Maternal Exercise before Pregnancy Alleviates Periventricular Leukomalacia by Lipopolysaccharide-Induced Maternal Intrauterine Inflammation in Infant Rat Brain

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Abstract

In the present study, we investigated the effects of maternal exercise before pregnancy on lipopolysaccharide (LPS)-induced hypomyelination and astrogliosis in neonatal rats. Female Sprague-Dawley rats (180 \pm 10 g, 8 weeks old, n = 45) were used for maternal rats in this experiment. Before pregnancy, rats were randomly divided into three groups: the non-ex-control (CON) group, non-ex-LPS (LPS) group, and the Pre-ex-LPS (Ex-LPS) group (n = 15 in each group). The rats in the Ex-LPS group were forced to run on a motorized treadmill for 30 min once a day for 4 weeks. After the completion of exercise, all rats were allowed to mate with male rats for a period of 24 h. After conforming of pregnancies (n = 5 in each group), the maternal rats were treated with 0.15 mg/kg LPS and/or the same amount of pyrogen-free saline (PFS) on the 15th, 17th, and 20th day of pregnancy. The expression of Cyclooxygenasee-2 (COX-2) and Tumor necrosis factor-a $(TNF-\alpha)$ in brain was analyzed by RT-PCR. Immunohistochemistry for the glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) in the corpus callosum were conducted. In results, the expressions of COX-2, TNF- α and GFAP was significantly increased while the expression of MBP was significantly decreased in the neonatal rat brain of the LPS group compared to those of the CON group (p < .05). This result showed that LPS might induce toxic effect in neonatal rat brain such as hypomyelination and astrogliosis. In contrast, the expressions of COX-2, TNF- α and GFAP was significantly decreased while the expression of MBP was significantly increased in the neonatal rat brain of the Ex-LPS group compared to those of the LPS group (p < .05). In conclusion, the present results revealed that regular maternal exercise before pregnancy might be effective to ameliorate the symptoms of PVL such as astrogliosis and hypomyelination in neonatal rat brain and it can be used as a therapeutic strategy for preventing the detrimental effects of CP.

Keywords: Cerebral palsy, hypomyelination, astrogliosis, lipopolysaccharide, maternal exercise

1. Introduction

Cerebral palsy (CP) is a neurological disorder which is leading cause of motor dysfunction in the developing fetal or infant brain. The motor dysfunction of CP is often

accompanied by persistent postural or movement dysfunctions, sensation disturbances, and cognitive impairment [1-4]. These severe disturbances by CP have been observed in approximately 10% of premature newborns [4] and it is postulated that the general prevalence of CP has slightly increased until recent years.

The causes of CP are multi-factorial with no single predominant etiology and main etiological factors are suggested to be periventricular leukomalacia (PVL), intrapartum asphyxia, cerebral dysgenesis, and intracranial hemorrhage [5]. Of them, PVL is closely associated with CP because most PVL is correlated with premature neonate brain damage [6]. Although the exact mechanisms underlying PVL have not been clearly understood, the primary causes of PVL involves cerebral ischemia and inflammation by maternal intrauterine or fetal systemic infection as well as asphyxia during delivery in premature infant brain [7-10] and it results in selective cerebral white matter injury such as microglial infiltration, astrogliosis, and hypomyelination in premature infant brain [11, 12].

White matter region of brain including the central nerve system (CNS) has abundant microglia, astroglia, and oligodendrocyte and these cells play crucial roles in responding to ischemia and inflammation [13, 14]. Reactive astrogliosis, typified by astrocyte proliferation, is increased in the CNS during neurodegenerative processes including PVL following brain injury and infection [15-17], which result in the increase of the astrocytespecific intermediate filament, glial fibrillary acidic protein (GFAP), expression [18]. Hypomyelination is also observed in the white matter impairment region of developing brain because of increase of damaged oligodendrocyte by PVL [6, 8, 11, 19]. Excessive microglia activation by PVL is associated with oligodendrocyte injury and myelin disturbance [13, 14]. Myelin sheath in brain is formed by oligodendrocyte and myelin basic protein (MBP) is the most abundant protein in the myelin sheath [20]. Thus, a change in the content of MBP is known to be an early sign of white matter abnormality like PVL by maternal infection [6]. In particular, maternal LPS administration during pregnancy could be used to develop an inflammatory model of CP in offspring by increasing the expression of GFAP and MBP [6, 21]. Based on the previous studies, the expression of GFAP and MBP in the white matter of premature infant brain is useful biomarker to confirm whether PVL including CP could be induced by maternal infection or not.

For now, there is no specific therapy for CP including PVL. Conventional therapies for CP are physical and mechanical treatment, pharmacologic and surgical treatment, and management for supportive and rehabilitative approaches [22]. Of them, it is well known that exercise induces several physiological and biochemical changes in the brain [23, 24]. In addition, physical exercise enhances neuronal plasticity and alters the transcription levels of various genes, in a manner associated with increased neuronal activity and synaptic remodeling [25]. Related to the pregnancy, regular physical exercise during pregnancy is known to be harmless to both the mother and the developing fetus [26]. Exercise during pregnancy promotes both muscle strength and endurance, alleviates excessive weight gain, mitigates backache, and ameliorates anxiety, depression, and other pregnancy-associated discomforts [27-29]. Moreover, exercise during pregnancy increases brain functions including learning and memory of rat pus under normal and abnormal maternal condition [30-32]. Recently, it has been reported that an adequate physical exercise among young women at reproductive age is especially important because it is one of the preconditions affecting their capability for pregnancy and delivery [33]. However, there is no study on the associations between regular maternal exercise before pregnancy and maternal infection-induced brain damage, especially PVL, in the premature infant brain. Thus, we investigated whether maternal exercise before pregnancy would have a protective effect on astrogliosis and hypomyelination in rat pups with PVL.

2. Materials and Methods

2.1. Animals and Treatments

Male Sprague-Dawley rats $(250 \pm 10 \text{ g} \text{ and } 10 \text{ weeks old}, n = 45)$ and female Sprague-Dawley rats $(180 \pm 10 \text{ g}, 8 \text{ weeks old}, n = 45)$ were used in this experiment. All experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences. Before pregnancy, female rats were randomly divided into three groups: the non-excontrol group, non-ex-LPS group, and the Pre-ex-LPS group (n = 15 in each group). The rats in the Pre-ex-LPS group were forced to run on a motorized treadmill for 30 min once a day for 4 weeks before pregnancy. The exercise load consisted of running at a speed of 2 m/min for the first 5 min, 5 m/min for the next 25 min, with a 0° inclination. After the completion of exercise program, all rats $(220 \pm 10 \text{ g}, 12 \text{ weeks old})$ were allowed to mate with male rats for a period of 24 h. Subsequently, the female rats were individually housed in plastic home cages for 2 weeks, under the controlled temperature $(20 \pm 2 \text{ °C})$ and a light–dark cycle consisting of 12 h of light and 12 h of darkness (lights on from 07:00 to 19:00 h). Food and water were made available *ad libitum*.

After conforming of pregnancies (n = 5 in each group), the maternal rats were treated with lipopolysaccharide (LPS) and/or the pyrogen-free saline (PFS). On the 15th, 17th, and 20th day of pregnancy, the pregnant rats in the LPS treated group received 1 ml intrauterine injections of 0.15 mg/kg LPS (from *Escherichia coli*, serotype 055:B5, Sigma Chemical Co., St. Louis, MO, USA) suspended in PFS, and the pregnant rats in the non-LPS treated group were injected with same amount of PFS.

After birth, 12 neonatal rats used in this experiment were randomly selected from each maternal rat. Each of them was in similar size and body weight. The average body weight of rat pups was 10.11 ± 1.65 g on the 2 days after birth. There was no statistically difference on body weight within groups. 10 rat pups were used for RT-PCR to determine Cyclooxygenasee-2 (COX-2) and Tumor necrosis factor- α (TNF- α) mRNA and immunohistochemistry to determine the glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) in neonatal rat brain.

2.2. RNA isolation and RT-PCR

In order to perform reverse transcription-polymerase chain reaction (RT-PCR), some part of corpus callosum the spanning from Bregma 2.28 mm to 0.00 mm were obtained from each brain and trimmed off. The samples were then minced and chopped using a pair of 0.1% diethyl pyrocarbonate (DEPC) water-treated tissue scissors on ice. After homogenization using RNAzolTMB (Tel-TEST, Friendswood, TX, USA), the RNA was isolated according to the manufacturer's instructions. The amount of RNA isolated was then quantified according to its absorbance at 260 nm (RNA/DNA calculator; Pharmacia, Uppsala, Sweden).

Single-strand cDNA was synthesized with a reaction mixture containing 2 μ g of RNA template, 1 μ l of random primer (Promega, Madison, WI, USA) and sterile H₂O to adjust the final volume to 10 μ l. The template was then denaturated at 65 °C for 10 min and maintained at room temperature for 5 min. One μ l of AMV reverse transcriptase (Promega), 5 μ l of 10 mM dNTP (Promega), 1 μ l of RNasin (Ribonuclease inhibitor; Promega), and 5 μ l of 10 x AMV RT buffer (Promega) were added to the mixture, and the final volume was adjusted to 40 μ l with DEPC water. The reaction mixture was then incubated for 2 h at 42 °C.

PCR amplification was performed in a reaction volume of 40 μ l containing 1 μ l of the appropriate cDNA, and 1 μ l of each set of primers at a concentration of 10 pM, 4 μ l of 10 x RT buffer, 1 μ l of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan).

The rat COX-2 primer sequences were 5'-CCAGATGCTATCTTTGGGGGAGAC-3' (a 23-mer sense oligonucleotide) and 5'-CTTGCATTGATGGTGGCTG-3' (a 19-mer antisense oligonucleotide). The rat TNF- α primer sequences were the primer sequences were 5'-TCATACCAGGGTTTGAGCTCAG-3' (a 22-mer sense oligonucleotide) and 5'-TCCCCAAA GGGATGAGAAGTT-3' (a 21-mer anti-sense oligonucleotide). GAPDH was used as the internal control. The GAPDH primer sequences were 5'-TGGTGCTGAGTATGTCGTCC-3' (a 20-mer sense oligonucleotide) and 5'-TTGTCATTGAGAGCAATGCC-3' (a 20-mer anti-sense oligonucleotide). The expected sizes of the PCR products were 583 bp for COX-2, 615 bp for TNF- α , and 650 bp for GAPDH.

The PCR procedure for COX-2 and TNF- α was conducted using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 40 amplification cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 56 °C for 45 sec, and extension at 72 °C for 45 sec, with an additional extension step at 72 °C for 5 min at the end of the procedure. The PCR procedure for GAPDH was conducted under identical conditions, except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using the Molecular AnalystTM version 1.4.1 software (Bio-Rad, Hercules, CA, USA). The expressions of COX-2 and TNF- α in each group were corrected according to GAPDH expression.

2.3. Tissue Preparation

For brain tissue preparation, the pups were sacrificed on the 2 days after birth. In brief, the animals were fully anesthetized using Zoletil $50^{\text{(B)}}$ (10 mg/kg, i.p.; Vibac Laboratories, Carros, France). The anesthetized rats were transcardially perfused with 50 mM phosphate-buffered saline (PBS), and then fixed with a freshly prepared solution consisting of 4% paraformaldehyde (PFA) in 100 mM phosphate buffer (PB) at pH 7.4. Brains were dissected, post-fixed in the same fixative overnight, and transferred to 30% sucrose solution for cryoprotection. Coronal sections of 40 µm thickness were made using a freezing microtome (Leica, Nussloch, Germany).

2.4. GFAP and MBP Immunohistochemistry

For immunolabeling of GFAP and MBP in the corpus callosum, GFAP and MBP immunohistochemistry was performed. An average of six sections within the corpus callosum spanning from Bregma 0.84 mm to 0.60 mm were obtained from each brain. Free-floating tissue sections were incubated overnight with rabbit anti-GFAP antibody or anti-MBP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:500. The sections were then incubated with biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA). The sections were subsequently incubated with avidin- biotin-peroxidase complex (1:100, Vector Laboratories) for 1 h at room temperature. Immunoreactivity was visualized by incubating the sections in a solution containing 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and 0.03% H_2O_2 in 50 mM Tris-HCl (pH 7.6) for approximately 5 min. The sections were then washed three times with PBS and mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature, and coverslips were mounted using Permount[®] (Fisher Scientific, New Jersey, NJ, USA).

2.5. Data Analysis

The area of corpus callosum from each slice was measured using Image-Pro[®] Plus computer-assisted image analysis system (Media Cyberbetics Inc., Silver Spring, MD) attached to a light microscope (Olympus, Tokyo, Japan). GFAP- and MBP-

immunoreactive optical density was measured in the corpus callosum and quantitatively assessed using a microdensitometrical method based on optical density (the mean gray scale) with the use of an image analyzer (Multiscan, Fullerton, CA, USA). Before starting the image analysis, the light source was adjusted to the brightness that generated the best possible contrast between immunopositive and immunonegative cells. To estimate GFAPand MBP-staining density, optical densities were corrected for the nonspecific background density. Statistical analysis was performed using SPSS 12.0 statistical software (SPSS Inc., Chicago, IL). The difference among the groups was determined by one-way analysis of variance (ANOVA) followed by Turkey HSD post-hoc test, and the results are expressed as the mean \pm standard error of the mean (S.E.M.). Significance was set as p < .05.

3. Results

3.1. Effect of Maternal Exercise before Pregnancy on the Expression of COX-2 and TNF-a in the Neonatal Rat Brain with the LPS-induced Maternal Intrauterine Infection

RT-PCR analysis of the mRNA levels of COX-2 (583 bp) and TNF- α (615 bp) in the neonatal rat brain was performed in order to provide the relative level of expressions of these genes (Fig. 1A). In the present study, the mRNA levels of COX-2 and TNF- α in the non-ex-control group were set as 1.00. The level of COX-2 mRNA was 1.48 ± 0.01 in the non-ex-LPS group, 1.25 ± 0.04 in the Pre-ex-LPS group (Fig. 1B). The level of TNF- α mRNA was 1.44 ± 0.01 in the non-ex-LPS group, 1.12 ± 0.17 in the Pre-ex-LPS group (Fig. 1C). These results showed that LPS significantly increased the COX-2 and TNF- α expression in neonatal rat brain and maternal exercise before pregnancy significantly reduced the LPS-induced increase of COX-2 and TNF- α expression in neonatal rat brain



Figure 1. Effect of Maternal Exercise before Pregnancy on the Expression of

Cyclooxygenase-2 (COX-2) and Tumor Necrosis Factor- α (TNF- α) in the Neonatal Rat Brain with the LPS-induced Maternal Intrauterine Infection. A: Results of Reverse Transcription-polymerase Chain Reaction (RT-PCR) Analysis of the mRNA Levels of COX-2 and TNF- α of Rat Pups on the 2 Days After Birth. GAPDH mRNA was used as the Internal Control. B: Relative mRNA Level of COX-2 Expression in the Corpus Callosum. C: Relative mRNA Level of TNF- α Expression in the Corpus Callosum. (Non-Ex-Con) no Exercise and No Lipopolysaccharide (LPS)-treated group, (Non-Ex-LPS) no Exercise and LPS-treated Group, (Pre-Ex-LPS) Pre-exercise and LPS-treated Group. Different Letters (a-b) Denote Statistically Significant Differences (p<.05) after Turkey HSD post-hoc. The Data are Presented as the Mean \pm Standard Error Mean (S.E.M)

3.2. Effect of Maternal Exercise before Pregnancy on GFAP Expression in the corpus Callosum with the LPS-induced Maternal Intrauterine Infection

Photomicrographs of GAFP expression in the corpus callosum are presented in Fig. 2A. The GFAP-positive fibers were 70.65 ± 0.94 in the non-ex-control group, 86.02 ± 1.00 in the non-ex-LPS group, and 72.31 ± 0.70 in the Pre-ex-LPS group (Fig. 2B). These results showed that LPS significantly increased GFAP expression in the corpus callosum and maternal exercise before pregnancy significantly reduced the LPS-induced increase of GFAP expression in the corpus callosum (p<.05).



Figure 2. Effect of Maternal Exercise before Pregnancy on Glial Fibrillary Acidic Protein (GFAP) Expression in the Corpus Callosum with the LPS-Induced Maternal Intrauterine Infection. A: Photomicrographs of GFAP-Positive Fiber in the Corpus Callosum of Neonatal Rat Brain on the 2 days After Birth. The Sections were Stained for GFAP (brown) and also showed White and Black Background Balance. The Scale Bar Represents 100 μm. B: Relative Optical Density of GFAP-positive Fibers in the Corpus Callosum among Groups. (Non-Ex-Con) No Exercise and No Lipopolysaccharide (LPS)-Treated Group, (Non-Ex-LPS) No Exercise and LPS-treated Group, (Pre-Ex-LPS) Pre-exercise and LPS-treated group. Different Letters (a-b) Denote Statistically Significant Differences (p<.05) after Turkey HSD posthoc. The Data are Presented as the mean ± standard Error Mean (S.E.M)

3.3. Effect of maternal exercise before pregnancy on MBP expression in the corpus callosum with the LPS-induced maternal intrauterine infection

Photomicrographs of MBP expression in the corpus callosum are presented in Figure 3A. The MBP-positive fibers were 91.45 ± 1.09 in the non-ex-control group, 84.62 ± 1.22 in the non-ex-LPS group, and 113.7 ± 2.64 in the Pre-ex-LPS group (Fig. 3B). These results showed that LPS significantly decreased MBP expression in the corpus callosum and maternal exercise before pregnancy significantly increased the LPS-induced decrease of MBP expression in the corpus callosum (p<.05).



Figure 3. Effect of Maternal Exercise before Pregnancy on Myelin Basic Protein (MBP) Expression in the Corpus Callosum with the LPS-induced Maternal Intrauterine Infection. A: Photomicrographs of MBP-positive fiber in the Corpus Callosum of Neonatal Rat Brain on the 2 Days after Birth. The Sections were stained for MBP (brown) and also showed White and Black Background Balance. The Scale Bar Represents 100 μm. B: Relative Optical Density of MBP-positive Fibers in the Corpus Callosum Among Groups. (Non-Ex-Con) No Exercise and No Lipopolysaccharide (LPS)-treated Group, (Non-Ex-LPS) No Exercise and LPS-treated Group, (Pre-Ex-LPS) Preexercise and LPS-treated Group. Different Letters (a-b) Denote Statistically Significant Differences (p<.05) after Turkey HSD post-hoc The Data are Presented as the Mean ± standard error mean (S.E.M)

4. Discussion

Prenatal brain damage which is strongly related PVL might be induced by immature babies, its results in life-long behavioral problems including movement, posture, and cognition. A lot of animal models have been established to study the mechanisms of PVL. One of the most popular animal models for PVL can be made by LPS administration during pregnancy because LPS exposure during pregnancy in maternal rat induces inflammation in the brain of rat pups [6, 21, 34]. LPS-induced inflammation may cause not only white matter injury, but also neuronal and axonal injury in the neonatal rat [35, 36]. In the present study, the animal model of PVL was induced by maternal intrauterine injection of LPS and we analyze the expression of pro-inflammatory factors such as COX-2 and TNF-a in the corpus callsom to confirm the LPS-induced maternal intrauterine infection in neonatal rat brain. In consistent with previous studies, our result showed that LPS significantly increased the COX-2 and TNF- α expression in neonatal rat brain. Thus, we confirm that PVL including CP is closely associated with early expression of pro-inflammatory factors by maternal infection in neonatal rat brain.

Another results showed that LPS-induced maternal inflammation significantly induced

astrogliosis and hypomyelination in the corpus callosum of the neonatal rat brain. It is well known that intrauterine inflammation induces astrogliosis by exposure of proinflammatory cytokines in the neonatal mouse brain [34, 37] as well as mental disability by impairing synaptic processes and hippocampal-dependent memories in developing rat brain [38-42]. Clinical studies also have reported that increased astrogliosis was observed in white matter lesion in extremely low birth weight infants [43]. Also, several studies suggested that hypomyelination is induced by maternal or fetal infection because of the increase of loss of oligodendrocytes and reduced both myelination and oligodendrocytes are concomitantly observed in the infant brains with PVL [44-46]. Together with previous studies, we confirm that LPS-induced maternal inflammation induced PVL which result from the astrogliosis and hypomyelination in neonatal rat brain following maternal LPS administration is an indication of potential role of cytokines in mediating maternal inflammation and the induction of PVL in newborn infants.

The main focus of this study is to investigate whether maternal exercise before pregnancy would have a protective effect on astrogliosis and hypomyelination in the corpus callosum of rats pups with PVL. We demonstrated that the maternal exercise before pregnancy significantly suppressed astrogliosis and alleviated hypomyelination by reducing LPS-induced inflammation in the corpus callosum of the rats with PVL. This is very interesting findings. However, in our knowledge, no study has examined the associations between maternal exercise before pregnancy and the symptom level of PVL in offspring to date. Although the precise mechanism on the effect of maternal exercise before pregnancy to the reduction of PVL symptoms in the offspring remains poorly understood, a few possible presumptions could be considered. Firstly, it is well established that physical exercise enhances brain function such as cognition and memory capability by increasing neurogenesis [47], preventing age-related decline in cell activity [48], and facilitating the recovery of brain damage [49-51]. Therefore, it is quite likely that physical exercise could be effective method for improvement of brain health. Secondly, several studies have reported that maternal exercise during pregnancy improves the cognitive function such as spatial learning in rat pups [30-32]. These studies provided the possibility that the effect of maternal exercise may be transmitted to the offspring after birth. Finally, a few studies showed that exercise exert anti-inflammatory effect, which result in enhancement of immune function [52, 53]. Handschin and Spiegelman (2008) suggested that exercise regulated the transcriptional coactivator PGC1alpha protein which suppresses a broad inflammatory response and mediates the beneficial effects of exercises [52]. Walsh et al., (2011) also reported that there is consensus that exercise enhances aspects of anti-tumor immunity and reduces inflammatory mediators [53].

Moreover, recent survey showed that an adequate level of physical activity among young women at reproductive age is especially important because physical activity is one of the preconditions affecting their capability for pregnancy and childbirth [33]. Together with these studies, our results showed the possibility that regular physical exercise at reproductive age might be effective method for reducing the risk of PVL by maternal infection in offspring.

5. Conclusion

Here, we have demonstrated the idea that maternal exercise before pregnancy reduces the risk of induction of PVL by maternal intrauterine inflammation in the infant rat brain, thereby results in preventing the motor dysfunction by CP in offspring. Therefore, the present results revealed that regular maternal exercise before pregnancy might be effective to ameliorate the symptoms of PVL such as astrogliosis and hypomyelination in neonatal rat brain and it can be used as a therapeutic strategy for preventing the detrimental effects of CP.

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