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Comparison of Brain and Pancreas Tissues Exosome derived -miRNA -9 and -146 levels in Healthy and Diabetes Rats

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Abstract: Small vesicles called exosomes have been found to regulate gene expression in tissues and play a role in the pathogenesis of many diseases. Therefore, this study aimed to determine the effects of exosomes on diabetes and associated microRNA (miRNA) alteration. For this purpose, nicotinamide (120 mg/kg) was administered intraperitoneally (i.p.) and 15 minutes later, Streptozotocin (50 mg/kg) was administered i.p. Rats with glucose levels of 126 mg/dL and above were considered to have Type 2 diabetes. At the end of 21 days, exosomes were obtained from the pancreas and brain tissues of diabetic patients and rats of healthy groups (n: 10). Then, biochemical analyses and oxidative stress parameters of both groups were examined. Additionally, miRNA changes were examined and the results were evaluated statistically. It was observed that the total antioxidant level (TAC) decreased compared to the control group (P<0.05). As a result of the examination by Real-Time PCR, it was determined that miRNA-9 levels were significantly increased and miR-146 gene levels were significantly down-regulated in both brain tissue and pancreas tissue (P<0.05).
As a result, significant changes occurred in miRNA -9 and -146 levels in brain and pancreatic tissue exosomes of diabetic rats. These results show that diabetic rat exosomes cause changes in miRNA levels and that this change may be related to neuroinflammation.

Keywords: Diabetes, LDH, miRNA-9, miRNA-146.

Sağlıklı ve Diyabetli Sıçanlarda Beyin ve Pankreas Dokusundan İzole Edilen Eksozom -miRNA -9 ve -146 Profillerinin Karşılaştırılması

Öz: Eksozom adı verilen küçük veziküllerin dokulardaki gen ekspresyonunu düzenlediği ve birçok hastalığın patogenezinde rol oynadığı bulunmuştur. Bu nedenle, bu çalışma ekzozomların diyabet ve buna bağlı mikroRNA (miRNA) değişimi üzerindeki etkilerini belirlemeyi amaçlamıştır. Bu amaçla intraperitoneal olarak nikotinamid (120 mg/kg) uygulandı (i.p.) ve 15 dakika sonra Streptozotosin (50 mg / kg) ip uygulandı. Glikoz seviyesi 126 mg/dL ve üzeri olan sıçanlar Tip 2 diyabet olarak kabul edildi. 21 Günün sonunda şeker hastalarının ve sağlıklı grupların sıçanlarının (n:10) pankreas ve beyin dokularından eksozomlar elde edildi. Daha sonra her iki grubun biyokimyasal analizleri ve oksidatif stres parametreleri incelendi. Ayrıca miRNA değişiklikleri incelenmiş ve elde edilen sonuçlar istatistiksel olarak değerlendirilmiştir. Total antioksidan düzeyinin (TAC) kontrol grubuna göre azaldığı gözlendi (P<0.05). Gerçek Zamanlı PCR ile yapılan inceleme sonucunda hem beyin dokusunda hem de pankreas dokusunda miRNA -9 düzeylerinin önemli ölçüde arttığı ve miR-146 gen düzeylerinin önemli ölçüde aşağı regüle edildiği belirlendi (P<0.05).
Sonuç olarak, diyabetik sıçanların beyin ve pankreas dokusu eksozomlarında miRNA -9 ve -146 seviyelerinde önemli değişiklikler meydana geldi. Bu sonuçlar, diyabetik rat eksozomların miRNA düzeyinde değişikliğe neden olduğu ve bu değşikliğin nöroinflamasyonla ilişkili olabileceğini göstermektedir.

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Anahtar kelimeler: Diabetes, LDH, miR-9, miR-146.

INTRODUCTION

There are two types of diabetes, Type 1 and Type 2. Type 1 diabetes occurs as a result of damage to pancreatic ß cells. In the etiology of type 2 (non-insulin-dependent diabetes) diabetes, there is amyloid deposition in the pancreas cells, which generally develops in the elderly. (Butler et al., 2003; Chatterjee et al., 2017; Westermark et al., 1987). Relative insulin lack in type 2 diabetes is characterized by impaired pancreatic beta-cell activity and insulin resistance in target organs. The incidence and prevalence of type 2 diabetes have recently increased fourfold due to an aging population, sedentary lifestyles, and global obesity rates (Collaboration, 2017). The main characteristic of diabetes is a high blood glucose (more than 200 mg/dL) concentration. High glucose concentrations lead to major damage to blood vessels endothelial, kidneys, liver, eyes, and brain tissue (Biessels & Reagan, 2015; Brownlee, 2005; Gilbert & Cooper, 1999; Li et al., 2002; Targher & Byrne, 2017). The increased incidence of dementia in type 2 diabetes (T2D) is best clarified at least in part, by neuroinflammation. Nowadays, the evidence for this claim is; it focuses on clinical and experimental data demonstrating that inflammation of the neurons contributes a more significant role than previously thought in the pathogenesis of neurodegenerative disorders, especially Alzheimer's disease (AD), as well as evidence that T2D is a condition with a significant inflammatory component that may increase neurological inflammation (Wong et al., 2018).

Exosomes were first discovered in the maturing mammalian reticulocyte (immature red blood cell) and nano-sized vesicles (40-150 nm), considered primary secretion products from many cells (Yeni et al., 2023). They can regulate cell-to-cell communication by transferring the mRNAs, miRNAs, and proteins they contain to target cells (Genc et al., 2023; Sıdıka et al., 2021)

Small interfering RNA (siRNA, a double-stranded RNA), known as short interfering RNA or silencing RNA, is similar to miRNA (Finnegan & Matzke, 2003; Sakshi et al., 2021). It is a class of non-coding RNA molecules generally 20-24 (normally 21) base pairs long. It interferes with the expression of nucleotide sequences by preventing translation by cleaving mRNA after the transcription of specific genes (Balasubramanyam et al., 2011; Sakshi et al., 2021). miRNAs are small, single-stranded, non-coding RNA molecules that inhibit protein production by binding to target mRNA. In addition, in different types of cancer like breast cancer, the expression of miRNA is suppressed. miRNA 123 can directly downregulate Integrin β1 (Balasubramanyam et al., 2011). The cytoplasmic domain of integrin beta-1 binds to the actin cytoskeleton. miR-146 is thought to be a mediator of inflammation. miRNA 226 down expression interference cell migration and innate immunity (Chen et al., 2012; Lopes et al., 2017; Taganov et al., 2006; Ye et al., 2020).

The current study aims to determine how the miRNA -9 and -146 profiles of exosomes belonging to brain and pancreatic tissue change in T2D rats. With this study, exosome miRNA level changes of rats with T2D will be studied for the first time, and it will be investigated that these exosomes may be mediators, especially in neuroinflammation.

MATERIAL AND METHOD

Chemicals And Reagent: Streptozotocin (18883-66-4) and Nicotinamide (98-92-0) were purchased from Sigma Aldrich (Berlin, Germany). Total Exosome RNA & Protein Isolation Kit (Catalog number: 4478545) was purchased Invitrogen™. Enzyme Linked-Immuno-Sorbent Assay (ELISA) kits were bought from Elabscience (Texas, USA).

Selection of Experimental Animals: For the study, 20 male Sprague-Dawley rats weighing 180-250 g were obtained from Atatürk University Medical Experimental Application and Research Center (ATADEM) and fed with standard rat chow, and drinking water bottles were changed daily. All rats were housed at room temperature of 24±2°C in a 12/12-hour dark/light period in the experimental animal laboratory before and until the end of the experiment. The study received ethical permission from Atatürk University on 29.09.2022 and E-77040475-641.04-2200302591 no.

Diabetes Induction of Rats: A single intraperitoneal injection of 50 mg/kg body weight streptozotocin was administered 15 minutes after administration of 120 mg/kg body weight nicotinamide intraperitoneal injection in type 2 diabetes mellitus, overnight fasted rats. Streptozotocin was dissolved in citrate buffer (pH 4.5) and nicotinamide was dissolved in saline (Masiello et al., 1998; Reed et al., 2000; Szkudelski, 2012). Hyperglycemia was confirmed by elevated plasma glucose levels, determined 72 hours after injection and then on day 7. Animals with a blood glucose concentration above 126 mg/dl were used for the study (Masiello et al., 1998; Reed et al., 2000).

Detection of the Hyperglycemic Effects: The night before the experiment, the rats were not fed with food, and only water was given. Before streptozotocin (STZ) injection, rats' baseline weights (g) were determined, and basal blood glucose values (mg/dL) were measured with a glucometer (On.Call® Plus, Acon Lab., Inc. America). Then, to induce diabetes in rats, STZ solution (1 mL) prepared in pH 4.5 cold citrate buffer just before administration was administered to rats i.p. was injected (50 mg/kg) after administration of 120 mg/kg body weight nicotinamide (NA) intraperitoneal injection in 0.9% NaCl (1 mL) prepared just before administration (1 mL) i.p. was given to animals (120 mg/kg).

Animals were given 4 mL i.p. of 20% dextrose solution in the first 4 hours (between the 2nd and 3rd hours). Animal waters were replaced with 5% dextrose solution for 24 hours. Those with a fasting blood glucose level >126 mg/dL in the blood sample taken from the tail vein on the 7th day of the application were considered diabetic (Type 2). Fasting and postprandial sugars were determined once a week during the 21-day waiting period to ensure the stability of the diabetes model in animals (Kaplan et al., 2019; Reed et al., 2000).

Working Groups and Application of Formulations: Group I: The group consists of healthy animals (n=10) to whom no substance was administered.

Group II: Rats considered to have Type 2 diabetes (n=10);

No treatment was applied after the diabetes model was created. The blood glucose level was measured using a glucometer in the blood sample taken from the tail vein at the specified time intervals (0., 1., 3., 6. hours) on the first day. The time point at which the blood glucose level was measured the lowest was determined, and the measurement was made only at that time point in the following days. During this time, all rats were given only water but no feed. Apart from these periods, animals were provided access to water and typical food. After definitive proof that the diabetes model was formed, the animals were exterminated within the framework of ethical rules, and brain and pancreatic tissue were taken.

Biochemical Analysis: Homogenates will be prepared from tissues of brain and pancreas tissues stored at -80 °C, and LDH, TAS, and TOS levels in the obtained supernatants were determined by taking measurements in the ELISA device with kits to be obtained from Elabscience (Germany).

Exosome Isolation: For exosome isolation, 1 ml of homogenized tissue was first taken and centrifuged at 300g for 10 minutes. The supernatant of the liquid was taken and centrifuged for 10 minutes at 2000g and 10000g, respectively. Then, the supernatant was born again, and ultracentrifugation was performed. The pellet was washed with HBSS by centrifugation at 100000-20000 x g for 70 minutes and then centrifuged again at 100000-20000 x g for 120 minutes to obtain pure exosomes. All centrifugation processes were carried out at 4 °C(Sıdıka et al., 2021).

Exosome Characterization:

SEM: A scanning electron microscope (SEM; Scanning Electron Microscope) is an electron microscope that obtains an image by scanning the sample surface with a focused beam of electrons. By interacting with the atoms in the exosome sample, it generates different signals containing information about the topography and composition of the sample surface. The change in the number of secondary electrons ejected from other parts of the sample primarily depends on the angle of encounter of the beam with the surface, that is, on the topography of the character. In addition to secondary electrons, backscattered electrons, characteristic X-rays, and signals are obtained, and appropriate topography and composition analyses are performed (Genc et al., 2023).

Real-Time PCR Analysis:

Obtaining genetic material: After the exosome isolation from the samples, the exosomes were kept at -80 until to RNA isolation step.

RNA isolation: miRNA isolation was obtained according to the procedure of Invitrogen™ Total Exosome RNA & Protein Isolation Kit (Catalog number: 4478545).

Gene expression: For miR-9 (forward primer 5’-CCGAGCTCAGAAAAGCAATAATGTCCAG-3’ and reverse primer 5’ -GCTCTAGATCCATTCACCAGGGAGCAG-3’) and miR-146 (forward primer GCGAGGTCAAGTCACTAGTGGT reverse primer CGAGAAGCTTGCATCACCAGAGAACG) genes, 0.25 µl of right and left primer, 0.15 µl of the probe, 3 µl of cDNA, 3 master mix (Roche, Germany), and 12.75 µl of distilled water were added to each strip (tube) at this stage. The final volume was adjusted to 20 µl. After 600 seconds at 95 0C, 10 seconds at 95 degrees, and 30 seconds at 60 degrees, 45 cycles were made. We normalized the miRNA expression of target miRNAs to the RNU6 reference control using the $^{\Delta\Delta}$Ct method, as previously described (Genc et al., 2023).

Statistical analysis: The results were analyzed with the SPSS 20.0 Windows program and given as mean ± standard error. Statistical comparison between groups was subjected to parametric and non-parametric tests. One Way ANOVA and Kruskal Wallis tests were applied, and data that were more significant than P<0.05 were considered statistically significant.

RESULTS

Exosome characterization via SEM: SEM visualized the resulting exosomes. Brain tissue exosomes are shown in Figure 1, and pancreatic tissue exosomes are shown in Figure 2. When SEM images were examined, it was determined that the exosomes obtained were between 24.80 nm and 129.2 nm. These results show that the isolation of exosomes was performed correctly.

Biochemical Results:

LDH Results: Since LDH is released by necrotic cells, it is an excellent metabolic marker of cell viability. The effect on LDH activity in rat brains and pancreatic tissues with diabetes mellitus was determined using an LDH kit. LDH activity expressed as % of standard (shown as 100%) is shown in Figure 3. LDH levels were relatively low in the brain and pancreatic tissue control groups. While the LDH level was 9.76% in the control group of the brain tissue, this

value was determined as 11.32 in the control group of the pancreatic tissue. LDH results of the diabetes group increased significantly (44.34% in brain tissue, 47.98% in pancreas tissue) ($p<0.05$ and $p<0.01$). These data show us that the cytotoxic effect increases significantly due to Type 2 diabetes.

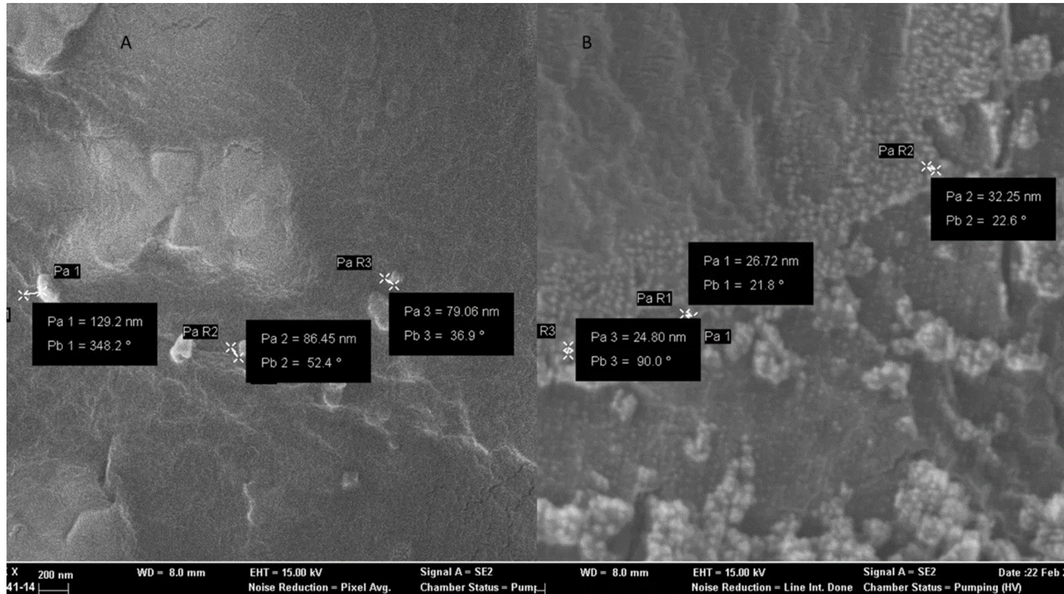

Figure 1. Controls (A) and Diabetes (B). Brain tissue exosomes were examined by SEM. The white arrow shows particle size. P: particle, Pa: particle size in nm, Pb: particle angle, and, Pa R: particle radius. (The images are taken by Carl Zeiss Evo 40 SEM; Jena Germany).

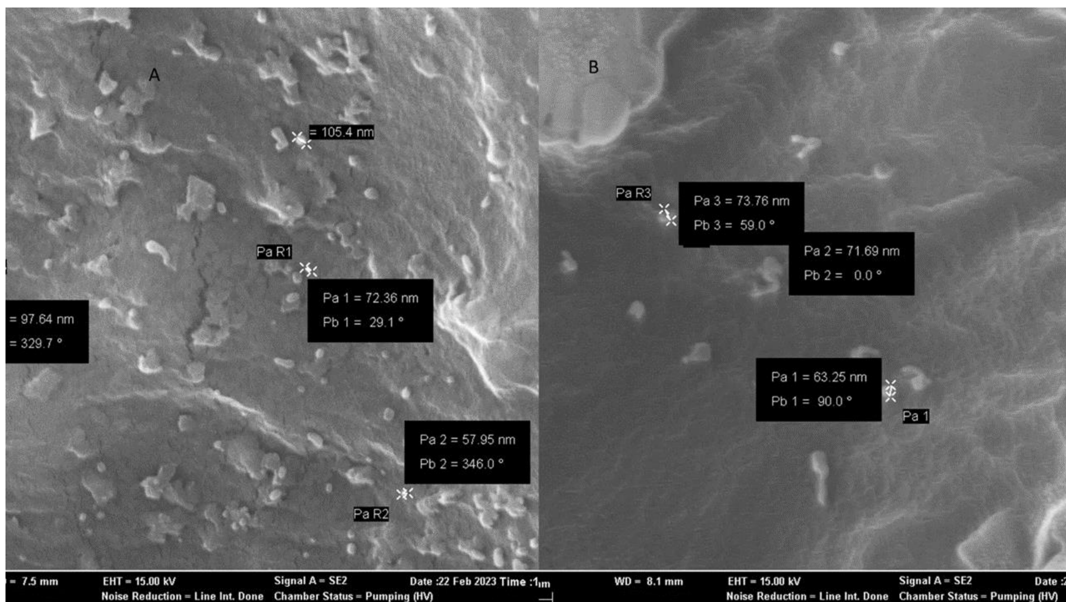

Figure 2. Controls (A) and Diabetes (B). Pancreas tissue exosomes examined by SEM. The white arrow shows particle size. P: particle, Pa: particle size in nm, Pb: particle angle, and, Pa R: particle radius. (The images are taken by Carl Zeiss Evo 40 SEM; Jena Germany).

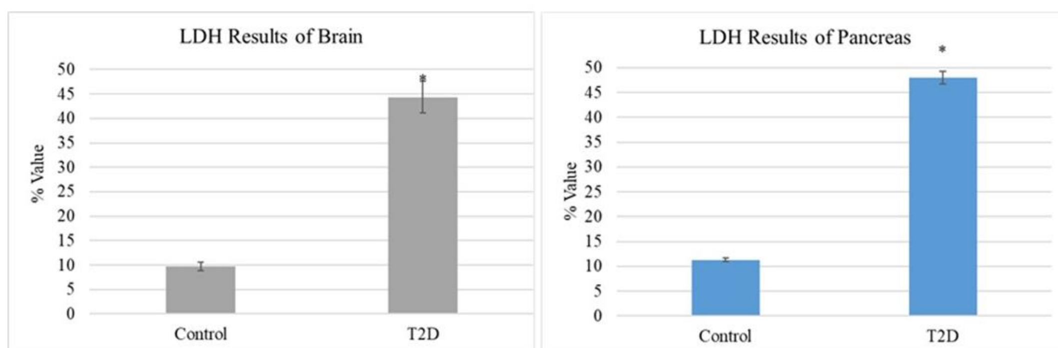

Figure 3. Results of LDH activity of experimental groups. Data, mean ± SD specified as. ** P values <0.05 were found to be very significant for the control group.

Redox Status of T2D Brain and Pancreas Tissues:

TOS results: The TOS value of the spectrophotometrically determined control group was 2.098 mmol H_2O_2 equivalent/$^-$L in the brain tissue, and the TOS value was 3.76 mmol H_2O_2 equivalent/$^-$L in the pancreatic tissue (Figure 4.) Compared to the control group, it increased the oxidant activity in the TD2 model and led the cells to cytotoxicity ($p<0.001$). While the oxidative stress level was found as 13.99 mmol H_2O_2 equivalent/$^-$L in the brain tissue, this value was found as 15.125 mmol H_2O_2 equivalent/$^-$L in the pancreatic tissue. TOS value increased significantly in both tissues.

TAC Results: When the TAC level was examined, the TAC value of the control group in the brain tissue was found to be 22.098 mmol Trolox equivalent/L, and the pancreatic tissue was 21.11 mmol Trolox equivalent/L (Figure 5.) Compared to the control group, antioxidant activity in the TD2 model decreased significantly in both tissues ($p<0.001$). While the oxidative stress level was 4.50 mmol Trolox equivalent/L in the brain tissue, this value was 5.22 mmol Trolox equivalent/L in the pancreatic tissue.

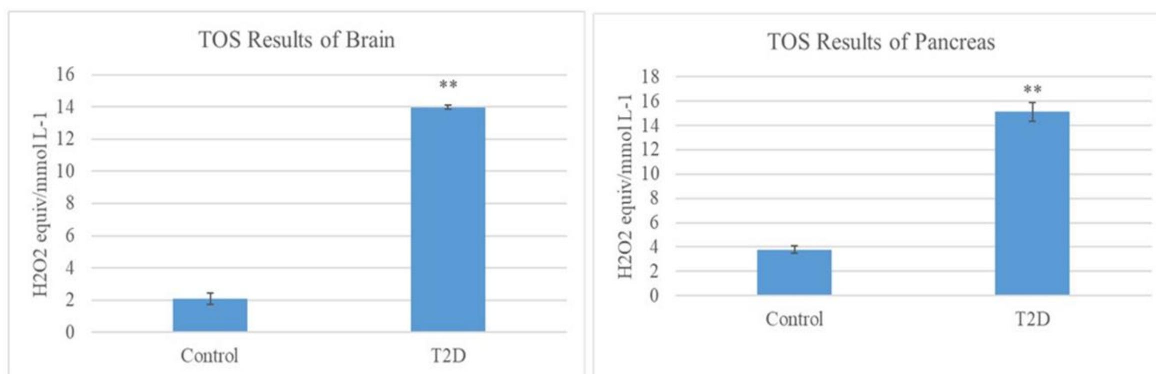

Figure 4. TOS results of brain and pancreas tissue. Data, mean ± SD specified as. ** P values <0.001 were found to be very significant for the control group.

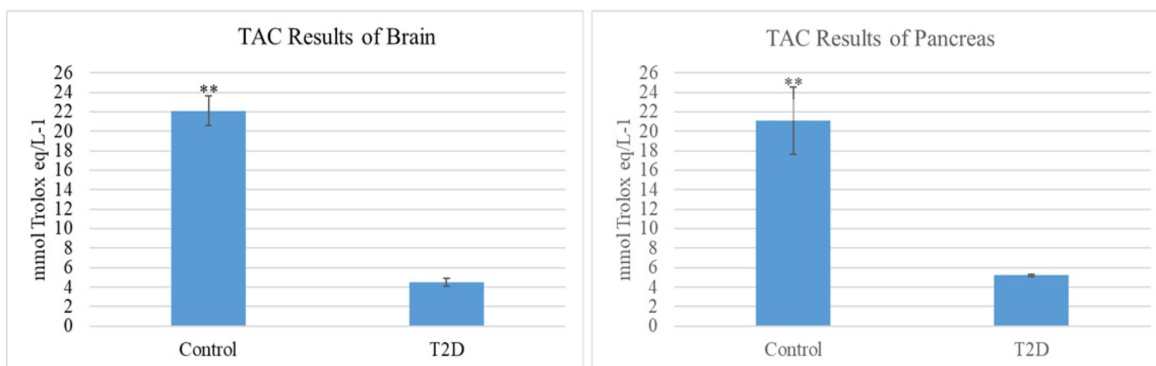

Figure 5. TAC level results of brain and pancreas tissue. Data, mean ± SD specified as. ** P values <0.001 were found to be very significant for the control group.

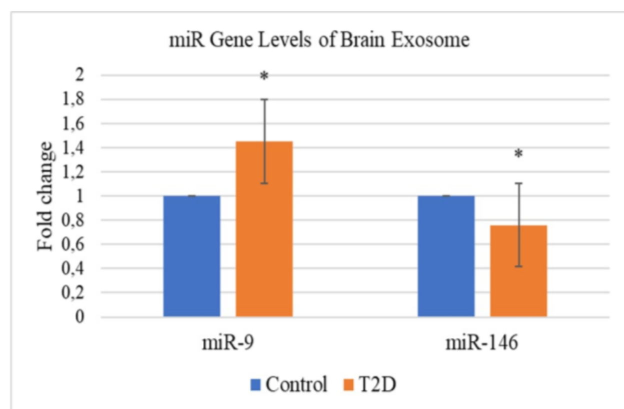

Figure 6. (A) miRNA-9 and (B) miRNA-146 gene expression levels in brain tissue exosomes (n = 10). Data are presented as means ± SD * p<0.05 vs. the control group.

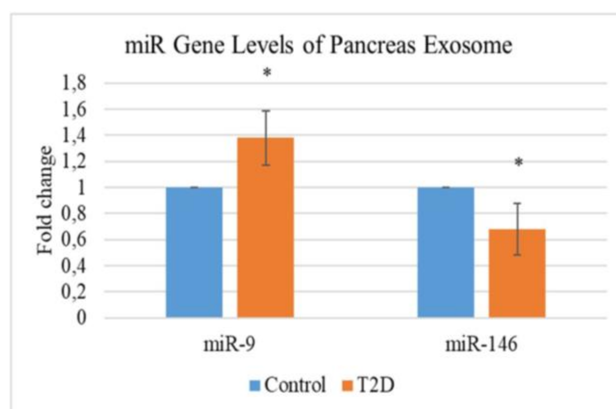

Figure 7. Effects of T2D on (A) miRNA-9 and (B) miRNA-146 gene expression levels in pancreas tissue exosomes (n = 10). Data are presented as means ± SD * p<0.05 vs. the control group.

RT-PCT Results: Exosomes obtained from brain and pancreas tissues of rats with diabetes model and gene expression levels of miRNA-9 and miRNA-146 genes were measured by Real-time analysis. The results are shown in Figures 4 and 5. When the exosomes obtained from the brain tissue were examined, it was determined that there was a significant increase in miRNA-9 level, on the contrary, the miRNA-146 gene was down-regulated. Similarly, while the miRNA-9 level increased in pancreatic tissue, miR-146 decreased significantly. ($p < 0.05$)

DISCUSSION

Diabetes has a high patient population worldwide. For this reason, the diabetes model, which has attracted the attention of scientists, has been the subject of much research. Although different methods are used to conduct diabetes research, induction of diabetes with the help of streptozotocin is more common. To increase the reliability and utility of this model, applications were made gradually with high care (Wang-Fischer & Garyantes, 2018; Wu & Huan, 2008).

When pancreatic tissue's beta cells are harmed, diabetes results. As a result, this tissue experiences initial injury and inflammation. Liver, eye, kidney, and brain tissue are affected afterward. A substantial amount of experimental and clinical data demonstrates that T2D negatively impacts brain physiology, cognition, and behavior and harms several organs, resulting in older people's daily lives (Kodl & Seaquist, 2008; Mijnhout et al., 2006). The duration, as well as the type of diabetes, impacts how cognitive impairment progresses and how severe it is. Diabetes-induced cognitive and behavioral impairments (DACD) have a complex multifactorial pathophysiology that has not yet been fully understood. On the other hand, it has been hypothesized that defective insulin signaling, oxidative stress, and inflammation may play a significant role in the emergence of diabetes-related cognitive impairment(Muriach et al., 2014). Inflammation and diabetes are inextricably interconnected, and NF-B has become a fundamental player in this relationship. Diabetes has been associated with increased downstream pro-inflammatory cytokines and NF-B activation.(Ganesh Yerra et al., 2013; Patel & Santani, 2009; Zhao et al., 2011) Understandably, NF-B regulates the expression of specific miRNA-related genes, given the fact that it has numerous pleiotropic signaling cascades. Studies have examined the possibility of miRNAs forming innate immune receptors that act through the activation of NF-kB signaling, and it has been found that there is a link between various miRNAs (miR-146a, miR-155, and miR-9) and the activation of NF-kB signaling (Bazzoni et al., 2009; Impey et al., 2004; Kluiver et al., 2007; Taganov et al., 2006). Therefore, our study, it was aimed to examine the changes in the exosomal miRNA (miRNA 9 and 146) levels, especially in the brain and pancreas tissues of rats for which the diabetes model was created. We think that the transport of these genes to the brain, which is a distant tissue, with the exosome may be effective in NF-kB activation or inhibition.

The study conducted by Mehmet Haligur et al (2012) shows degenerative and necrotic beta cells in Langerhans islets of the pancreas, in the diabetes study group (Haligur et al., 2012). In another study, the researcher showed that in diabetic rats, pancreas weight decreased near to 50% of the control group because of inflammation and beta cell necrosis (Shawky et al., 2019). This data correlates with our oxidative studies results (figs 3, 4, and 5). According to our results, LDH and TOS (oxidative stress markers) show a significant increase. Increasing degeneration and damage to cells leads to secrete both LDH and free radicals. In the paradox, total antioxidant capacity shows a decrease in severe degeneration and tissue damage process. According to our result, the TAC level decreased sharply.

Exosomes are small nanosized vesicles. Recently an increase was seen in the number of studies showing exosome role in disease pathogenesis majorly in Brain, cancer, and liver disease (Guo et al., 2020; L Isola & Chen, 2017; Zhang et al., 2021). in addition, exosomes can be used as a marker for diagnosis and prognosis (Samanta et al., 2018). In diabetes, exosomes have dual functions in mediating insulin resistance/sensitivity. For example, M1 macrophage-derived exosomes inhibit insulin secretion. Also, exosomes can carry miRNAs, and by transferring among cells to regulate various molecular pathways such as AMPK and PI3K/Akt, to affect DM progression (Ashrafizadeh et al., 2022). In the current study, we evaluate miRNA-9 and miRNA-146. The result shows an increase in the miRNA-9 level and a decrease in the miRNA-146 gene expression level. Lei Kong et al (2011) shows blood and pancreas miR-9, miR-29a, miR-30d, miR34a, miR-124a, miR-146a, and miR-375 level was up-regulated in newly diagnosed diabetes patient (Kong et al., 2011). This study shows a correlation with our study miRNA -9 shows an increase. Also, we did this evaluation in rat brain tissue, and we have seen an upregulation. Lucy Baldeón R (2014) et al serum miRNA-146a level as a sign of chronic inflammation in type 2 diabetic patients shows a significant decrease (Baldeón R et al., 2014). Degeneration and necrosis of the pancreas and brain as a result of DM are responsible for the decrease in miRNA-146a expression. In our study exosome origin miRNA-146a expression was down-regulated whereas miRNA-9 was upregulated.

CONCLUSION

In conclusion, exosomes can be used as a biomarker for the screening of DM prognosis. Exosomes can carry miRNA and by evaluating them we can find different tissue inflammation statuses and is the treatments are capable of overcoming diabetes or not.

Informed Consent Statement: Not applicable.
Data Availability Statement: Not applicable.
Conflicts of Interest: The authors declare no conflict of interest.

Authors Contributions

AT and SG designed the experiments.AT and SG performed the experiments.AT and SG analyzed the data. AT and SG wrote and revised the manuscript. All authors have read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study was approved by the Ataturk University Animal Experiments Local Ethics Committee (decision number: E-77040475-641.04-2200302591; dated 29.09.2022), which operates in line with European (EU) Directive 2010/63/EU.

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