Original Article

JER

https://doi.org/10.12965/jer.2550186.093

Journal of Exercise Rehabilitation 2025;21(2):47-52

Impact of maternal exercise on neurodevelopment and gut microbiota in offspring from advanced-age mice

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The effects of maternal exercise on hippocampal neurogenesis, synaptic protein expression, and gut microbiome composition in the offspring of older females were investigated. Male offspring from female C57BL/6 mice were divided into four groups: offspring of young female group (CON), offspring of exercised young female group, offspring of advanced-age female group (AMA), and offspring of exercised advanced-age female group (AMA+EX). The exercised group received 8 weeks of treadmill training before and during pregnancy. Male offspring were assessed at 4 weeks of age. Hippocampal neurogenesis was assessed by 5-bromo-2'-deoxyuridine/neuronal double immunofluorescence staining. Expression of synaptic plasticity-related proteins, including brain-derived neurotrophic factor (BDNF) and postsynaptic density protein 95 (PSD-95), was analyzed by Western blot. Gut microbiome composition and diversity were assessed using 16S rRNA sequencing of fecal samples. Offspring born to AMA females had significantly reduced hippocampal neurogenesis and lower expression levels of BDNF and PSD-95 compared to the CON group. In the AMA+EX group, maternal treadmill exercise significantly improved these deficits, restoring both neurogenesis and synaptic protein expression. In contrast, gut microbiota analysis showed that microbial richness and alpha diversity were reduced in the offspring of exercised females, despite the relatively high diversity in the CON and AMA groups, especially in the AMA+EX group. Older mothers impair hippocampal neurogenesis and synaptic protein expression in offspring, and alter gut microbial diversity. Maternal exercise may alleviate age-related neurodevelopmental disorders, but may also reduce microbial diversity in the offspring's gut.

Keywords: Maternal exercise, Advanced maternal age, Neurogenesis, Microbiome, Mice

INTRODUCTION

Advanced maternal age (AMA), generally defined as maternal age at delivery of 35 years or older, has become increasingly common in recent decades. The rise in AMA pregnancies has raised concerns about maternal and fetal health. Numerous studies have shown that AMA is associated with an increased risk of adverse obstetric outcomes, including increased rates of gestational diabetes, preeclampsia, preterm birth, and cesarean section (Cleary-Goldman et al., 2005). There is also growing evidence that AMA may affect the long-term developmental trajectories of offspring, including increased vulnerability to metabolic, neurodevelopmental, and psychiatric disorders (Myrskylä and Fenelon, 2012).

Physical activity during pregnancy has been highlighted as a modifiable factor that may mitigate some of the risks associated with AMA. Maternal exercise not only supports maternal metabolic health, but may also positively influence placental function, fetal brain development, and the establishment of the infant gut microbiome (Gomes da Silva et al., 2016). Maternal metabolic health plays an important role in shaping the physical and neurological development of the offspring, including cognitive and behavioral outcomes (Page et al., 2009). Maternal obesity or insulin resistance during pregnancy impairs synaptic plasticity and spatial learning in the offspring by reducing learning and expression of

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Received: February 11, 2025 / Revised: March 18, 2025 / Accepted: March 31, 2025

brain-derived neurotrophic factor (BDNF) (Tozuka et al., 2010).

The gut microbiome has been highlighted as a key mediator of maternal metabolic influences on fetal brain development, forming a bidirectional communication system known as the gut-brain axis (Lana and Giovannini, 2023; Vuong et al., 2020). Imbalances, or dysbiosis, of the maternal gut microbiome have been associated with increased inflammation, altered metabolite profiles, and neurodevelopmental impairments in offspring. Given that maternal age is associated with changes in the composition of the gut microbiome, these changes may contribute to the neurodevelopmental deficits observed in offspring born to older mothers.

Importantly, maternal physical activity is emerging as an important intervention to modulate maternal metabolism and gut microbiota composition. Exercise improves whole-body metabolism (Hawley et al., 2014) and promotes beneficial microbial diversity, while enhancing hippocampal neurogenesis and increasing BDNF levels in offspring (Gomes da Silva et al., 2016). Exercise-induced changes in maternal gut and brain environment may be transmitted to offspring, improving cognitive outcomes and reducing the risk of long-term neurological disorders (Monda et al., 2017). Taken together, these findings suggest that interventions targeting maternal metabolism and gut microbiota, particularly through prenatal exercise, may be effective strategies to mitigate the neurodevelopmental risks associated with AMA. Therefore, the purpose of this study was to investigate whether maternal exercise during pregnancy can alleviate the negative effects of AMA on offspring, and to investigate changes in gut microbiota composition, hippocampal neurogenesis, and expression of synaptic plasticity-related proteins such as BDNF and postsynaptic density protein 95 (PSD-95).

MATERIALS AND METHODS

Experiment animals

All animal experiments were conducted in compliance with the ethical guidelines established by the National Institutes of Health and the Korean Academy of Medical Sciences. The study protocol was approved by the Animal Experiment Ethics Committee of Kyung Hee University (approval number: KHUASP[SE]-20-497). Female C57BL/6 mice were randomly divided into four groups (n = 6 per group): (1) young female group (8 weeks old), (2) young and exercised female group, (3) old female group (10 months old), and (4) exercised old female group. Each female was paired with an 8-week-old male and housed together for 1 week during a dark cycle to facilitate mating. After delivery, the male offspring were divided into four experimental groups (n = 10 per group): offspring of young female group (CON), offspring of exercised young female group (CON+EX), offspring of advanced-age female group (AMA), and offspring of exercised advanced-age female group (AMA+EX). All subsequent analyses were performed using 4-week-old male offspring. To assess neurogenesis, 5-bromo-2′-deoxyuridine (BrdU; 100 mg/kg/day, intraperitoneal injection; Sigma, St. Louis, MO, USA) was administered daily from gestational days 14 to 18.

Exercise protocol

The treadmill exercise protocol was adapted from a previously established method (Kim et al., 2022a). Mice in the exercise group were trained on an animal treadmill once a day, 6 days a week for 8 consecutive weeks before mating and throughout pregnancy. To minimize stress and promote adaptation, all exercised animals underwent a 1-week acclimation period before starting the main regimen. A standard exercise session consisted of a 5-min warm-up at 3 m/min, followed by 30 min of running at 0° incline, followed by 5 min at 5 m/min, and 30 min at 8 m/min. Control animals in the nonexercised group were placed on a stationary treadmill for the same period without running to control for environmental exposure.

Immunofluorescence

BrdU/neuronal nuclei (NeuN)-positive cells in the dentate gyrus were assessed by immunofluorescence according to a previously described method (Kim et al., 2020b). Briefly, brain sections were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 20 min, incubated in 50% formamide in 2× normal saline citrate at 65°C for 2 hr, denatured in 2 N HCl at 37°C for 30 min, and rinsed twice in 100 mM sodium borate buffer (pH 8.5). Sections were then incubated overnight with rat anti-BrdU antibody (1:200; Abcam, Cambridge, UK) and mouse anti-NeuN antibody (1:200; Millipore, Temecula, CA, USA). After washing with phosphate-buffered saline, sections were incubated with appropriate secondary antibodies for 1 hr. The secondary antibodies used were Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 560-conjugated anti-rat IgG.

Western blotting for BDNF and PSD-95

Hippocampal tissues were placed on ice and homogenized using a mechanical homogenizer according to the method reported by Park et al. (2023). The homogenates were lysed in a buffer solution. Total protein concentration was measured using a colorimet-

https://doi.org/10.12965/jer.2550186.093

ric protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. For immunodetection, membranes were incubated with primary antibodies specific for β-actin (mouse, 1:3,000; Santa Cruz Biotechnology), BDNF (rabbit, 1:1,000; Bioss), and PSD-95 (rabbit, 1:1,000; Cell Signaling Technology). The corresponding primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies, anti-mouse IgG and anti-rabbit IgG.

Collection of fecal samples and sequencing/metagenomic analysis

Stool samples were collected as described previously (Park et al., 2024), placed into sterile test tubes, and immediately stored at -80°C until further processing. Total genomic DNA was extracted from 200 mg of each stool sample using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. To amplify the V3-V4 hypervariable region of the bacterial 16S rRNA gene, 2 ng of input DNA was used in a polymerase chain reaction (PCR) mixture containing 5× reaction buffer, 1 mM dNTP, 500 nM universal forward and reverse primers, and Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA). Thermal cycling conditions for the first PCR consisted of an initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 5 min. Amplicons were purified using AMPure XP beads (Agencourt Bioscience, Beverly, MA, USA). For indexing, 2 μL of purified PCR product was applied to a second PCR using Nextera XT index primers to generate dual-indexed libraries. The second PCR followed the same cycling parameters but was limited to ten cycles. The final amplicons were purified again using AMPure XP beads.

Library quantification was performed by quantitative PCR using the KAPA Library Quantification Kit (Roche, Basel, Switzerland), and library quality and fragment size were assessed using the Agilent TapeStation D1000 ScreenTape system (Agilent Technologies, Waldbronn, Germany). Sequencing of barcoded 16S rRNA gene amplicons was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) by Macrogen Inc. (Seoul, Korea). In parallel, total metagenomic DNA extracted from stool samples was subjected to paired-end shotgun sequencing on an Illumina HiSeq 2000 platform, also performed by Macrogen Inc.

Data analysis

Cell counting and optical density quantification were performed using Image-Pro Plus software (Media Cybernetics Inc., Rockville, MD, USA) in combination with a light microscope (Olympus, Tokyo, Japan). Statistical analysis was performed using IBM SPSS Statistics ver. 26.0 (IBM Co., Armonk, NY, USA). Differences between groups were assessed using one-way analysis of variance followed by Tukey *post hoc* test. Data are expressed as the mean±standard error of the mean, and a P-value <0.05 was considered statistically significant.

RESULTS

The effects of maternal exercise with AMA on neurogenesis in the hippocampus

To evaluate the hippocampal neurogenesis of the offspring, double-labeling immunofluorescence for NeuN and BrdU was performed, and NeuN/BrdU-positive cells in the dentate gyrus were quantified (Fig. 1). Compared with the CON group, the AMA group showed a significant decrease in the number of NeuN/BrdU-positive cells in the hippocampus ($P<0.05$). This suggests that neurogenesis is impaired in older mothers. In contrast, the offspring of the AMA+EX group showed a significant increase in NeuN/BrdU-positive cells compared with the AMA group ($P<0.05$). This suggests that maternal exercise effectively counteracts the negative effects of maternal aging on neurogenesis. Taken together, these results suggest that older mothers decrease hippocampal neurogenesis in their offspring, while maternal treadmill exercise during pregnancy can alleviate this decrease and promote hippocampal neurogenesis.

The effects of maternal exercise with AMA on BDNF and PSD-95 expression in the hippocampus

Fig. 2 shows the hippocampal expression levels of BDNF and PSD-95, which are neuroplasticity-related proteins in offspring. For comparative analysis, the expression levels in the CON group were normalized to 1.00. Offspring born to older females (AMA group) showed significantly decreased levels of BDNF and PSD-95 compared to the CON group ($P<0.05$). This suggests that neurotrophic signaling associated with maternal aging is impaired. In contrast, maternal exercise in the AMA+EX group significantly restored the expression of both BDNF and PSD-95 compared to the AMA group ($P<0.05$). This suggests that maternal physical activity can alleviate the age-related decline in fetal hippocampal neurotrophic factors. These results highlight the protective role of maternal exercise in preserving molecular markers of synaptic plasticity in the offspring brain.

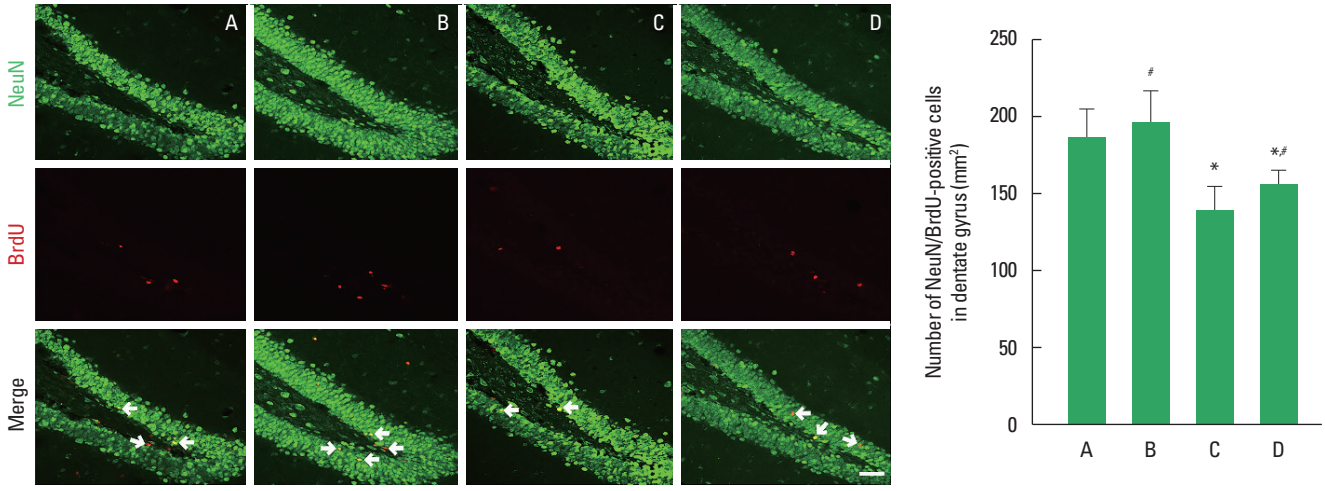

Fig. 1. Effect of maternal exercise in advanced maternal age on hippocampal neurogenesis in offspring. Left panel: representative images and quantification of neurogenesis markers in the dentate gyrus of offspring. Arrows indicate newly formed cells. Right panel: data of neurogenesis. Data are presented as mean ± standard error of the mean. *$P<0.05$ compared to offspring of young female group. #$P<0.05$ compared to offspring of advanced-age female group. (A) Offspring of young female group. (B) Offspring of exercised young female group. (C) Offspring of advanced-age female group. (D) Offspring of exercised advanced-age female group.

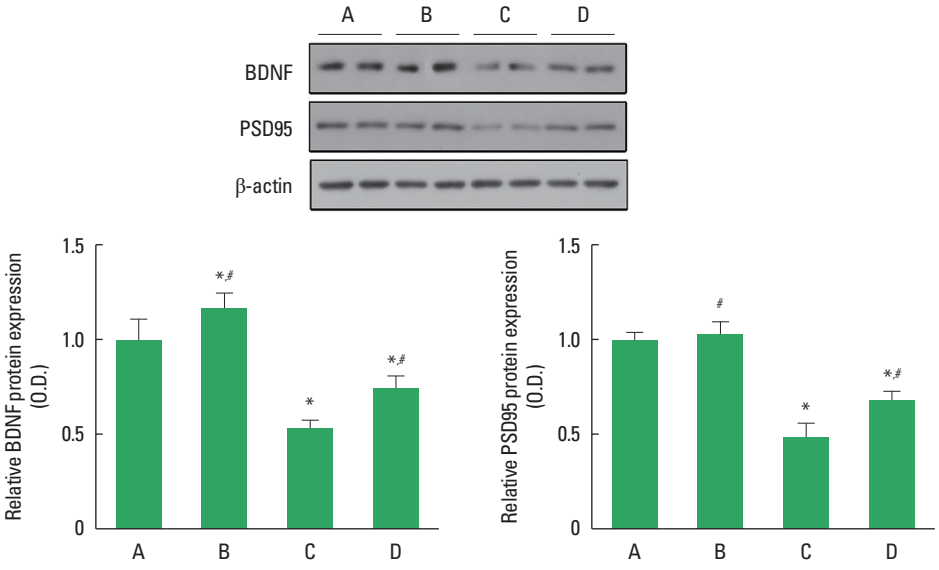

Fig. 2. Effects of maternal exercise on brain-derived neurotrophic factor (BDNF) and postsynaptic density protein 95 (PSD-95) expression in the hippocampus. Upper panel: representative BDNF and PSD-95 expression. Left lower panel: relative BDNF expression. Right lower panel: relative PSD-95 expression. Data are presented as mean ± standard error of the mean. *$P<0.05$ compared to offspring of young female group. #$P<0.05$ compared to offspring of advanced-age female group. (A) Offspring of young female group. (B) Offspring of exercised young female group. (C) Offspring of advanced-age female group. (D) Offspring of exercised advanced-age female group.

The effects of maternal exercise with AMA on gut microbiomes

Operational taxonomic unit (OTU) analysis revealed that offspring of control mothers had the highest microbial richness within each group (Fig. 3). In contrast, offspring of exercised mothers had a significantly reduced number of OTUs, suggesting that maternal exercise may affect the gut microbial richness of offspring. Similarly, Shannon and Inverse Simpson diversity indices both showed consistent patterns. Offspring of CON and AMA groups generally had higher microbial diversity and had the highest Shannon indices across all samples. In contrast, offspring of exercised mothers had lower diversity indices across samples, which may indicate a decrease in the evenness and dominance of certain taxa. These results suggest that although older mothers may be associated with

https://doi.org/10.12965/jer.2550186.093

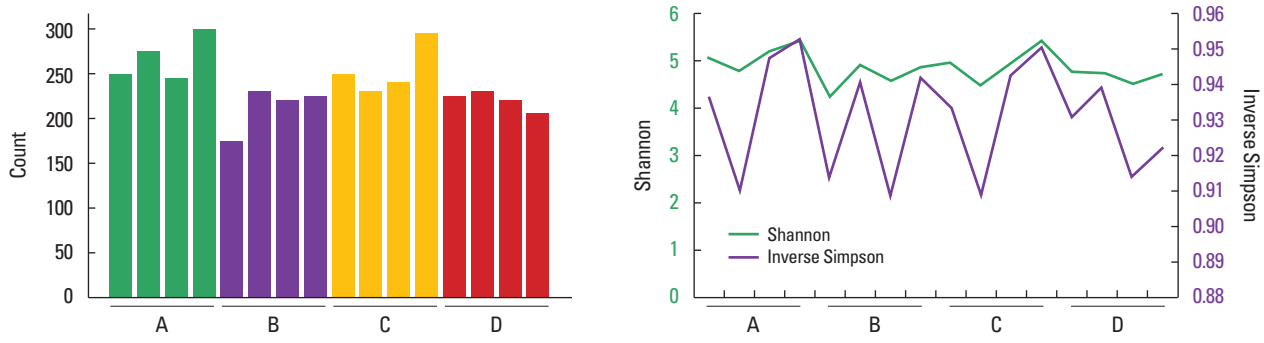

Fig. 3. Effect of maternal exercise in advanced maternal age on operational taxonomic units and community diversity in the gut microbiome of offspring. Left panel: number of operational taxonomic units. Right panel: microbial community diversity measured by Shannon and Inverse Simpson indices. Shannon index accounts for both species' richness and evenness. Inverse Simpson index reflects the probability that two randomly selected individuals belong to the same species. (A) Offspring of young female group. (B) Offspring of exercised young female group. (C) Offspring of advanced-age female group. (D) Offspring of exercised advanced-age female group.

increased gut microbial diversity in some offspring, maternal exercise may decrease both microbial richness and diversity in offspring, especially in older mothers.

DISCUSSION

This study aimed to investigate the effects of maternal age and exercise during pregnancy on the gut microbiota, hippocampal synaptic protein expression, and neurogenesis in offspring. BrdU immunostaining of the hippocampal dentate gyrus showed a significant decrease in BrdU-positive cells in the AMA group, indicating impaired hippocampal neurogenesis in offspring of older mothers. The dentate gyrus is one of the few brain regions where neurogenesis persists into adulthood and plays a central role in memory formation, pattern separation, and emotional regulation (Aimone et al., 2014). The decrease in BrdU-labeled cells reflects decreased proliferation of neural progenitor cells, which may lead to the cognitive impairment observed later in life.

Age-related epigenetic changes in maternal tissue can be passed on to the offspring, reprogramming hippocampal gene expression and limiting neuronal proliferation (Aiken and Ozanne, 2014). Consistent with these findings, we observed decreased expression of BDNF, a neurotrophic factor essential for neuronal growth and synaptic plasticity, in the AMA group. The parallel decrease in BDNF and BrdU signals suggests that synaptic and neuronal damage may be interdependent and reflect a broader disruption of hippocampal plasticity (Lu et al., 2013).

In addition to changes in neurogenesis, we found that maternal exercise significantly influenced the composition of the offspring's gut microbiome. Offspring from the control and AMA groups had relatively higher microbial richness and diversity, as reflected by the number of OTUs and alpha diversity indices (Shannon and Inverse Simpson). These results are consistent with previous studies showing that maternal factors such as age and health significantly shape the early microbial colonization of offspring (Chu et al., 2017; Koren et al., 2012). However, maternal exercise was found to decrease microbial richness and diversity, particularly in the AMA+EX group. Although physical activity is widely known to have beneficial effects on maternal and fetal health (Mourtakos et al., 2015), its specific effects on the neonatal gut microbiome remain complex and context-dependent.

One potential explanation is that maternal exercise may alter hormonal and metabolic profiles, thereby influencing offspring microbial transmission and colonization patterns. In older mothers, these physiological changes may interact with age-related stressors to produce less favorable offspring microbial outcomes. These findings highlight the importance of considering maternal age as a key biological variable that may modulate the efficacy of prenatal lifestyle interventions, particularly those that support gut-brain development.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Education of the Republic of Korea and the National Research Foundation of Korea (NRF-2018R1D1A1B07051213).

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https://doi.org/10.12965/jer.2550186.093