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N-3 PUFA improved post-menopausal depression induced by maternal separation and chronic mild stress through serotonergic pathway in rats—effect associated with lipid mediators

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Abstract

Early life maternal separation (MS) increases the vulnerability to depression in rats with chronic mild stress (CMS). N-3 polyunsaturated fatty acids (PUFA) improved depressive behaviors in rats with acute stress; however, their effects on rats with MS+CMS were not apparent. The purpose of the present study was to investigate the hypothesis that lifetime n-3 PUFA supplementation improves post-menopausal depression through the serotonergic and glutamatergic pathways while modulating n-3 PUFA-derived metabolites. Female rats were fed diets of either 0% n-3 PUFA during lifetime or 1% energy n-3 PUFA during pre-weaning, post-weaning, or lifetime periods. Rats were allocated to non-MS or MS groups and underwent CMS after ovariectomy. N-3 PUFA increased brain n-3 PUFA-derived endocannabinoid/oxylipin levels, and reversed depressive behaviors. N-3 PUFA decreased blood levels of adrenocorticotropic hormone and corticosterone, and brain expressions of corticotropin-releasing factor and miRNA-218, which increased the expression of the glucocorticoid receptor. N-3 PUFA decreased the expression of tumor necrosis factor-α, interleukin (IL)-6, IL-1β, and prostaglandin E_2, while increased the expression of miRNA-155. N-3 PUFA also increased brainstem serotonin levels and hippocampal expression of the serotonin-1A receptor, cAMP response element-binding protein (CREB), phospho-CREB, and brain-derived neurotrophic factor. However, n-3 PUFA did not affect brain expression of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor subtype 1, N-methyl-D-aspartate receptor subtype 2B, or miRNA-132. Moreover, n-3 PUFA exposure during lifetime caused greater effects than pre- and post-weaning periods. The present study suggested that n-3 PUFA improved depressive behaviors through serotonergic pathway while modulating the metabolites of n-3 PUFA in post-menopausal depressed rats with chronic stress.

Keywords: Endocannabinoid/oxylipin; Maternal separation; N-3 PUFA; Post-menopausal depression; Serotonin.

Abbreviations: ACTH, adrenocorticotrophic hormone; ALA, α-linolenic acid; ANCOVA, analysis of covariance; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element binding protein; cGR, cytosolic glucocorticoid receptor; CMS, chronic mild stress; CORT, corticosterone; CRF, corticotrophin releasing factor; DAPI, fluoroshield containing 4′, 6-diamidino-2-phenylindole; DHA, docosahexaenoic acid; DHEA, docosahexaenoyl ethanolamidel; DiHDoPE, dihydroxydocosapentaenoic acid; DiHETE, dihydroxyeicosatetraenoic acid; ED, embryonic day; ELISA, enzyme-linked immunosorbent assay; en %, energy percent; EPA, eicosapentaenoic acid; EpDoPE, epoxydocosapentaenoic acid; EPEA, eicosapentaenoyl ethanolamide; FDR, false discovery rate; FST, forced swimming test; GC, gas chromatography; GluR1, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor subtype 1; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HPA, hypothalamic-pituitary-adrenal; IL, interleukin; MS, maternal separation; nGR, nuclear glucocorticoid receptor; NR2B, N-methyl D-aspartate receptor subtype 2B; OVX, ovariectomy; PGE_2, prostaglandin E_2; PLSr, partial least square regression; PND, postnatal day; PreW, pre-weaning; PostW, post-weaning; PUFA, polyunsaturated fatty acids; RT-PCR, real time-polymerase chain reaction; SI, sucrose preference index; SPT, sucrose preference test; TBST, Tris-buffered saline with 0.1% Tween 20; TLC, thin layered chromatography; TNF-α, tumor necrosis factor-α; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; VIP, variable importance in projection; 5-HT, serotonin; 5-HT_{1A}R, serotonin 1A receptor.

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1. Introduction

Supplementation of n-3 polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid (EPA; 20:5n3) and docosahexaenoic acid (DHA; 22:6n3), reduced the severity of depression in post-menopausal women [1]. Menopausal transition increased susceptibility to stressful life events [2], and interaction between low levels of ovarian hormone and chronic mild stress (CMS) rather than a deficit in ovarian secretion alone, induced depression in mice with ovariectomy (OVX) [3]. Women who experienced early life stress were more likely to have a first onset of depression during the menopause transition [4]. In addition, female rats with maternal separation (MS), one of the most common paradigms for early life stress in rodents, were more vulnerable to develop depressive behavior after exposure to CMS in adulthood [5].

MS enhances CMS-induced hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis system, including corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), corticosterone, and glucocorticoid receptor (GR) [5]. Impaired GR inhibits the cAMP response element-binding protein (CREB)/brain-derived neurotrophic factor (BDNF) pathway and increases neuroinflammation, which regulates serotonergic and glutamatergic neurotransmission [6]. Both MS and CMS can modulate the expression of miRNA, which regulates translation and stability of target mRNA [7]. Previous studies have shown that GR, CREB, and BDNF are regulated by miRNA-218, -132, and -155 in depressed humans [8,9] and rodents [10].

Supplementation of n-3 PUFA has been shown to increase brain levels of serotonin, CREB, and BDNF in post-menopausal depressed rat induced by acute stress [11,12], and in male rodents with CMS [13,14]. n-3 PUFA supplementation also modified the blood level of corticosterone and prefrontal cortical expression of the GR in male rodents with chronic restraint stress [15] and chronic social defeat stress [16]. It is well-known that n-3 PUFA has anti-inflammatory and neuroprotective effect that is linked to the modulation of n-3 PUFA-derived endocannabinoids and oxylipins [17,18], which drives the production of inflammation mediator such as resolvins, protectins, and maresins via enzymatic oxidation [19,20,21]. Supplementation of n-3 PUFA increased the blood level of EPA-derived endocannabinoids, which was associated with clinical remission of depression in patients with major depressive disorder, suggesting a relationship between n-3 PUFA and depression through n-3 PUFA-derived endocannabinoids and oxylipins [22].

Furthermore, n-3 PUFA deficiency during critical period of brain development up-regulated HPA axis response to stress in adult rat offspring [23]. However, there is an inconsistency regarding the best timing of n-3 PUFA supplementation to exert the greatest antidepressant-like effects. Previous studies have shown that n-3 PUFA supplementation during the pre-weaning period, but not the post-weaning period, have antidepressant-like effects in non-stressed rats [23,24]. However, Ferraz et al. [25] have reported that both pre- and post-weaning n-3 PUFA supplementation has similar antidepressant properties in non-stressed rats. We have also previously reported that post-weaning n-3 PUFA supplementation has an antidepressant-like effect in post-menopausal rats with acute stress-induced depression [11,12], and in male rats with CMS [13], chronic restraint stress [15] and acute stress [26]. However, these studies did not compare the period-dependent effect of n-3 PUFA.

The purpose of the present study was to investigate the hypothesis that n-3 PUFA supplementation improves depressive behaviors induced by CMS with and without MS in post-menopausal rats, and that changes in n-3 PUFA metabolites correspond to modulations of the serotonergic and glutamatergic pathways via HPA axis regulation. The present study also evaluated if a lifetime supplementation of n-3 PUFA had a greater antidepressant-like effect than supplementation during the pre- and post-weaning period.

2. Materials and methods

2.1. Animals and diet

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University (HY-IACUC-18-0031). All rats were housed in a ventilated air-conditioned room maintained 22±1°C with a 12 h light-dark cycle and 40–50% humidity. The rats were weighed weekly, and food intake was measured daily.

For mating, 10-week-old female and 14-week-old male Wistar rats were purchased from Orient Bio Inc. (Seongnam, Korea). The experimental diets were isocaloric modified American Institute of Nutrition-93G diets with 0 energy percent (en %) of n-3 PUFA (control diet) or 1 en % of n-3 PUFA (n-3 PUFA diet). Previous studies showed that this physiological dose had antidepressant-like effect in depressed rats [11,12,27] and depressed human [28]. The experimental diets were made up of grape seed oil (Sajo Haepyo, Seoul, Korea) instead of soybean oil due to the high level of α-linolenic acid in soybean oil, or a combination of grape seed oil and fish oil (Cenovis Health Company, Turrella, Australia) containing 0.6 en % EPA and 0.4 en % DHA. The ingredient compositions of diet were shown in our previous study [27].

2.2. Experimental design

After 1 week of acclimatization, the phase of the estrous cycle was identified in each female rat for 1 week by vaginal cytology according to the methodology previously described [29]. The gestational day was indicated, with the day after a mating period specified as embryonic day (ED) 0, and the day of delivery was designated postnatal day (PND) 0. The rats that had not given birth until ED 35 were identified as not pregnant and were euthanized. Thirteen out of 14 rats were classified as pregnant (92.8%). To minimize the bias from different delivery date, the pups whose birth dates differed by 1 day were individually grouped, intervened, and executed.

On ED 0, dams (female rat parents; n=7 per diet) were randomly assigned to control or n-3 PUFA diets and maintained under the assigned diet until PND 20 (Fig. 1A). On PND 2–14, pups (baby rats) were assigned randomly to the non-MS group or MS group. On PND 20, pups were weaned; only female pups were used in the study. Sixty-four female pups were caged in groups of two or three after weighing, and randomly assigned to control or n-3 PUFA diets during the post-weaning period (PND 20–112).

On PND 84, when rats are sexually matured, bilateral ovariectomy (OVX) was performed under anesthesia with zoletil (25 mg/kg) and rompun (10 mg/kg). After OVX, antibiotics (Baytril, Bayer AG, Germany) diluted in water were provided to prevent any complication. One week after the surgery, rats were allocated to CMS on PND 91–105 to induce post-menopausal stress. Thus, there were eight groups (n=8 per group): CMS and lifetime control diet (CMS-Never), CMS and pre-weaning n-3 PUFA diet (CMS-PreW), CMS and post-weaning n-3 PUFA diet (CMS-PostW), and CMS and lifetime n-3 PUFA diet (CMS-Lifetime); CMS with MS and lifetime control diet (MS+CMS-Never), CMS with MS and pre-weaning n-3 PUFA diet (MS+CMS-PreW), CMS with MS and post-weaning n-3 PUFA diet (MS+CMS-PostW), and CMS with MS and lifetime n-3 PUFA diet (MS+CMS-Lifetime).

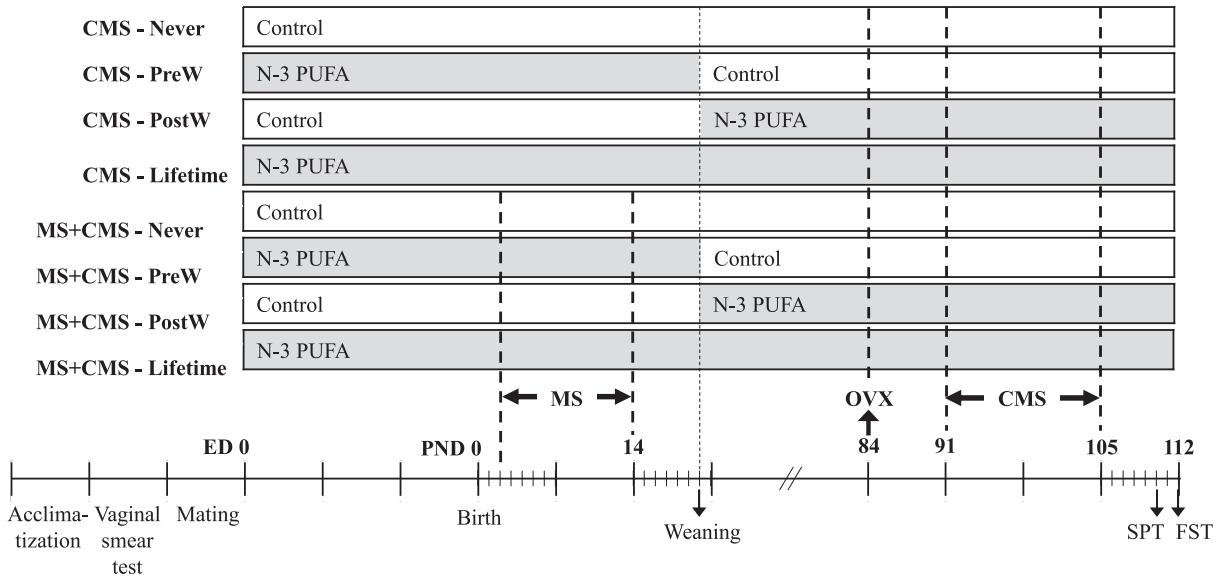
2.3. Maternal separation

The procedure of MS was performed for 180 min (9:30–12:30) on the MS group [30], and 15 min (9:30–9:45) on the non-MS group using similar handling treatment during PND 2–14, as previously described [31]. The dams were first removed from the nest to a separate cage, and pups were transferred to a room adjacent to the colony room. The home cages, which the separated pups were in as a group, were lined with bedding material, and they were placed on a heating pad (Ilwoul, Gwangju, Korea) maintained at a temperature of 32±1°C. After separation, the dams were returned to the home cage. All dams and pups were left undisturbed until weaning on PND 20.

2.4. Chronic mild stress

On PND 91–105, the CMS protocol was performed based on Bravo et al. [32] and involved exposure to a series of different mild stress that was changed daily, using a weekly rotation plan (Fig. 1B). The stresses included: food deprivation, water deprivation, cage tilting (45 degrees), soiled cage (300 mL of water with 25±1°C), group housing (5–6 adult male pups per cage) after a period of water deprivation, stroboscopic illumination (130 flashes/min), and intermittent illumination every 2 h.

A



B

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
08:30-18:30	Water Deprivation		Grouped Housing	Water Deprivation		Soiled Cage	
18:30-08:30	Intermittent Illumination	Water Deprivation	Cage Tilting	Food Deprivation	Stroboscopic Illumination	Intermittent Illumination	Food Deprivation

Fig. 1. Study design and chronic mild stress (CMS) procedure. (A) Schematic representation of the experimental design. (B) Stressors in the CMS procedure. CMS-Never, -PreW, -PostW, and -Lifetime: CMS and control diet during the lifetime, n-3 polyunsaturated fatty acid (PUFA) diet during pre-weaning period, n-3 PUFA diet during post-weaning period, and n-3 PUFA diet during the lifetime, respectively; MS+CMS-Never, -PreW, -PostW, -Lifetime: CMS with maternal separation (MS) and control diet during the lifetime, n-3 PUFA diet during pre-weaning period, n-3 PUFA diet during post-weaning period, and n-3 PUFA diet during the lifetime, respectively; ED, embryonic day; PND, postnatal day; SPT, sucrose preference test; FST, forced swimming test.

2.5. Sucrose preference test

Anhedonia, which is one of the core symptoms of depression, was assessed by a standard two-bottle choice procedure, as described previously [33]. After completion of CMS on PND 105, all rats were individually housed and were adapted to two bottles containing tap water and a 1% sucrose solution for 4 days, respectively. Positions of the bottles within each cage were switched daily to avoid side bias. On PND 110, the sucrose preference was assessed for 12 h by using two bottles of 1% sucrose solution and tap water after 10 h of food and water deprivation. The sucrose preference index (%) was calculated as (gram of sucrose solution intake/gram of total liquid intake) × 100.

2.6. Forced swimming test

On PND 112, rats underwent a forced swimming test (FST), as previously described, to measure depression-related behavior [34]. Briefly, rats were individually placed inside the cylinder (height 50 cm, diameter 20 cm) containing 25±1°C water up to a height of 30 cm. During the pre-test, the rats were forced to swim for 15 min, and 24 h later, each animal was re-exposed to an FST for 5 min. The test was videotaped with a camera positioned at the side of the cylinder, and the duration of immobility, swimming, and climbing were measured by three raters who were unaware of the treatment conditions.

2.7. Milk, blood and tissue collection

On PND 20, each dam was injected with oxytocin (4 IU/kg body weight; Ubtech unibio, Anyang, Korea) to stimulate milk production after 10 min of separation. Milk was collected by massaging the nipples until at least 200 μL of milk was obtained.

On PND 112, adult rats were anesthetized, and serum and plasma were obtained by using serum separator tubes and ethylenediaminetetraacetic acid (EDTA) tubes, respectively, after centrifugation at 1,610 g for 10 min. Organs were dissected, rinsed with saline, weighed, and frozen in liquid nitrogen. Milk, tissue, and blood samples were stored at -80°C for further analysis.

2.8. Tissue and milk fatty acid analysis

Phospholipid fatty acids were quantified as methyl esters of the brain cortex (n=8 per group) and milk (n=3–4 per group) by gas chromatography (GC) and used to determine fatty acid compositions as previously described with a modification [35]. Brain cortical tissue (100 mg) was homogenized with 5 mL of chloroform:methanol:distilled water (2:2:1 v/v). The homogenates were centrifuged at 2,862 g (Hanil Science Inc., Gimpo, Korea) for 10 min. The solvents of the bottom layer were dissolved in chloroform and separated using thin layer chromatography (TLC; SIL G-25, Macherey-nagel GmbH & Co, Duren, Germany) by development with n-hexane:diethyl ether:acetic acid (40:10:1, v/v). After developing the TLC plate, phospholipids were scraped off. Brain phospholipids and milk were directly methylated by adding boron trifluoride methanol (B1252; Sigma-Aldrich, St. Louis, MO, USA) and heated at 100°C for 10 and 45 min, respectively. Fatty acid methyl esters of brain phospholipid and milk were analyzed using gas chromatography (Shimadzu 2010AF; Shimadzu Scientific Instrument, Tokyo, Japan) with a 100 m × 0.25 mm inner diameter and 0.20 μm film capillary column (SP2560; Supelco, PA, USA). Hydrogen was used as the carrier gas at a flow rate of 40 mL/min. The injector and flame ionization detector temperatures were 230°C and 240°C, respectively. The run temperature began at 180°C and then was increased at a rate of 5°C/min up to 200°C, and then by 10°C/min to 240°C. The split ratio was 10:1. The GC was calibrated with an external standard composed of defined fatty acids (GLC-OQA; Nu-Check Prep, MN, USA). Qualified GC evaluation was carried out by measuring the coefficient of variation of the sum of EPA and DHA composition in quality control material; the coefficient of variation was 2.8%.

2.9. Tissue oxylipins, endocannabinoids, and PUFA analysis

Non-esterified oxylipins, endocannabinoids, and polyunsaturated fatty acids were quantified using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) in the prefrontal cortex (n=6 per group), where degradation of oxylipins was reportedly increased by inflammation [36]. Metabolites were isolated from 41±8 mg of the prefrontal cortex tissue by protein precipitation using methanol/acetonitrile mixture (1:1 v/v), and UPLC-MS/MS was quantified using standard internal methods [37]. Tissue aliquots were homogenized using the GenoGrider 2000 sample homogenizer (SPEX Sample Prep; Metuchen, NJ, USA) using the following: 5 μL BHT/EDTA (1:1 methanol:water); 5 μL of 1,250 nM deuterated oxylipin and endocannabinoid surrogates in methanol; 5 μL 1-cyclohexyl ureido 3-dodecanoic acid (Sigma-Adlrich, MO, USA); 1-phenyl ureido 3-hexanoic acid (kind gift from Dr. B.D. Hammock, University of California - Davis, Davis CA, USA) at 5 μM in 1:1 methanol:acetonitrile; and 185 μL of 1:1 methanol:acetonitrile. They were kept at -20°C for 30 min and centrifuged at 15,000 g for 10 min to remove protein precipitates. The supernatant was further filtered through 0.2 μm PVDF 96-well filter plates and collected for LC-MS/MS analysis. Residues in extracts were separated on a 2.1 mm x 150 mm, 1.7 μm BEH C18 column (Waters, MA, USA) and detected by electrospray ionization with multi reaction monitoring on an API 6500 QTRAP (Sciex, CA, USA). Unless otherwise indicated, they were quantified against 7–9 point calibration curves of authentic standards using minor modifications of previously reported methods [37]. Levels of the eicosapentaenoyl ethanolamide, palmitoleic acid ethanolamide, linoleic acid, alpha-linolenic acid, arachidonic acid, EPA, and DHA were measured using specific mass transitions, but without comparison to authentic standards. The levels of these residues are reported as the percent relative abundance such that the sum of each compound across all experimental samples is equal to 1.

2.10. Tissue prostaglandin E_2 and serotonin, and plasma hormone measurements

Commercially available enzyme-linked immunosorbent assay (ELISA) was used to quantify a set of small molecule targets. Prostaglandin E_2 (PGE_2) and serotonin were respectively measured in brain parietal-occipital cortical tissues and brain stem tissues (n=8 per group) after homogenization in PBS buffer. Concentrations were determined using ELISA kits from Aviva Systems Biology (CA, USA) following the manufacturer's protocol. ELISA kits for corticosterone (Enzo Life Science, NY, USA) and adrenocorticotropic hormone (Phoenix Pharmaceuticals, CA, USA) were used to measure plasma hormones according to the manufacturer's instructions. All measurements were performed in duplicate and quantified against authentic standards by spectrophotometer (Multiscan GO, Thermo Scientific, MA, USA).

2.11. Western blot analysis

Levels of various receptors, transcription factors, and cytokines were determined using western blot analyses. For the analyses, hypothalamic tissue (n=8 per group) was homogenized in an ice-cold radioimmunoprecipitation buffer containing Complete EDTA-free protease and PhosSTOP inhibitor cocktails (Roche Diagnostics GmbH, Mannheim, Germany); the homogenates were centrifuged at 10,000 g for 15 min at 4°C. The cytoplasmic/nuclear fractionation of hippocampal tissues (n=8 per group) were also completed using nuclear/cytosol fractionation kits (Bio-Vision, CA, USA) that contained protease and phosphatase inhibitor cocktails (Bio-Vision, CA, USA), according to the manufacturer's instruction. Bicinchoninic acid assay (Pierce Biotechnology, IL, USA) was applied for protein quantification in the supernatants of whole hypothalamic and hippocampal tissues, and the hippocampal nucleus and cytosol, respectively. Protein (30 μg) in the supernatants of whole hypothalamic and hippocampal tissues, and the hippocampal nucleus and cytosol was separated on a 12% SDS-PAGE, transferred to polyvinylidene fluoride membranes (0.45 μm, Merck Millipore, Billerica, MA, USA), and blocked for 1 h at room temperature with 5% skim milk or 3–5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBST). The membrane was then incubated with primary antibodies against corticotropin-releasing factor (1:2,000), nuclear glucocorticoid receptor (nGR; 1:250), cytosolic GR (cGR; 1:250), cAMP response element-binding protein (CREB; 1:1,000), phosphorylated CREB (pCREB; 1:250), brain-derived neurotrophic factor (BDNF; 1:5,000), tumor necrosis factor-α (TNF-α; 1:500), interleukin-1β (IL-1β; 1:1,000), IL-6 (1:250), serotonin 1A receptor (5-HT_{1A}R; 1:5,000), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor subtype 1 (GluR1; 1:2,000), pGluR1 (1:5,000), N-methyl D-aspartate (NMDA) receptor subtype 2B (NR2B; 1:1,000), and pNR2B (1:1,000), with 5% skim milk or 3–5% bovine serum albumin in TBST overnight at 4°C. Antibodies against CREB and pCREB were purchased from Cell signaling (New England Biolabs, Beverly, MA, USA), and antibodies against the GR, CRF, TNF-α, IL-1β, IL-6, BDNF, 5-HT_{1A}R, GluR1, pGluR1, NR2B, and pNR2B were purchased from Abcam (Cambridge, UK). After washing in TBST, membranes were incubated with a horseradish-peroxidase-conjugated secondary antibody, that was either anti-rabbit IgG (Cell signaling, New England Biolabs, Beverly, MA, USA) or anti-mouse IgG (Enzo Life Science, Farmingdale, NY, USA), with 5% skim milk in TBST for 1 h at room temperature. Immunoreactive bands were visualized on the UV setting in the Chemidoc MP Imaging System (Bio-rad, Hercules, CA, USA) to estimate total protein per lane; β-actin was used for normalization. The hypothalamus was used to analyze the corticotropin releasing factor (CRF) expression, while the hippocampus was used to analyze the other proteins. Each western blot was repeated three times.

2.12. Immunofluorescence staining

Rats were anesthetized and perfused through the aorta with saline followed by 4% paraformaldehyde. The brain was extracted and placed in the same fixative at 4°C overnight. After fixation, tissues (n=1 per group) were dehydrated in increasing concentrations of alcohol, cleared with xylene, and then embedded in paraffin. Tissues were cut into 10 μm sections and mounted on glass slides. The sections were immersed in xylene followed by alcohol and rehydrated in a wash buffer. Antigen retrieval was performed by heating the sections in sodium citrate buffer (0.01 M, pH 6.0). DAKO peroxidase block solution (DAKO, Produktionsvej, Denmark) was applied to block endogenous peroxidase activity, and anti-GR antibody (1:30, Abcam, Cambridge, UK) was added and incubated at 4°C overnight. The sections were incubated for 2 h at room temperature with goat anti-rabbit IgG conjugated with DyLight 488 (Abcam, Cambridge, UK) for the GR. After washing, the slides were mounted with a fluoroshield medium containing 4′, 6-diamidino-2-phenylindole (DAPI; Abcam, Cambridge, UK). A Leica AF6000 fluorescent microscope and Leica AF imaging software (Leica Microsystems, Wetzlar, Germany) were used to evaluate immunofluorescence images of the CA1, CA2, and CA3 gyrus region of the hippocampus.

2.13. Quantitative real time-polymerase chain reaction (RT-PCR) of microRNA

Total RNA was extracted from the hippocampus (n=8 per group) using the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's institutions. RNA concentration was determined using the microspectrophotometer (NanoDrop One, Thermo Fisher Scientific, WI, USA). Complementary DNA was synthesized from small RNA-enriched total RNA using the iScript Select cDNA synthesis kit (Bio-rad, CA, USA). Gene-specific primers of miRNA-218 (Assay ID: 000521), miRNA-155 (Assay ID: 002571), miRNA-132 (Assay ID: 000457), and small nuclear RNA U6 (U6 snRNA; Assay ID: 001973) (Applied Biosystems, Carlsbad, CA, USA) were also used. Quantitative RT-PCR reactions for the detection of miRNA-218, miRNA-155, miRNA-132, and U6 snRNA expressions were performed using the Thunderbird Probe qPCR Mix (Toyobo, Osaka, Japan) and the CFX96 Real-Time Detection system instrument (Bio-rad, Hercules, CA, USA). The PCR conditions were as follows; 95°C for 60 s, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Each sample was analyzed in triplicate. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with the U6 snRNA as an internal control.

2.14. Statistical analyses

Brain levels of oxylipin, endocannabinoids, and PUFAs were assessed for dietary treatment timing effects using the partial least square regression (PLSr) analysis (Supplemental Fig. 1 and 2). The MS effect was evaluated using the analysis of covariance (ANCOVA). Before the analyses, missing values were imputed for variables with >80% of the data present. Two variables out of 54 were excluded. The imputed data constituted <1% of the remaining data. The resulting data set was Johnson transformed, and variable normality was confirmed with the Shapiro-Wilk test. PLSr was performed using the NIPALS algorithm with leave-one-out cross-validation. For this analysis, dietary treatment timing was converted into a continuous variable in the order of no n-3 PUFA<pre-weaning<post-weaning<lifetime. The ANCOVA test was applied as a fixed effect and the dietary treatment timing as a covariate. Additionally, data were tested for the Diet × MS interaction, followed by a *post hoc* least squared mean t-test. Mean differences were considered significant at P<.05. The P value for each effect test was subjected to the Benjamini and Hochberg false discovery rate (FDR) corrections at a q=0.2 to account for multiple comparisons [38]. Both PLSr and ANCOVA were performed in JMP Pro v 14 (SAS Institute, NC, USA).

Other data from GC, ELISA, western blot, and RT-PCR were analyzed with a two-way analysis of variance (ANOVA) with factors of diet and MS, followed by the least significant difference *post hoc* test. Values were expressed as the mean and standard deviation of the mean, and differences were considered significant at P<.05. ANOVA was performed using SPSS for Windows, version 24.0 (SPSS Inc., IL, USA).

3. Results

3.1. Dietary intake, body weight, and fatty acid composition

Neither n-3 PUFA diet nor MS significantly affected dietary intake amount, and body and organ weight (Supplemental Table 1). MS had no effect on the fatty acid composition of milk, but n-3 PUFA diet increased proportion of n-3 PUFA and decreased n-6 PUFA in fatty acid composition of milk (Supplemental Table 2). In both CMS and MS+CMS, brain levels of 18:2n6, 20:4n6, 22:4n6,

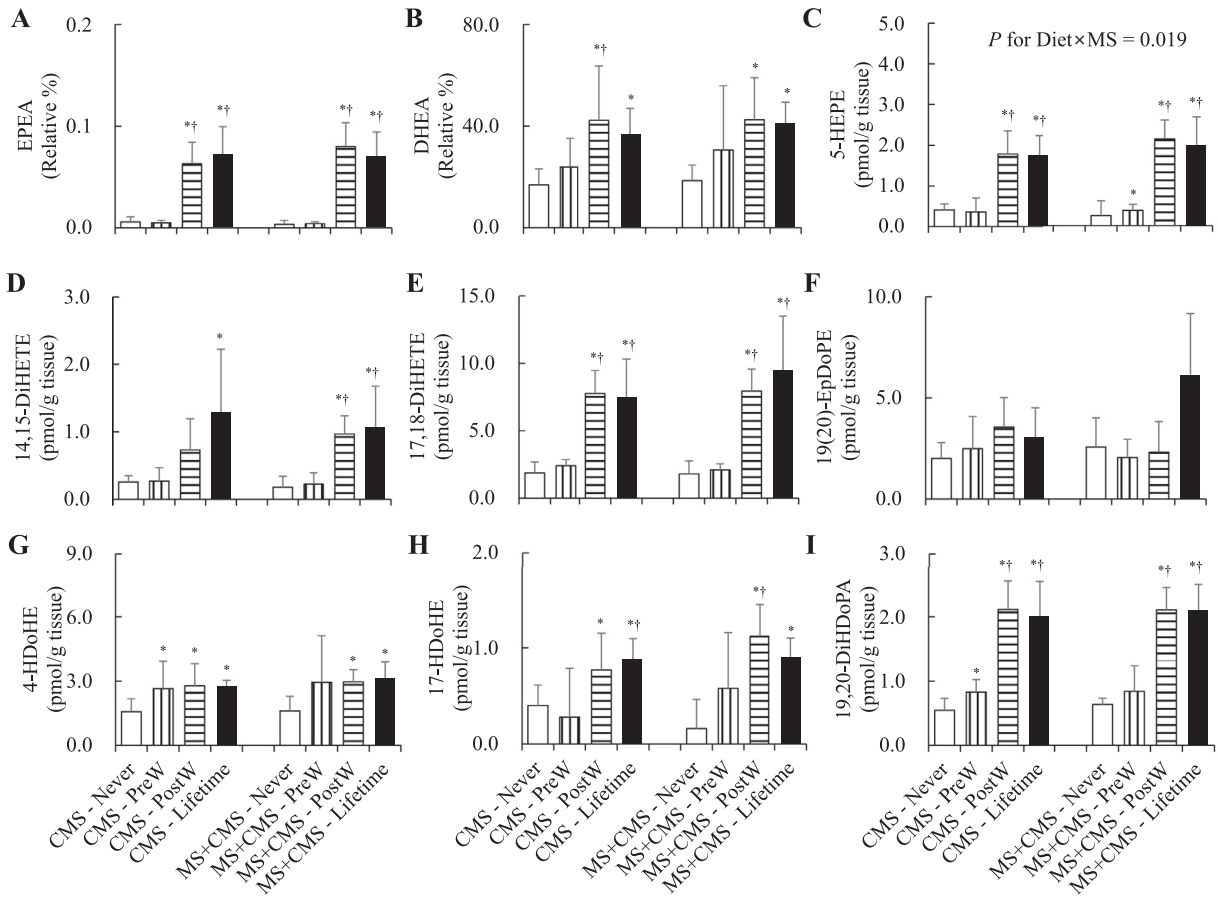


Fig. 2. Effect of n-3 PUFA on n-3 PUFA-derived endocannabinoid and oxylipin. (A–I) Prefrontal cortex level of eicosapentaenoyl ethanolamide (EPEA), docosahexaenoyl ethanolamide (DHEA), 5-hydroxyeicosapentaenoic acid (HEPE), 14,15-dihydroxyeicosatetraenoic acid (DiHETE), 17,18-DiHETE, 19(20)-epoxydocosapentaenoic acid (EpDoPE), 4-hydroxydocosahexaenoic acid (HDoHE), 17-HDoHE, and 19,20-dihydroxydocosapentaenoic acid (DiHDoPA). Values are mean and standard deviation (n=6); *Values are significantly different between control diet and n-3 PUFA diet within the same stress condition; †Values are significantly different between pre-weaning period and post-weaning or lifetime within n-3 PUFA diet with the same stress condition.

and 22:5n6 were decreased by n-3 PUFA diet as compared to control diet, and were lower by n-3 PUFA diet during post-weaning and lifetime than during pre-weaning (Supplemental Table 3). In both rat with CMS and MS+CMS, brain levels of 20:5n3, 22:5n3 increased by n-3 PUFA diet during post-weaning and lifetime as compared to control diet and n-3 PUFA diet during pre-weaning. On the other hand, in both rat with CMS and MS+CMS, brain level of 22:6n3 increased by n-3 PUFA diet as compared with control diet, and was higher by n-3 PUFA diet during post-weaning and lifetime than during pre-weaning.

3.2. Prefrontal cortex endocannabinoid and oxylipin profiles

There was no difference on endocannabinoids and oxylipins in parietal-occipital cortex of rats between MS+CMS and CMS (Fig. 2; Supplemental Fig. 1). In both CMS and MS+CMS, n-3 PUFA diet during post-weaning period and lifetime increased levels of eicosapentaenoyl ethanolamide (EPEA), docosahexaenoyl ethanolamide (DHEA), 5-hydroxyeicosapentaenoic acid (HEPE), 14,15-dihydroxyeicosatetraenoic acid (DiHETE), 17,18-DiHETE, 19(20)-epoxydocosapentaenoic acid (EpDoPE), 4-hydroxydocosahexaenoic acid (HDoHE), 17-HDoHE, and 19,20-dihydroxydocosapentaenoic acid (DiHDoPE) as compared with the control diet and n-3 PUFA diet during pre-weaning period (Fig. 2; Supplemental Fig. 1 and 2). Moreover, the n-3 PUFA diet had significant interaction with MS on the level of 5-HEPE (Fig. 2C).

3.3. Depressive behaviors

MS+CMS decreased sucrose preference and swimming duration, and increased immobility duration as compared to CMS (Table 1). In CMS, n-3 PUFA diet during lifetime increased climbing duration, while decreased immobility duration as compared with control diet. In MS+CMS, n-3 PUFA diet during pre- and post-weaning and lifetime decreased immobility duration as compared to control diet, and the effect of n-3 PUFA diet during lifetime was greater than during pre- and post-weaning period. Moreover, in MS+CMS, n-3 PUFA diet during lifetime increased sucrose preference and climbing duration as compared to control diet.

3.4. Hypothalamic-pituitary-adrenal (HPA) axis activity

As compared to CMS, MS+CMS increased blood level of CORT and ACTH, and hypothalamic expression of CRF, while decreased hippocampal ratio of nGR/cGR (Fig. 3A–D). MS+CMS increased hippocampal expression of GR-regulating miRNA-218 (Fig. 3E). In CMS, n-3 PUFA diet during lifetime period decreased blood levels of CORT and ACTH, and the expressions of CRF and miRNA-218 as

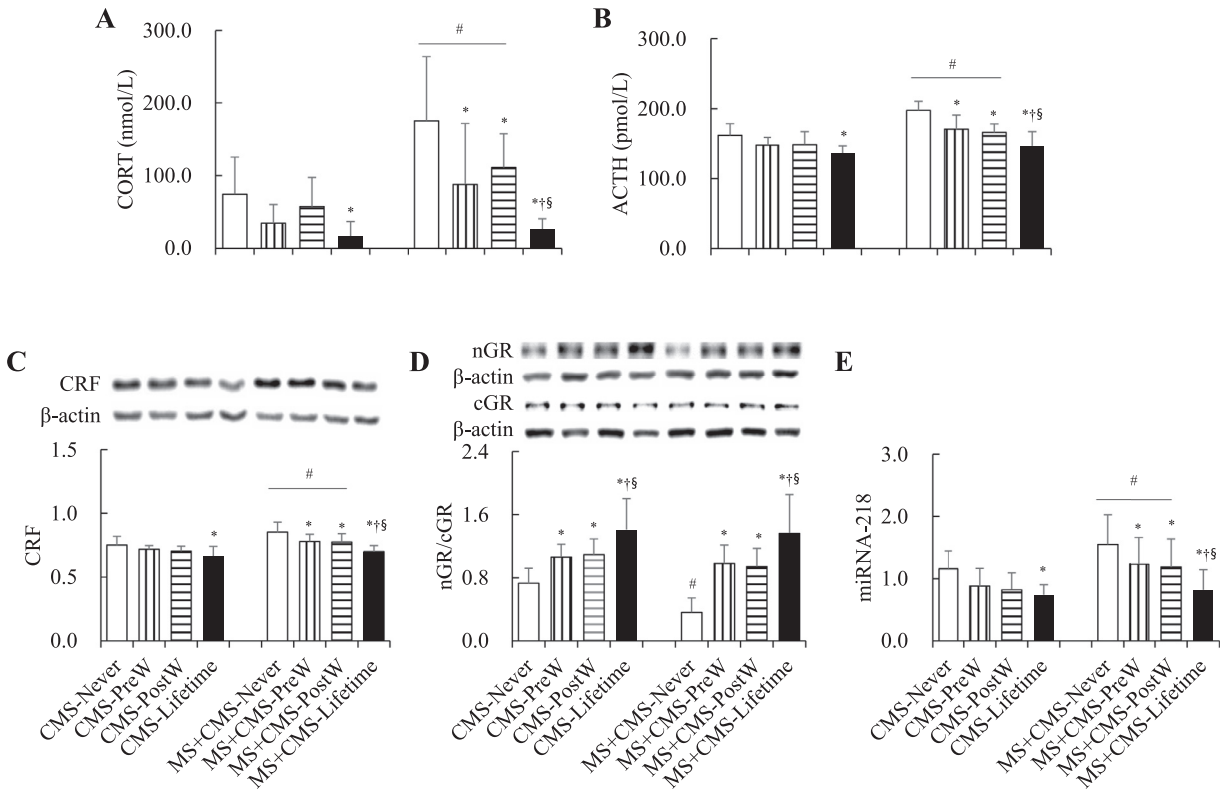


Fig. 3. Effect of n-3 PUFA on hypothalamic-pituitary-adrenal (HPA) axis activity. (A–B) Blood level of corticosterone (CORT) and adrenocorticotropic hormone (ACTH). (C) Hypothalamic expression of corticotropin-releasing factor (CRF). (D) Hippocampal ratio of nuclear glucocorticoid receptor (nGR)/cytosolic GR (cGR). (E) Hippocampal expression of miRNA-218. Values are mean and standard deviation (n=8); *Values are significantly different between control diet and n-3 PUFA diet within the same stress condition; †Values are significantly different between pre-weaning period and post-weaning or lifetime within n-3 PUFA diet with the same stress condition; §Values are significantly different between post-weaning period and lifetime within n-3 PUFA diet with the same stress condition; #Values are significantly different between CMS and MS+CMS within the same diet and feeding period.

compared with control diet and n-3 PUFA diet during pre- and post-weaning period (Fig. 3A–C, E). In CMS, n-3 PUFA diet during pre- and post-weaning and lifetime increased hippocampal ratio of nGR/cGR as compared to control diet, and the effect of n-3 PUFA on GR activation was greater during lifetime than pre- and post-weaning period (Fig. 3D). In MS+CMS, n-3 PUFA decreased blood levels of CORT and ACTH, and the expressions of CRF and miRNA-218, while increased hippocampal ratio of nGR/cGR as compared to control diet, and the effects of n-3 PUFA diet were greater during lifetime than pre- and post-weaning period (Fig. 3A–E).

Accordingly, MS+CMS decreased the expression of nGR in hippocampal CA1, CA2, and CA3 region as compared to CMS, regardless of diet (Fig. 4). On the other hand, n-3 PUFA diet increased the expression of nGR in hippocampal CA1, CA2, and CA3 region in both CMS and MS+CMS (Fig. 4).

3.5. Neuroinflammation

As compared to CMS, MS+CMS increased expression of TNF-α, IL-1β, IL-6, and miRNA-155, parietal-occipital cortex level of PGE_2 (Fig. 5A–E). In CMS, n-3 PUFA diet during lifetime decreased TNF-α. IL-1β, miRNA-155, and PGE_2 as compared to control diet (Fig. 5A–B, D–E). In CMS, n-3 PUFA diet during pre- and post-weaning period and lifetime decreased expression of IL-6 as compared to control diet, and the effect of n-3 PUFA diet during lifetime was greater than during pre- and post-weaning period (Fig. 5C). In MS+CMS, n-3 PUFA diet during pre- and post-weaning period and lifetime decreased TNF-α, IL-1β and IL-6, and PGE_2 as compared to control diet, and the effects of n-3 PUFA diet during lifetime were greater than during pre- and post-weaning period (Fig. 5A–D). In MS+CMS, n-3 PUFA diet during lifetime increased miRNA-155 as compared with control diet (Fig. 5E). Additionally, n-3 PUFA diet had an interaction with MS on TNF-α (Fig. 5A).

3.6. cAMP response element binding protein (CREB)-brain derived neurotrophic factor (BDNF)-serotonergic pathway

As compared to CMS, MS+CMS decreased hippocampal ratio of pCREB/CREB, expressions of BDNF and 5-HT_{1A}R, and brainstem level of 5-HT (Fig. 6A–D). In CMS, n-3 PUFA diet during pre- and post-weaning and lifetime increased expression of 5-HT_{1A}R as compared to control diet, and the effect of n-3 PUFA diet during lifetime was greater than during pre- and post-weaning period (Fig. 6C). In CMS, n-3 PUFA diet during lifetime increased hippocampal ratio of pCREB/CREB and expression of BDNF, and brainstem level of 5-HT as compared to control diet (Fig. 6A–B, D). In MS+CMS, n-3 PUFA diet during pre- and post-weaning period and lifetime increased pCREB/CREB, BDNF, 5-HT_{1A}R, and 5-HT as compared to control diet, and the effects of n-3 PUFA diet during lifetime were greater than during pre- and post-weaning period (Fig. 6A–D).

3.7. Glutamatergic pathway

As compared to CMS, MS+CMS increased hippocampal ratios of pGluR1/GluR1, and pNR2B/NR2B, and the expression of

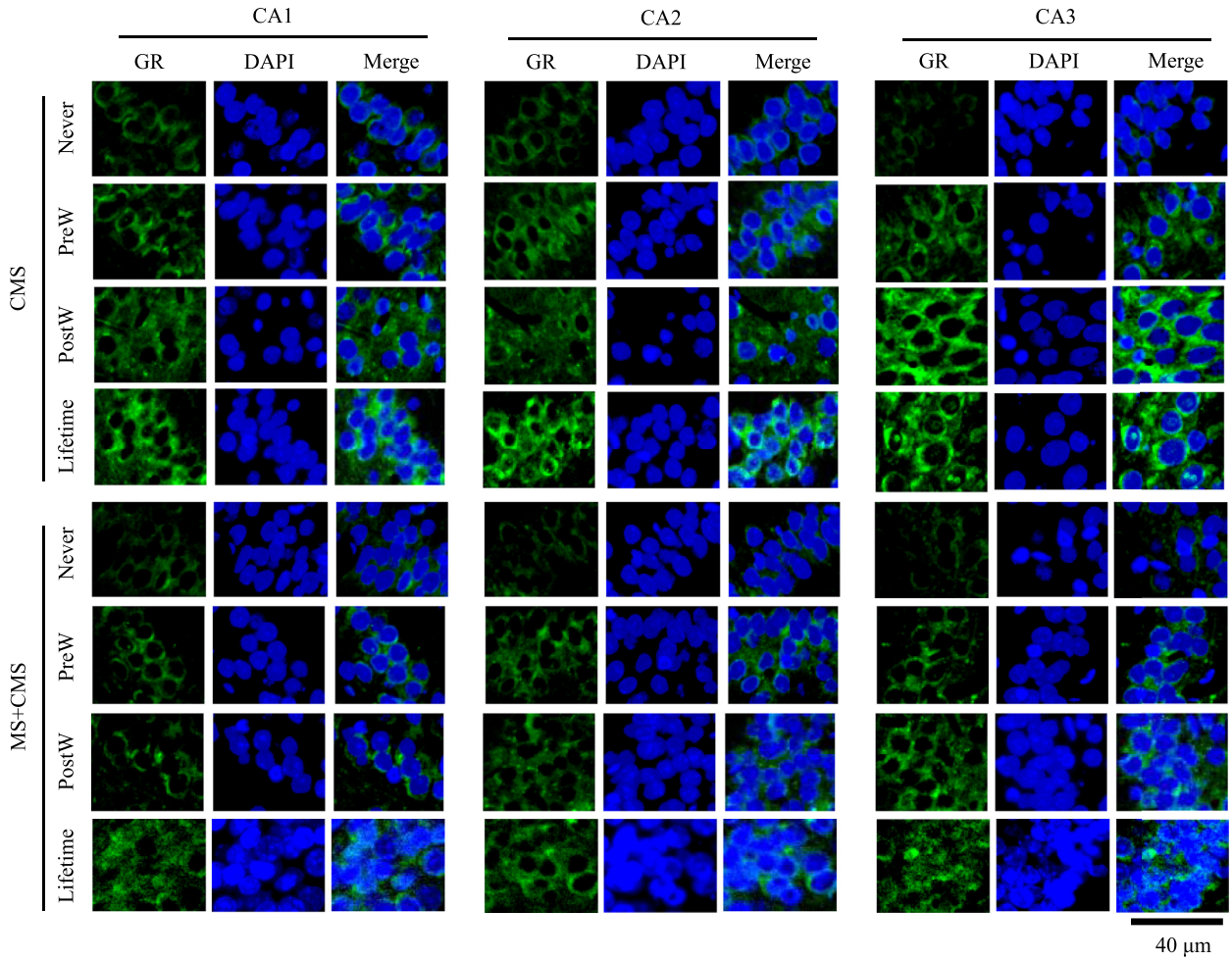


Fig. 4. Immunofluorescence staining of glucocorticoid receptor (GR) in the CA1, CA2, CA3 regions in hippocampus of the rats. GR expression was visualized with DyLight 488 (green), and DNA was stained blue (DAPI). Each staining of GR, DAPI, and merged images was shown. Scale bar=40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

miRNA-132 (Fig. 7). In both CMS and MS+CMS, n-3 PUFA diet did not significantly affect hippocampal ratios of pGluR1/GluR1 and pNR2B/NR2B, and expression of miRNA-132.

4. Discussion

The present study demonstrated that n-3 PUFA supplementation improved depressive behaviors induced by CMS with and without MS in post-menopausal rats. This effect was associated with changes in n-3 PUFA metabolites and serotonergic, but not glutamatergic pathways, as made evident by changes in the HPA axis (Fig. 8). As far as we are aware, the antidepressant-like effects of n-3 PUFA have not been studied in a post-menopausal depression model induced by MS+CMS. Previous studies have reported that n-3 PUFA improves depressive behaviors by decreasing the duration of immobility and increases climbing in acute stress-induced post-menopausal depressed rats [11,12]. Similar effects of n-3 PUFA have also been identified in male rats with depression induced by either acute stress [26] or CMS [13]. Consistent with our study, previous studies showed that n-3 PUFA had no significant effect on swimming in post-menopausal female rats [11,12] and male rats with acute stress [26]. Swimming behavior was shown to be increased by inhibition of glutamatergic neurotransmission in rats with severe CMS [39], while climbing behavior was regulated by norepinephrine, the release of which was increased by 5-HT_{1A}R in the hippocampus [40,41]. Accordingly, n-3 PUFA increased hippocampal level of serotonin, but not glutamate in acute stress-induced post-menopausal depressed rats [11], suggesting that climbing but not swimming could be modified by n-3 PUFA since serotonin rather than glutamate was dominantly influenced by n-3 PUFA. Tang et al. [13] have also shown that oral administration of n-3 PUFA improves sucrose preference, indicating depressive symptoms in rats with CMS. However, there has been no previous study regarding the effect of n-3 PUFA on sucrose preference in MS-induced depression.

It has been reported that vulnerability to CMS is elevated by MS, as shown by increased immobility time and decreased sucrose preference in female rats with CMS+MS as compared with those with CMS alone [5,42], consistent with the results of the present study. In the present study, n-3 PUFA increased sucrose preference. There were also increases in the prefrontal cortex levels of n-3 PUFA derived oxylipins obtained from cytochrome P450/soluble epoxide hydrolase metabolism (epoxides and diols) and multiple lipoxygenase dependent metabolites (PUFA mid-chain alcohols). n-3 PUFA feeding also decreased hippocampal expression of TNF-α to a greater extent in rats with MS+CMS than with CMS alone. How-

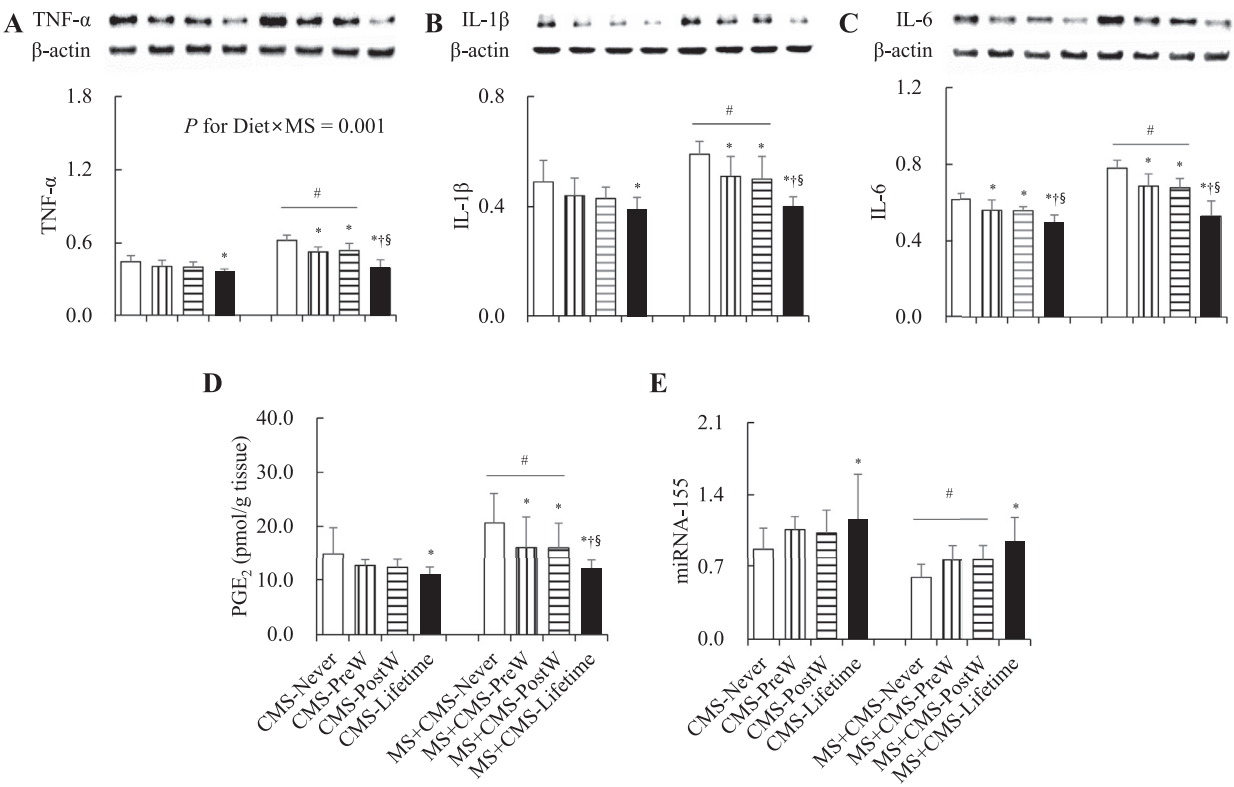


Fig. 5. Effect of n-3 PUFA on neuroinflammation. (A–C) Hippocampal expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). (D) Parietal-occipital cortex level of prostaglandin E_2 (PGE_2). (E) Hippocampal expression of miRNA-155. Values are mean and standard deviation (n=8); *Values are significantly different between control diet and n-3 PUFA diet within the same stress condition; †Values are significantly different between pre-weaning period and post-weaning or lifetime within n-3 PUFA diet with the same stress condition; §Values are significantly different between post-weaning period and lifetime within n-3 PUFA diet with the same stress condition; #Values are significantly different between CMS and MS+CMS within the same diet and feeding period.

ever, the effects of n-3 PUFA on most behavioral and biochemical outcomes were essentially the same in both rats with CMS+MS and with CMS alone, suggesting that n-3 PUFAs could not reverse the depression-related modifications induced by MS.

It is suggested that depression occurs through dysregulation of the serotonergic and glutamatergic pathway [43]. In rats with MS- [44] and CMS- [45] induced depression, the levels of serotonin and 5-HT_{1A}R decrease, which in turn inhibits CREB phosphorylation, leading to a reduction in BDNF expression. However, supplementation with n-3 PUFA increases serotonin levels, 5-HT_{1A}R, BDNF, and CREB in the blood and brain of post-menopausal acute stress-induced depressed rats [11,12], and male rodents with CMS [13,14] and MS [46]. Neuronal accretion of n-3 PUFA also enhances membrane fluidity, which increases the density and binding affinity of 5-HT_{1A}R [47]. Previous studies have shown that miRNA-218 modulates brain expression of CREB in rats with pharmacologically induces depression [10]. In the present study, n-3 PUFA feeding also increased hippocampal expressions of miRNA-218, suggesting that n-3 PUFA up-regulated the serotonergic pathway and related miRNAs.

Synthesis of serotonin is inhibited by pro-inflammatory cytokines [48], which are increased by MS and CMS [42]. Consistent with the results of the present study, the down-regulating effects of n-3 PUFA on the expressions of TNF-α, IL-1β, and IL-6 have been reported in post-menopausal rats with acute stress-induced depression [11,12] and mice with CMS [49]. Moreover, treatment with EPA increases miRNA-155 expression in human leukemic monocytes [50], which inhibits pro-inflammatory cytokine synthesis [51]. n-3 PUFA and their metabolites, including oxylipins and endocannabinoids, are well-known modulators of inflammation and cognitive function [52-54]. For instance, the anti-inflammatory effect of n-3 PUFA in rodents has been suggested based on the up-regulation of n-3 PUFA-derived endocannabinoids and oxylipins associated with changes in inflammatory gene expression [18]. Previous studies have reported that as compared to 20:4 n-6-derived AEA, DHA-derived DHEA has a higher binding affinity to cannabinoid receptor 1 [55,56], which up-regulates the BDNF-serotonergic pathway [57]. Consistent with the results of the present study, n-3 PUFA supplementation increases blood levels of metabolites derived from EPA such as EPEA, 5-HEPE, 14,15-DiHETE, and 17,18-DiHETE, and from DHA such as DHEA, 19(20)-EpDoPE, 4-HDoHE, and 19,20-DiHDoPA in inflamed rodents [17] and depressed patients [22].

Furthermore, inflammation is down-regulated by activation of the GR, which switches off the production of pro-inflammatory cytokines [6]. MS and chronic restraint stress have previously been found to decrease the hippocampal density of the GR, which increases the release of hypothalamic CRF, pituitary ACTH, and adrenal corticosterone [5]. However, consistent with the results of the present study, n-3 PUFA increases brain expression of the GR [5,16,58] and decreases the corticosterone blood level in male rodents exposed to either chronic restraint stress [15] or chronic social defeat stress [16]. Dwivedi et al. [10] have reported that brain expression of GR decreases with the elevation of miRNA-218 in rats with pharmacologically induced depression. The present study showed that n-3 PUFA decreased the hippocampal expression of

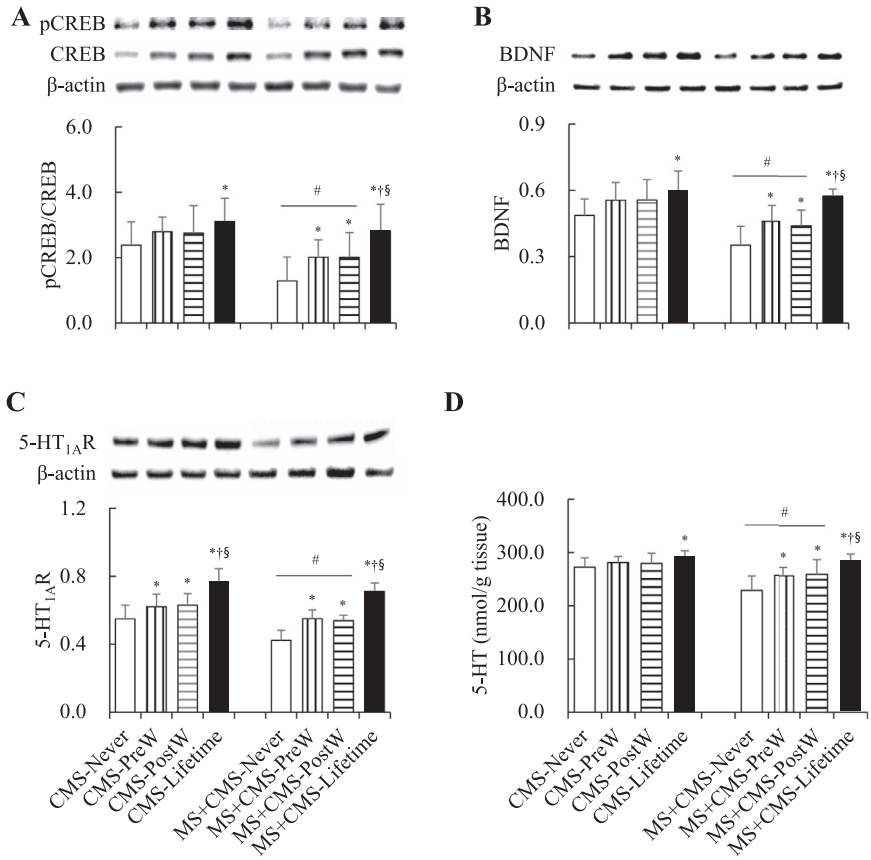


Fig. 6. Effect of n-3 PUFA on cAMP response element binding protein (CREB)/brain derived neurotrophic factor (BDNF)-serotonergic pathway. (A) Hippocampal ratio of pCREB/CREB. (B–C) Hippocampal expression of BDNF and serotonin 1A receptor (5-HT_{1A}R). (D) Brainstem level of serotonin (5-HT). Values are mean and standard deviation (n=8); *Values are significantly different between control diet and n-3 PUFA diet within the same stress condition; †Values are significantly different between pre-weaning period and post-weaning or lifetime within n-3 PUFA diet with the same stress condition; §Values are significantly different between post-weaning period and lifetime within n-3 PUFA diet with the same stress condition; #Values are significantly different between CMS and MS+CMS within the same diet and feeding period.

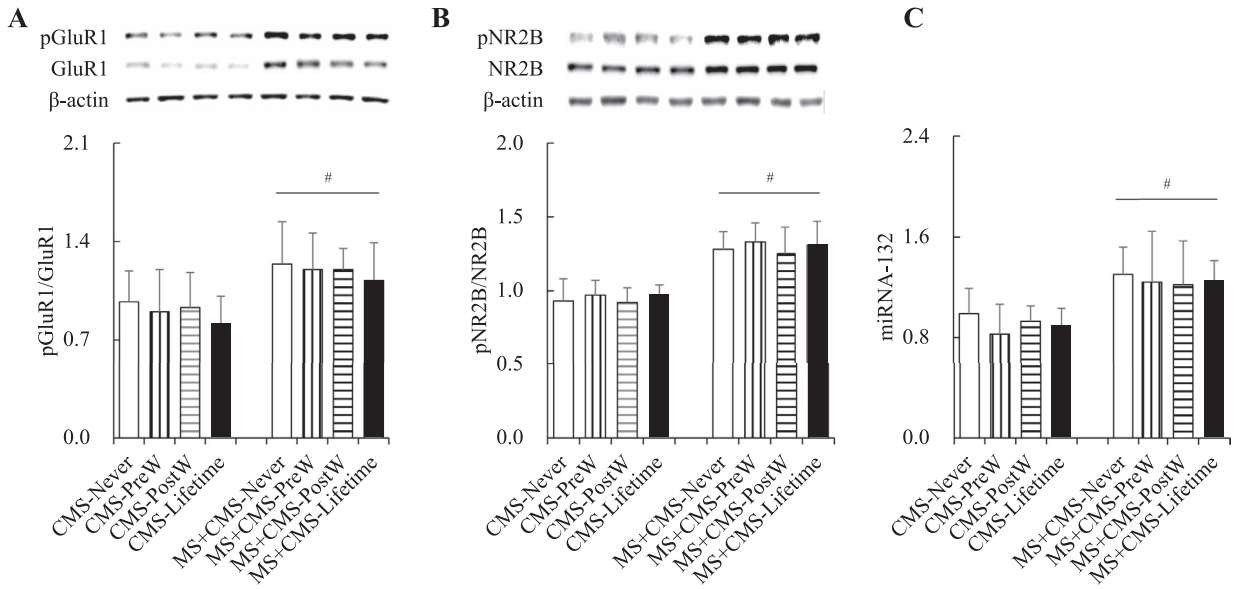


Fig. 7. Effect of n-3 PUFA on glutamatergic pathway. (A–B) hippocampal ratio of phosphorylated α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor subtype 1 (pGluR1)/GluR1, and phosphorylated N-methyl D-aspartate receptor subtype 2B (pNR2B)/NR2B. (C) Hippocampal expression of miRNA-132. Values are mean and standard deviation (n=8); #Values are significantly different between CMS and MS+CMS within the same diet and feeding period.

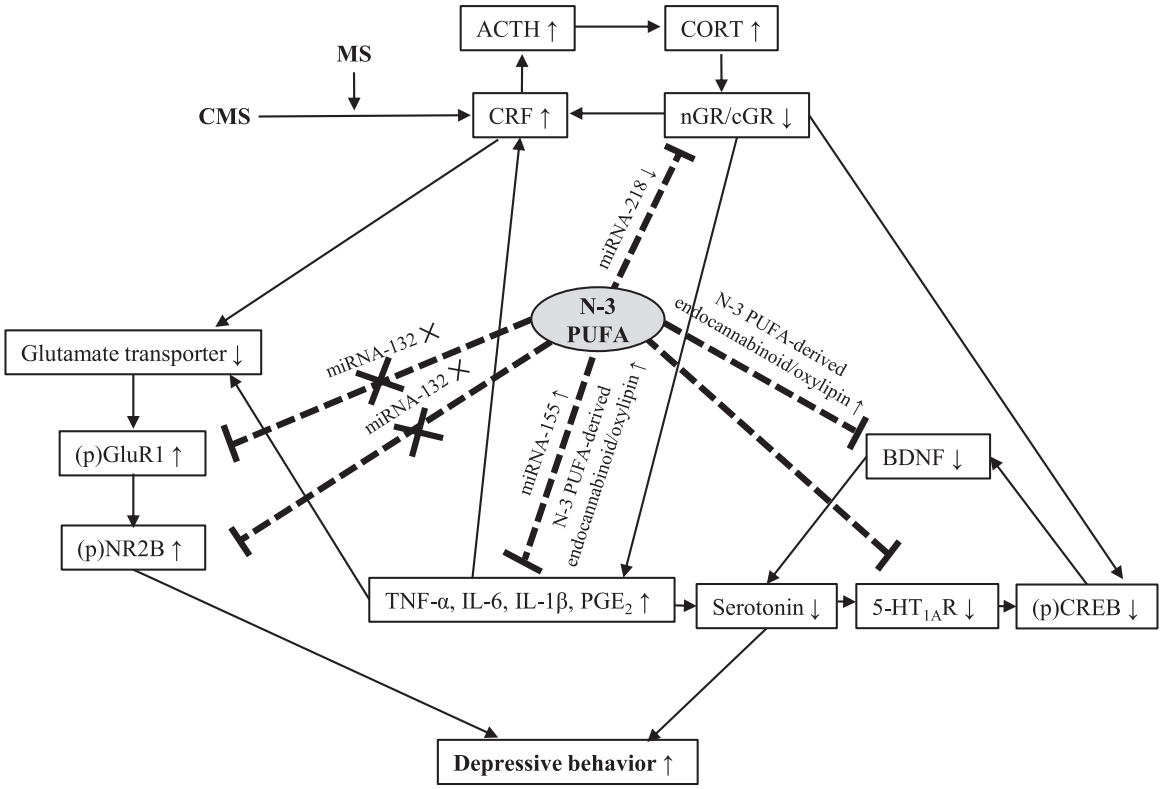


Fig. 8. Abridged general view of the potential pathway for the antidepressant-like effect of n-3 PUFA. N-3 PUFA and n-3 PUFA-derived metabolites increased the ratio of nuclear glucocorticoid receptor (nGR)/cytosolic GR (cGR), and decreased the expression of miRNA-218, leading to down-regulation of corticotrophin releasing factor (CRF)-adrenocorticotropic hormone (ACTH)-corticosterone, and modulation of cAMP response element binding protein (CREB) phosphorylation and cytokine transcription. N-3 PUFA increased expression of serotonin 1A receptor ($5\text{-HT}_{1A}\text{R}$), which in turn up-regulated CREB phosphorylation followed by increase in BDNF and serotonin. In addition, n-3 PUFA and n-3 PUFA-derived metabolites decreased level of prostaglandin E_2 (PGE_2), and expression of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), while increased expression of miRNA-155, which inhibited production of pro-inflammatory cytokines. However, n-3 PUFA had no effect on the phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor subtype 1 (GluR1) and N-methyl D-aspartate receptor subtype 2B (NR2B), and miRNA-132, a regulator of GluR1 and NR2B.

miRNA-218, suggesting the modulation of HPA axis activity by n-3 PUFA at both the protein and miRNA levels.

The up-regulation of CRF leads to glutamatergic neurotoxicity by modulating the release and uptake of glutamate and the expression of AMPA and NMDA glutamate receptors [59]. Both MS [60] and CMS [61] dysregulate glutamate uptake and NMDA and AMPA receptor expression in the rat brain. In contrast, n-3 PUFA supplementation decreases the level of glutamate in the prefrontal cortex [62], but not in the thalamus [62] and hippocampus [11], where regulation of the number and activity of NMDA receptors occurs [63]. Menard et al. [62] have reported that the EPA treatment does not alter total protein expressions of GluR1 of the AMPA receptor and NR1 of the NMDA receptor in the hippocampus of rats. Expressions of GluR1 and NR2B have also been shown to increase by the transfection of miRNA-132 in rat cortical neurons [64]. The present study also showed that n-3 PUFA did not affect the hippocampal expression of GluR1, NR2B, and miRNA-132, suggesting that the antidepressant-like effects of n-3 PUFA might not be related to protection from glutamatergic neurotoxicity.

Consistent with the results of the present study, Weiser et al. [24] have reported that lifetime DHA supplementation has a greater effect on depressive behaviors than supplementation during the pre-weaning period in non-stressed male rats. Ferraz et al. [25] have also shown that pre- and post-weaning supplementation of n-3 PUFA has similar antidepressant properties in non-stressed rats. In the present study, post-weaning supplementation with n-3 PUFA increased the brain level of n-3 PUFA to a similar extent as lifetime supplementation. However, it had a lower antidepressant-like effect than lifetime supplementation but a similar effect to pre-weaning supplementation. Deficiency of n-3 PUFA during the neurodevelopmental period (ED 0-PND 21) could lead to irreversible serotonergic neurotransmission damage [65] since this period coincides with the perinatal maturation of central serotonergic synapses [66]. Thus, previous studies have suggested that the timing of n-3 PUFA supplementation could play an essential role in the pathophysiology of depression.

There are a few limitations of the present study. The expression of the gene targeted by miRNA was not measured. The antidepressant-like mechanism of n-3 PUFA was also not proven in the knockout model. The groups subjected to MS alone or no stress at all were not included. However, as far as we know, this was the first study showing that lifetime exposure of n-3 PUFA had more antidepressant-like effects in rats with post-menopausal depression than during pre- or post-weaning exposure of n-3 PUFA. The effects are associated with the up-regulation of the serotonergic pathway and related miRNAs, as well as changes in the endocannabinoid and oxylipin systems in post-menopausal rats with CMS, regardless of the experience of MS. Further studies are warranted to confirm the anti-depressant effect of n-3 PUFA in post-menopausal depressed patients who experienced early life stress.

Table 1
Depressive behaviors of female rat[1]

	CMS				MS+CMS				*P*		
	Never	PreW	PostW	Lifetime	Never	PreW	PostW	Lifetime	Diet	MS	Diet × MS
SI (%)	79.27±18.61	88.05±7.32	86.86±9.30	95.88±1.89	61.23±27.78[#]	68.20±19.20[#]	69.70±22.21[#]	79.37±13.27[*]	.047	<.001	.998
Immobility (s)	30.10±7.54	17.44±8.73	18.70±8.61	12.73±6.68[*]	51.19±29.26[#]	32.85±8.88[#*]	33.51±9.31[#*]	11.88±4.89[*†§]	<.001	<.001	.100
Climbing (s)	68.63±11.42	78.44±25.49	79.95±12.91	90.62±15.98[*]	74.37±21.89	85.01±16.54	89.00±24.31	96.21±25.26[*]	.028	.182	.994
Swimming (s)	201.27±15.72	204.11±25.89	201.34±16.07	196.63±12.58	174.43±22.15[#]	182.13±15.62[#]	177.48±21.93[#]	191.91±27.29	.785	<.001	.417

[1] Values are mean and standard deviation (n=8); data were analyzed with a two-way analysis of variance with factors of n-3 PUFA supplementation period and MS, followed by the least significant difference *post hoc* test; CMS-Never, -PreW, -PostW, and -Lifetime: CMS and control diet during the lifetime, n-3 polyunsaturated fatty acid (PUFA) diet during pre-weaning period, n-3 PUFA diet during post-weaning period, and n-3 PUFA diet during the lifetime, respectively; MS+CMS-Never, -PreW, -PostW, -Lifetime: CMS with maternal separation (MS) and control diet during the lifetime, n-3 PUFA diet during pre-weaning period, n-3 PUFA diet during post-weaning period, and n-3 PUFA diet during the lifetime, respectively; SI, sucrose preference index.
[*] Values are significantly different between control diet and n-3 PUFA diet within the same stress condition.
[†] Values are significantly different between pre-weaning period and post-weaning or lifetime within n-3 PUFA diet with the same stress condition.
[§] Values are significantly different between post-weaning period and lifetime within n-3 PUFA diet with the same stress condition.
[#] Values are significantly different between CMS and MS+CMS within the same diet and feeding period.

Author Contributions

Jeong-Eun Choi: Methodology, Investigation, Formal analysis, Writing – Original draft preparation; Kamil Borkowski: Investigation, Formal analysis, Writing – Review & Editing; John W. Newman: Formal analysis, Writing – Review & Editing, Funding acquisition; Yongsoon Park: Conceptualization, Supervision, Writing – Review & Editing, Funding acquisition.

J.-E.C. performed experiments and prepared the manuscript. K.B. performed UPLC-MS/MS analyses. K.B. and J.W.N. performed statistical analysis and interpretation of UPLC-MS/MS data and revised the manuscript. Y.P. supervised the study design and data analysis and revised the manuscript. All the authors have read and approved the final manuscript.

Declaration of competing interests

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that any aspect of the work covered in this manuscript that has involved experimental animals has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2021.108599.

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