Changes in cognitive ability and serum microRNA levels during aging in mice

KEISUKE YAMAMOTO[1], KOHTA MIYANO[2], MINAMI FUJITA[2], WAKANA KURATA[2], HIROYA OHTA[2], KANA MATSUMOTO[1] and MITSURU CHIBA[1,3]

[1]Department of Bioscience and Laboratory Medicine, Graduate School of Health Sciences; [2]Department of Medical Technology, School of Health Sciences; [3]Research Center for Biomedical Sciences, Hirosaki University, Hirosaki, Aomori 036-8564, Japan

Received August 30, 2023; Accepted January 8, 2024

DOI: 10.3892/etm.2024.12408

Abstract. Mild cognitive impairment (MCI) is an early stage that can result in dementia. MCI can be reversed, and diagnosis at an early stage is crucial to control the progression to dementia. Dementia is currently diagnosed based on interviews and screening tests; however, novel biomarkers must be identified to allow early MCI detection. Therefore, the present study aimed to identify novel biomarkers in the form of blood microRNAs (miRNAs/miRs) for the diagnosis of MCI or early dementia. Blood samples were collected from C57BL/6NJcl male mice at four time points, including 4-week-old (4W), 8-week-old (8W), 36-week-old (36W) and 58-week-old (58W), and serum was isolated. Body weight and blood total cholesterol levels were increased, and blood alkaline phosphatase was decreased with aging. The 8W mice exhibited the highest cognitive ability in the Morris water maze test, whereas the 58W mice demonstrated decreased cognitive ability. The serum RNA concentrations of the 4W, 8W, 36W and 58W mice demonstrated no significant differences. Furthermore, small RNA levels were detected in the serum of all mice. miRNA microarray analysis revealed a >1.5-fold increase in the serum expression of two miRNAs (miR-21a-5p and miR-92a-3p) and a >1.5-fold decrease in the serum expression of two other miRNAs (miR-6769b-5p and miR-709) in 58W mice compared with those in 8W mice. In the future, we aim to further analyze aged mice to discover novel MCI biomarkers.

Correspondence to: Dr Mitsuru Chiba, Department of Bioscience and Laboratory Medicine, Graduate School of Health Sciences, Hirosaki University, 66-1 Hon-cho, Hirosaki, Aomori 036-8564, Japan
E-mail: mchiba32@hirosaki-u.ac.jp

Key words: aging, microRNA, gene expression, microarray, serum

Introduction

The global prevalence of dementia has exponentially increased due to prolonged life expectancy and an aging population. Currently, >55 million people are suffering from dementia globally (1). The syndrome of dementia is caused by a various diseases that eventually destroy nerve cells, thereby damaging the brain, and causing cognitive ability deterioration compared with that expected from the normal biological aging course (1,2). Mild cognitive impairment (MCI), can be considered the 'transitional zone' between normal cognition and dementia and has become a novel topic in clinical research (3).

MCI is an early stage that can result in dementia. MCI can be reversed and diagnosis at an early stage is crucial to control the progression to dementia. Currently, neuropsychological testing (4), transcranial ultrasonography (5), near-infrared spectroscopy that enables measurement of hemoglobin concentration changes in the brain (6), magnetic resonance imaging (7), detection of cerebrospinal fluid biomarkers, detection of such as amyloid beta and phosphorylated tau protein in spinal fluid (8,9), electroencephalography (10), and olfaction tests (11) are the diagnostic modalities used to diagnose dementia. However, all of these testing methods are useful in diagnosing dementia, but they cannot detect MCI with sensitivity.

MicroRNAs, which are small RNA molecules that regulate gene expression in cells, are found in blood and are mainly encapsulated in extracellular vesicles such as exosomes (12,13). In recent years, many researchers have revealed serum microRNAs as biomarkers for early disease detection (14,15). This study aimed to clarify changes in serum microRNAs in cognitively impaired mice.

Materials and methods

Mice. We purchased male C57BL/6NJcl mice from CLEA Japan. All mice were provided solid diet CE-2 (CLEA Japan) and water *ad libitum* and were housed in a conventional animal room with 12-h light/dark cycles. Mice were housed up to 5 mice per gauge, and bedding, feed, and water were changed weekly. In this study, 10 4-week-old (4W), 9 8-week-old (8W), 5 36-week-old (36W) and 6 58-week-old (58W) mice were used.

We consulted the report of Ackert-Bicknell *et al* in determining the age groups (16). The weight of each mouse was measured using an electronic balance and is shown in the results. Mice were observed 2-3 times per day for monitoring, and no abnormalities in mouse health or behavior were observed during the rearing period. Blood samples from all mice were collected by cardiac blood sampling was collected under anesthesia with the inhalation anesthetic solution isoflurane (Pfizer) at the end of the 4W, 8W, 36W and 58W time points. Small animal anesthesia machines (Muromachi Kikai) were used to anesthetize the mice, and isoflurane vaporized to a concentration of 4-5% was inhaled into the mice and maintained at 2-3% throughout the experiment, and blood was drawn from the mouse hearts. After anesthesia, approximately 0.5-1.0 ml of blood was drawn from the heart, and mice were promptly cervically dislocated to minimize distress as a humane endpoint. From the start of anesthesia to the end of blood collection took less than 10 min per animal. Death was confirmed by respiratory arrest and cardiac arrest. All mice used were euthanized immediately after the experiment. Blood samples were then placed in a Microtainer (Becton Dickinson), and the coagulated blood was centrifuged at 6000 G for 3 min for serum separation. The Hirosaki University Ethics Committee for Animal Experiments approved this experiment which was conducted under the Hirosaki University Guidelines for Animal Experiments (Approval No. AE01-2023-004).

Biochemical examination. Serum alkaline phosphatase (ALP) and total cholesterol levels were measured to evaluate age-related biochemical changes. LabAssay™ ALP (Fujifilm Wako Shibayagi Co., Ltd.) was used to measure ALP, following the kit protocol. LabAssay™ Cholesterol (Fujifilm Wako Shibayagi Co., Ltd.) was used to measure total cholesterol following the kit protocol.

Morris water maze test. The Morris water maze test, which was conducted in a circular pool of 120 cm in diameter and 80 cm high, was used to assess the cognitive ability of mice (17,18). Markers were placed around the pool, and the mice were allowed to swim in the pool filled with water with a platform for evacuation under the water surface at one location only. The maximum time to reach the platform was 60 sec. Mice that reached the platform were returned to the gauge after letting them stay on the platform for 20 sec. Mice which failed to reach the platform within 60 sec were led to the platform and allowed to stay for 20 sec to memorize the platform location. This was repeated for five days, starting with three locations per day, and the reduced time taken to reach the platform was used to evaluate cognitive ability.

Total RNA extraction. Isogen II reagent and ethachinmate (Nippongene) were used to extract total RNAs from 200-μl of sera following the manufacturer's instructions. A Qubit 4 Fluorometer (ThermoFisher Scientific) and Qubit™ microRNA Assay kits (ThermoFisher Scientific) were used to measure the total RNA concentration extracted from sera. An Agilent 2100 Bioanalyzer and an Agilent RNA 6000 Pico Kit (Agilent Technologies) were used to determine and confirm the peaks characteristic of the total RNAs, following the manufacturer's instructions.

Microarray analysis. SurePrint G3 Mouse miRNA 8 x 60-K Microarray (Agilent Technologies) was used to analyze serum microRNA expression. Cyanine 3 labeling was conducted with 1 ng of serum microRNA using the miRNA Complete Labeling and Hyb kit (Agilent Technologies), following the manufacturer's instructions. Hybridization, microarray slide washing, and fluorescence image scanning were performed using the procedure described in our previous report (19). MicroRNAs whose expression varied >1.5-fold ($P<0.05$) were screened using fold change analysis and Student's t-test (unpaired) at 58W compared with 8W using GeneSpring 14.9 software (Agilent Technologies). The obtained microarray data were registered with Gene Expression Omnibus (GSE249248).

Statistical analysis. Statcel 3 software (OMS Publishing Inc.) was used for statistical analyses. One-way analysis of variance was performed to compare the results of the three groups and the Tukey-Kramer test was used for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Changes in body weight, serum ALP, serum total cholesterol and cognitive ability during aging. An electronic balance was used to weigh 4, 8, 36 and 58W mice. The weight of the mice increased with aging, with a statistically significant difference in body weight between 8W and 58W (each n=4) ($P<0.01$) (Fig. 1A). The mice underwent serum biochemical tests serum was conducted on the mice, which demonstrated highest serum ALP in 4W mice and decreased with aging, with a statistically significant difference in ALP between 4W or 8W and 58W mice (each n=3-4) ($P<0.05$) (Fig. 1B). Serum total cholesterol was highest in 58W mice and was observed to increase with aging, with a statistically significant difference in total cholesterol between 8W and 58W mice (each n=3-4) ($P<0.01$) (Fig. 1C). The other parameters, including red blood cell count, white blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, neutrophil count, lymphocyte count, and monocyte count, between the groups did not demonstrate statistically significant differences (Fig. S1).

The Morris water maze test revealed a significantly delayed case reaching the platform in 58W compared to 8W on the third and fourth day (each n=5-6) ($P<0.05$) (Fig. 1D). These results indicated that the 58W mice demonstrated signs of aging and were beginning to decline in cognitive ability.

Changes in microRNA expression in mouse serum with aging. The total RNA was extracted from the serum and analyzed to identify the aging-associated changes occurring in serum RNA. Serum RNA concentrations in 4W, 8W, 36W and 58W mice were evaluated, and statistically significant differences were not noted in serum RNA concentrations respectively (each n=4) (Fig. 2A). Next, RNA electrophoresis was conducted to determine the size of RNA in the serum of 4W, 8W, 36W and 58W mice using an Agilent bioanalyzer. A single peak of small RNA ranging from 25 to 200 nucleotides in size was detected in the serum RNA of all 4W, 8W, 36W and 58W mice (Fig. 2B).

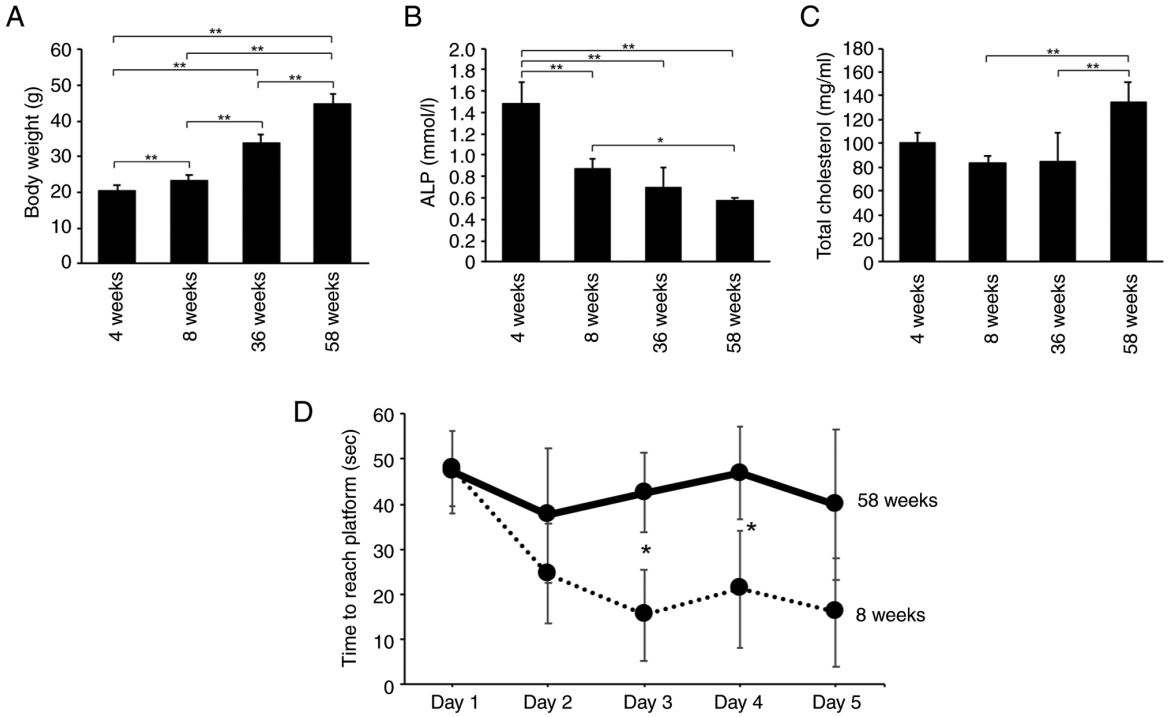

Figure 1. Changes in body weight, serum ALP, serum total cholesterol, and cognitive ability with aging in male C57BL/6NJcl mice. We measured the following parameters in 4W, 8W, 36W and 58W male C57BL/6NJcl mice: (A) Body weight (each n=4); (B) serum ALP (each n=3-4); (C) serum total cholesterol (each n=3-4); (D) Morris water maze test (each n=5-6). Values are presented as mean ± 2 standard deviation. *P<0.05, **P<0.01. ALP, alkaline phosphatase; W, weeks-old.

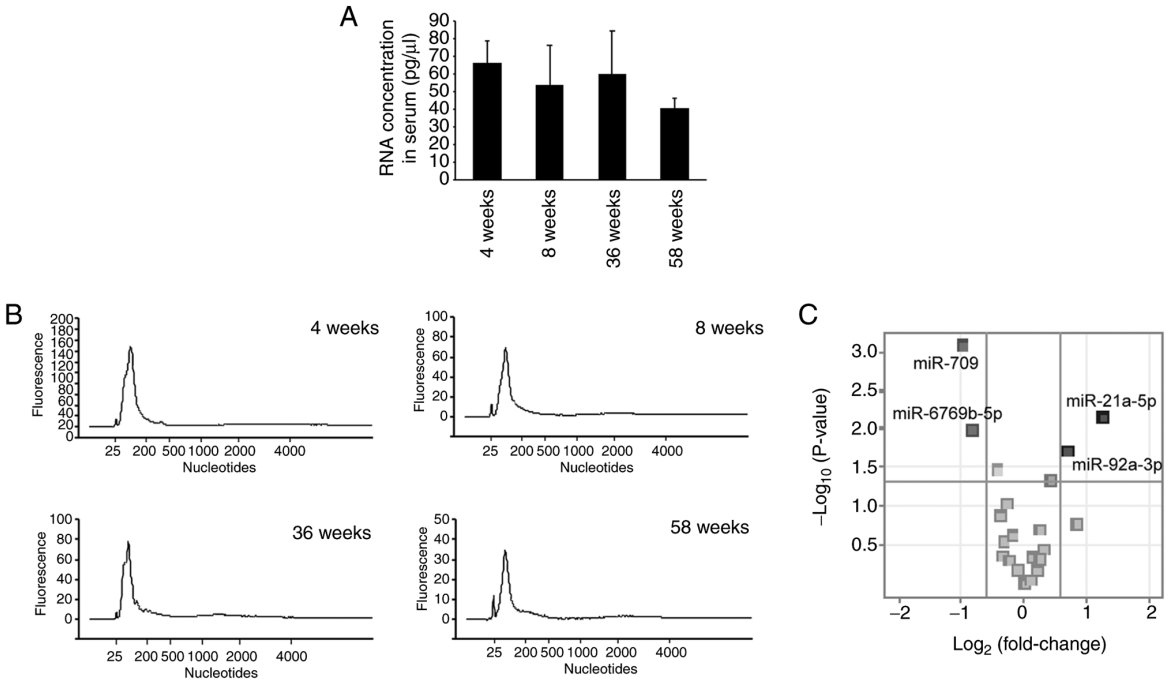

Figure 2. Changes in microRNA expression in mouse serum with aging. (A) Serum RNA concentrations in 4W, 8W, 36W and 58W mice (each n=4). Values are presented as mean ± 2 standard deviation. (B) RNA electrophoresis by Agilent bioanalyzer. (C) Volcano plot. miRNA microarray was conducted using serum RNA from 8W and 58W mice (each n=4). miRNAs whose expression varied more than 1.5-fold (P<0.05) were screened using fold change analysis and Student's t-test (unpaired) at 58W compared to 8W. miR/miRNA, microRNA; W, weeks-old.

We then performed miRNA microarrays using serum RNA from 8W and 58W mice (each n=4). The results indicated that the serum of the 58W mice demonstrated 1.5 times greater expression of two microRNAs (miR-21a-5p and miR-92a-3p) and 1.5 times lowered expression of two microRNAs (miR-6769b-5p and miR-709) compared with the serum of 8W mice (Fig. 2C). These results indicate the presence of microRNAs in serum with altered expression corresponding to a decline in cognitive ability.

Discussion

This study revealed microRNAs whose expression was altered in the serum of middle-aged 58W mice, which demonstrated reduced cognitive ability compared with young 8W mice with normal cognitive ability.

In general, women are at higher risk of developing dementia than men, but we used male mice in this study to examine changes in blood composition during aging because of the article by Hahm *et al* (20). Although not included in the present data, we conducted experiments using mice older than 58W, but because we could not obtain sufficient samples of older mice due to spontaneous mouse death and other factors, we used mice up to 58W in this study for this study.

Changes in body weight, serum biochemistry, and cognitive performance with aging were examined in 4W, 8W, 36W and 58W mice. A statistically significant increase in body weight was found among each mouse as they aged (Fig. 1A). Previous reports revealed that the body weight of mice increased with age (21). Further, serum ALP is higher in young age, and serum total cholesterol is higher in old age (21,22). Hence, serum ALP and serum total cholesterol were measured to infer the age of the mice used. Serum ALP was statistically significantly lower in the 58W group than in the 4W or 8W group (Fig. 1B). Furthermore, serum total cholesterol was significantly higher in the 58W group than in the 8W group (Fig. 1C). These results are consistent with those of previous studies. A decrease in cognitive ability was observed in 58W mice compared with 8W mice when the cognitive abilities of 8W and 58W mice with statistically significant differences in serum ALP and serum total cholesterol were confirmed (Fig. 1D). We then examined the difference in serum microRNA expression between 8W and 58W mice with significant differences in cognitive ability (Fig. 2C). We identified miR-21a-5p and miR-92a-3p as microRNAs that were upregulated and miR-6769b-5p and miR-709 as microRNAs that were downregulated in the serum of 58W mice compared with 8W mice. In this study, swimming time was assessed in the Morris water maze test. In order to fully evaluate cognitive function, it is necessary to measure and evaluate swim path, swim speed, and crossing number. In the future, we would like to evaluate cognitive function using mice aged more than 58W as well as various measurement methods in the Morris water maze test.

This study revealed elevated serum miR-21a-5p expression in mice with impaired cognitive ability (Fig. 2C). Very few studies have revealed the association between cognitive ability and serum miR-21a-5p levels. Yuan *et al* revealed an increased expression of serum miR-21 in patients with cognitive impairment following the incidence of stroke compared with patients with normal cognition following stroke (23). Other previously conducted studies have revealed that miR-21 plays a crucial role in cognitive ability regulation because an increase in miR-21 in the brains of Alzheimer's model mice was observed to restore cognitive ability (24,25). This result indicates that the loss of miR-21 in the brain and its leakage into the serum is a factor associated with cognitive decline. Further studies that explore the relationship between cognitive ability and miR-21 expression should be conducted in the future.

Several studies reported on the association between serum miR-92a-3p levels and dementia, and several investigators have reported an association between miR-92a-3p levels and cognitive ability. Siedlecki-Wullich *et al* demonstrated a statistically significant increase in serum miR-92a-3p in the patient group with Alzheimer's disease compared with the group with normal cognitive abilities and indicated an increasing trend in the incidence of this serum in the MCI group (26). However, Peña-Bautista *et al* contradicted this claim by revealing a decrease in plasma miR-92a-3p levels in patients with early Alzheimer's disease (27).

This study revealed that a decrease in serum miR-709 levels reduces in mice with cognitive decline. This is a novel result because previous reports of an association between serum miR-709 levels and cognitive ability are limited. Further studies are warranted to confirm and elaborate the association between serum miR-709 levels and cognitive ability. In the future, we would like to further analyze aged mice to reveal novel MCI biomarkers.

Acknowledgements

Not applicable.

Funding

This work was supported by JST SPRING (grant no. JPMJSP2152).

Availability of data and materials

The microarray data generated in the present study may be found in the Gene Expression Omnibus under accession number GSE249248 or at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE249248. The other data generated in the present study may be requested from the corresponding author.

Authors' contributions

KY and MC confirm the authenticity of all the raw data. KY, KoM, MF and MC were major contributors in performing the experiments and writing the manuscript. WK, HO and KaM helped conduct the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed in accordance with The Guideline for Animal Experimentation of Hirosaki University. The Animal Research Committee of Hirosaki University (approval no. AE01-2023-097-1) approved and monitored the procedures.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. World Health Organization (WHO): Dementia. https://www.who.int/en/news-room/fact-sheets/detail/dementia. Accessed August 19, 2023.
2. Błaszczyk JW: Pathogenesis of dementia. Int J Mol Sci 24: 543, 2022.
3. Wolf A, Tripanpitak K, Umeda S and Otake-Matsuura M: Eye-tracking paradigms for the assessment of mild cognitive impairment: A systematic review. Front Psychol 14: 1197567, 2023.
4. Del Bene VA, Gerstenecker A and Lazar RM: Formal neuropsychological testing: Test batteries, interpretation, and added value in practice. Clin Geriatr Med 39: 27-43, 2023.
5. Di Lazzaro V, Bella R, Benussi A, Bologna M, Borroni B, Capone F, Chen KS, Chen R, Chistyakov AV, Classen J, *et al*: Diagnostic contribution and therapeutic perspectives of transcranial magnetic stimulation in dementia. Clin Neurophysiol 132: 2568-2607, 2021.
6. Devezas MÂM: Shedding light on neuroscience: Two decades of functional near-infrared spectroscopy applications and advances from a bibliometric perspective. J Neuroimaging 31: 641-655, 2021.
7. Botz J, Lohner V and Schirmer MD: Spatial patterns of white matter hyperintensities: A systematic review. Front Aging Neurosci 15: 1165324, 2023.
8. Papaliagkas V, Kalinderi K, Vareltzis P, Moraitou D, Papamitsou T and Chatzidimitriou M: CSF biomarkers in the early diagnosis of mild cognitive impairment and Alzheimer's disease. Int J Mol Sci 24: 8976, 2023.
9. Hansson O, Blennow K, Zetterberg H and Dage J: Blood biomarkers for Alzheimer's disease in clinical practice and trials. Nat Aging 3: 506-519, 2023.
10. Ulbl J and Rakusa M: The importance of subjective cognitive decline recognition and the potential of molecular and neurophysiological biomarkers-A systematic review. Int J Mol Sci 24: 10158, 2023.
11. Fatuzzo I, Niccolini GF, Zoccali F, Cavalcanti L, Bellizzi MG, Riccardi G, de Vincentiis M, Fiore M, Petrella C, Minni A, *et al*: Neurons, nose, and neurodegenerative diseases: Olfactory function and cognitive impairment. Int J Mol Sci 24: 2117, 2023.
12. Jahromi FNA, Dowran R and Jafari R: Recent advances in the roles of exosomal microRNAs (exomiRs) in hematologic neoplasms: Pathogenesis, diagnosis, and treatment. Cell Commun Signal 21: 88, 2023.
13. Jain G, Das P, Ranjan P, Neha, Valderrama F and Cieza-Borrella C: Urinary extracellular vesicles miRNA-A new era of prostate cancer biomarkers. Front Genet 14: 1065757, 2023.
14. Bielak C, Arya A and Savill S: Circulating microRNA as potential diagnostic and prognostic biomarkers of well-differentiated thyroid cancer: A review article. Cancer Biomark 36: 193-205, 2023.
15. Nestler T, Schoch J, Belge G and Dieckmann KP: MicroRNA-371a-3p-The novel serum biomarker in testicular germ cell tumors. Cancers (Basel) 15: 3944, 2023.
16. Ackert-Bicknell CL, Anderson LC, Sheehan S, Hill WG, Chang B, Churchill GA, Chesler EJ, Korstanje R and Peters LL: Aging research using mouse models. Curr Protoc Mouse Biol 5: 95-133, 2015.
17. Othman MZ, Hassan Z and Che Has AT: Morris water maze: A versatile and pertinent tool for assessing spatial learning and memory. Exp Anim 71: 264-280, 2022.
18. Vorhees CV and Williams MT: Morris water maze: Procedures for assessing spatial and related forms of learning and memory. Nat Protoc 1: 848-858, 2006.
19. Chiba M, Monzen S, Iwaya C, Kashiwagi Y, Yamada S, Hosokawa Y, Mariya Y, Nakamura T and Wojcik A: Serum miR-375-3p increase in mice exposed to a high dose of ionizing radiation. Sci Rep 8: 1302, 2018.
20. Hahm JR, Jo MH, Ullah R, Kim MW and Kim MO: Metabolic stress alters antioxidant systems, suppresses the adiponectin receptor 1 and induces Alzheimer's like pathology in mice brain. Cells 9: 249, 2020.
21. Mazzaccara C, Labruna G, Cito G, Scarfò M, De Felice M, Pastore L and Sacchetti L: Age-related reference intervals of the main biochemical and hematological parameters in C57BL/6J, 129SV/EV and C3H/HeJ mouse strains. PLoS One 3: e3772, 2008.
22. Hu Y, Wang R, Wang S, Yuan C, Yuan Q, Zhang Y, Ren J, He Y, Wu X, Dai W, *et al*: Selenium-enriched polysaccharides from Pyracantha fortuneana (SePFP) ameliorated hepatic lipid accumulation through enhancing mitochondrial fatty acid β-oxidation in aging mice. J Oleo Sci 72: 929-938, 2023.
23. Yuan M, Guo YS, Zhang XX, Gao ZK, Shen XY, Han Y and Bi X: Diagnostic performance of miR-21, miR-124, miR-132, and miR-200b serums in post-stroke cognitive impairment patients. Folia Neuropathol 60: 228-236, 2022.
24. Cui GH, Wu J, Mou FF, Xie WH, Wang FB, Wang QL, Fang J, Xu YW, Dong YR, Liu JR and Guo HD: Exosomes derived from hypoxia-preconditioned mesenchymal stromal cells ameliorate cognitive decline by rescuing synaptic dysfunction and regulating inflammatory responses in APP/PS1 mice. FASEB J 32: 654-668, 2018.
25. Gao X, Xiong Y, Li Q, Han M, Shan D, Yang G, Zhang S, Xin D, Zhao R, Wang Z, *et al*: Extracellular vesicle-mediated transfer of miR-21-5p from mesenchymal stromal cells to neurons alleviates early brain injury to improve cognitive function via the PTEN/Akt pathway after subarachnoid hemorrhage. Cell Death Dis 11: 363, 2020.
26. Siedlecki-Wullich D, Català-Solsona J, Fábregas C, Hernández I, Clarimon J, Lleó A, Boada M, Saura CA, Rodríguez-Álvarez J and Miñano-Molina AJ: Altered microRNAs related to synaptic function as potential plasma biomarkers for Alzheimer's disease. Alzheimers Res Ther 11: 46, 2019.
27. Peña-Bautista C, Tarazona-Sánchez A, Braza-Boils A, Balaguer A, Ferré-González L, Cañada-Martínez AJ, Baquero M and Cháfer-Pericás C: Plasma microRNAs as potential biomarkers in early Alzheimer disease expression. Sci Rep 12: 15589, 2022.