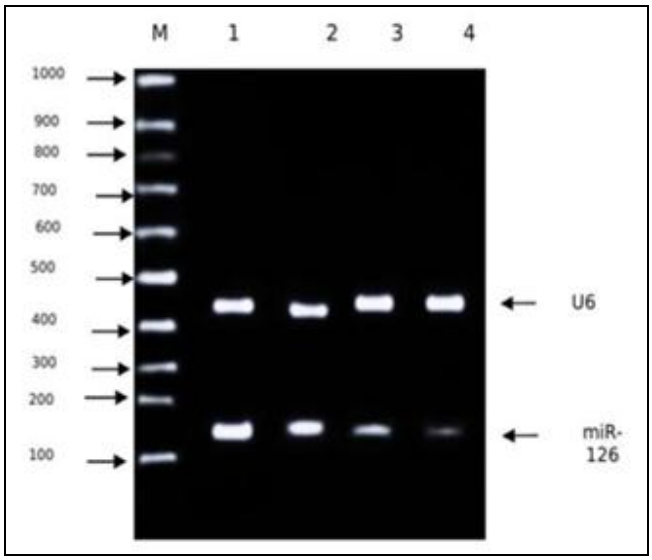

FIG. 1A: miR-126 EXPRESSION LEVELS BY RT-qPCR. Lane M- Marker lane (100-1000 bp); Lane 1-Normal control; Lane 2-STZ; Lane 3-HFD; Lane 4-HFD+STZ.As shown in the Figure 1, miR-126 expression was seen in normal control group. In STZ showed slightly moderate expression of miR-126 and less expression of miR-126 seen in HFD and STZ+HFD group.

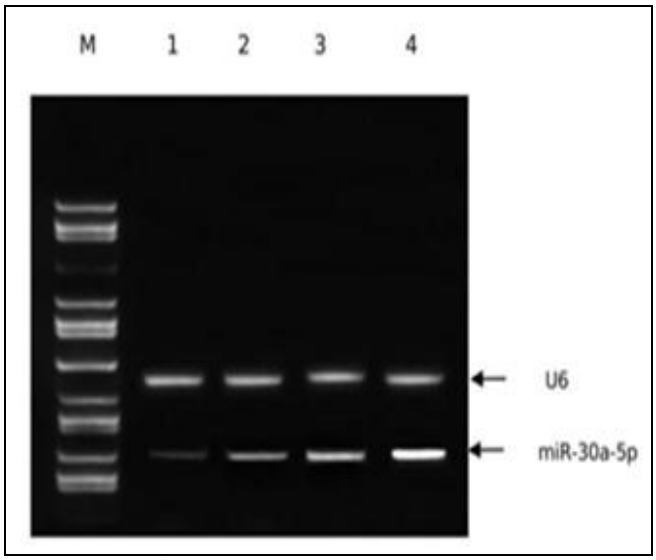

FIG. 1B: miR-30a-5p EXPRESSION LEVELS BY RT-qPCR. Lane M-Marker lane (100-1000bp); Lanel-Normal control; Lane 2-HFD; Lane3-STZ; Lane 4-HFD+STZ. As shown in the Figure 1 level of miR-30a-5p expression was upregulated in STZ group as compared with normal group. In STZ+HFD showed increased expression of miR-30a-5p as compared with HFD group.

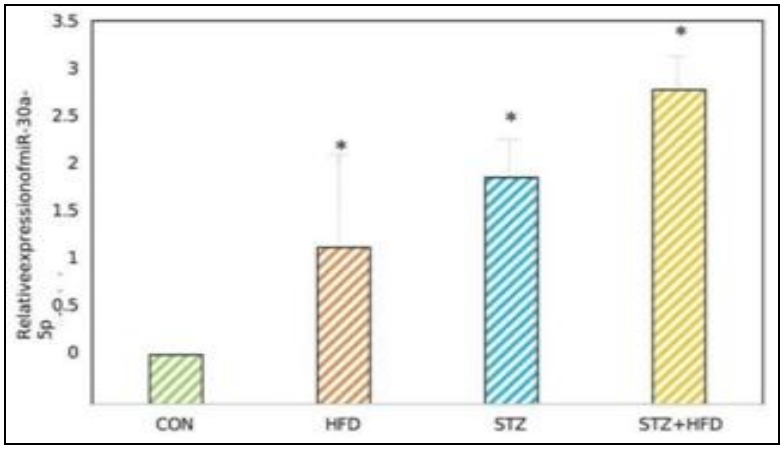

FIG. 2: GRAPHICAL REPRESENTATION OF miR-30a-5p EXPRESSION LEVELS. The figure illustrates the expression levels of miR-30a-5p across different experimental groups. The expression levels were determined by quantitative real-time PCR (qRT-PCR) with a sample size of n = 8. Data are presented as mean ± SEM. The analysis of relative gene expression was conducted using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and the 2^-AACq method.

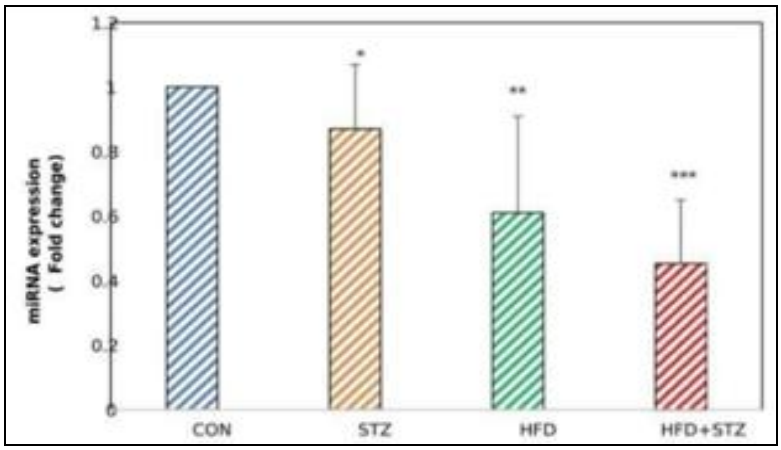

FIG. 3: GRAPHICAL REPRESENTATION OF EXPRESSION miR-126 LEVELS. Fig 1: The expression levels of the miR-126 were determined by qRT-PCR (n = 8) Data are expressed as mean ± SEM. Analysis of relative gene expression data was performed using reverse transcription-quantitative polymerase chain reaction and the 2-AACq method. $p < 0.05$ ** $p < 0.01$ and * $P < 0.001$ vs. respective control.

DISCUSSION: MicroRNAs (miRNAs) play a pivotal role in regulating gene expression and have been implicated in numerous diseases, including cancer, cardiovascular diseases, neurodegenerative disorders, and diabetes mellitus [1]. In this study, we explored the expression profiles of selected miRNAsmiR-126, miR-30a-5p in a rat model of type 2 diabetes mellitus (T2DM) induced by high-fat diet (HFD) and low-dose streptozotocin (STZ). Our findings provide valuable insights into the involvement of these miRNAs in metabolic dysfunction, platelet reactivity, and endothelial impairment [11].

Emerging evidence suggests that miRNA dysregulation is intricately linked to the pathogenesis of both type 1 and type 2 diabetes [4]. miRNAs influence critical processes such as insulin signalling, glucose metabolism, and β-cell function. Specifically, in type 1 diabetes, miRNAs contribute to pancreatic β-cell destruction, while in T2DM, they mediate insulin resistance by interfering with key metabolic signalling pathways [1].

Obesity, a major risk factor for T2DM, exacerbates insulin resistance through a complex interplay of factors including adipokine dysregulation, chronic inflammation, and impaired insulin signalling. miRNAs regulate these pathological processes, and research has shown promising results in non-human primate models, laying the groundwork for potential miRNA-based therapeutics [4]. These targeted therapies may revolutionize diabetes management by modulating disease-specific miRNAs. In our study, the downregulation of miR-126 in diabetic groups aligns with previous

findings suggesting its protective role in vascular integrity and endothelial function [7]. Plasma miR-126 levels were inversely correlated with fasting blood glucose, supporting its potential utility as a non-invasive biomarker for early detection and monitoring of T2DM progression. Similarly, the upregulation of miR-30a-5p observed in the STZ and HFD+STZ groups corroborates findings from the CORDIOPREV trial and other studies, which identified elevated miR-30a-5p levels in individuals at risk of developing diabetes [8, 9]. miR-30a-5p has been shown to mediate β-cell dysfunction and glucotoxicity in rodent models, highlighting its relevance in diabetic pathology [10, 15].

Platelets, which serve as reservoirs of miRNAs, are deeply involved in the development of atherosclerosis and thrombotic complications associated with diabetes [15]. Platelet hyperactivity, driven by hyperglycemia and chronic inflammation, contributes to vascular complications and the pro-thrombotic state observed in diabetic patients [7]. The altered expression profiles of circulating miRNAs observed in our study suggest their potential utility as biomarkers of diabetes-associated vascular dysfunction. For diabetes and cardiovascular risk stratification. Furthermore, miRNAs' biochemical stability and tissue specificity make them attractive candidates for biomarker development [13].

As precise regulators of gene expression, miRNAs contribute to complex biological networks, and their comprehensive profiling can enhance our understanding of disease mechanisms and aid in the development of personalized therapeutic strategies. It is noteworthy that the variability in miRNA expression reported across different studies could be attributed to differences in study populations, disease stages, and methodological approaches [10]. Additionally, the dynamic nature of miRNA expression in response to metabolic and inflammatory stimuli highlights the need for standardized protocols and longitudinal studies to validate their clinical utility [15]. Our study contributes to the growing body of evidence supporting the role of circulating miRNAs in T2DM and its vascular complications. Specifically, we identified miR-126, miR-30a-5p as key regulators potentially involved in glucose metabolism, insulin signalling, platelet activation, and endothelial dysfunction [11].

CONCLUSION: The present study highlights the critical role of specific circulating microRNAs (miRNAs) in the regulation of endothelial dysfunction within the context of diet- and chemically-induced type 2 diabetes mellitus (T2DM). Our findings demonstrate that miR-126, miR-30a-5p, exhibit distinct expression patterns in response to metabolic stress induced by a high-fat diet and low-dose streptozotocin administration.

Downregulation of miR-126 and miR-30c-5p in diabetic groups underscores their potential protective roles in maintaining vascular homeostasis and metabolic regulation. Conversely, the upregulation of miR-30a-5p, points to their involvement in promoting inflammatory responses, platelet activation, and glucose metabolism dysfunction. These findings reinforce the hypothesis that miRNAs not only serve as biomarkers but may also actively participate in the pathogenesis of diabetes and its vascular complications. The intricate network of miRNA-mediated regulation presents both challenges and opportunities. While technical and methodological limitations persist, advances in high-throughput sequencing and quantitative PCR technologies have significantly improved the sensitivity and specificity of miRNA detection.

Integrating these technologies with human disease-related miRNA profiling holds great promise for unraveling the molecular underpinnings of cardiometabolic diseases. Future research should focus on validating these findings in clinical settings, exploring the therapeutic potential of miRNA modulation, and elucidating the complex interplay between miRNAs and their target genes. Such efforts will pave the way for the development of innovative diagnostic tools and personalized treatment strategies aimed at mitigating the burden of diabetes and its associated cardiovascular risks. In conclusion, our study provides valuable insights into the regulatory roles of miRNAs in T2DM, supporting their potential as novel biomarkers and therapeutic targets. By deepening our understanding of miRNA-mediated pathways, we move closer to achieving more precise and effective interventions for metabolic diseases.

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Ethical Declarations:

Compliance with Ethical Standards: All procedures performed in this study were in accordance with the ethical standards of the Institutional Animal Ethics Committee and with national guidelines for the care and use of laboratory animals.

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Ethical Approval: Ethical approval for the animal study was obtained from the Institutional Animal Ethics Committee (IAEC), Saveetha Medical College and Hospital, Saveetha Institute of Medical and Technical Sciences (SIMATS), Chennai, in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (IAEC Approval No.: SU/CLAR/RD/001/2022; CPCSEA Registration No.: 1183/PO/Re/S/08/CPCSEA).

Informed Consent: This article does not contain any studies with human participants, and therefore informed consent is not applicable.

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

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