Objectives  The loss of maternal care during early postnatal period may increase development of mood-related disorders, such as depression, anxiety, and personality disorders. In this study, the effect of acupuncture on depression in relation with cell proliferation in the hippocampal gyrus dentatus was investigated using maternal-separated rat pups.

Methods  On the postnatal 14th day, rat pups from six dams were grouped into following groups: maternal care group, maternal separation group, maternal separation and non-acupoint-acupunctured group, maternal separation and Zusanli-acupunctured group, and maternal separation and fluoxetine-treated group. Acupuncture was performed from postnatal 28th day to postnatal 37th day. The rat pups that belong in the maternal separation and fluoxetine-treated group were injected subcutaneously with 5 mg/kg fluoxetine hydrochloride once a day for the same period of time. To evaluate activity of the rat pups, open field test was performed, immunohistochemistry for serotonin (5-hydroxytryptamine, 5-HT) and tryptophan hydroxylase (TPH) in the dorsal raphe and for 5-bromo-2'-deoxyuridine (BrdU) in the hippocampal gyrus dentatus was conducted.

Results  The present results reveal that the activity was decreased by maternal separation. In contrast, acupuncture at Zusanli overcame maternal separation-induced hypoactivity. Maternal separation suppressed TPH expression and 5-HT synthesis in the dorsal raphe and decreased cell proliferation in the hippocampal gyrus dentatus of rat pups. In contrast, acupuncture at Zusanli alleviated maternal separation-induced decrease of 5-HT synthesis and TPH expression.

Conclusions  The present results demonstrate that acupuncture at Zusanli ameliorated depressive state through increasing cell proliferation and enhancing 5-HT synthesis. (J Korean Med Rehabil 2019;29(2):91-99)

Key words  Maternal separation, Acupuncture, Depression, Cell proliferation, Serotonin, Zusanli

Introduction

Children that are subjected to neglect and physical or emotional abuse may cause changes in the central nervous system and are associated with the development of psychological alterations and neuropsychiatric...
disorders\textsuperscript{1}. Increased rate of depression, post traumatic stress disorder, and attention-deficit hyperactivity disorder in adulthood are caused by maltreatment in the childhood\textsuperscript{2}. Abnormal mother-infant interaction is a key factor, in both the mother and their offspring, increasing weakness to psychological stress\textsuperscript{3}. Maternal separation and social isolation in young animals induce alteration of neurotransmitters in their brains. In particular, the dopaminergic and serotonergic systems are suppressed by maternal separation and social isolation. Therefore, maternal separation has been applied as the animal model of early life stress and depression\textsuperscript{4}.

Depression is associated with dysfunction of the serotonergic neurotransmitter system, such as serotonin (5-hydroxytryptamine, 5-HT), which are involved in the regulation of mood\textsuperscript{5}. 5-HT plays an important role in depressive disorders, Tryptophan hydroxylase (TPH), is an enzyme that limits rate in the biosynthesis of 5-HT\textsuperscript{6}.

Depression is also a common medical condition associated with neurochemical changes in the hippocampus\textsuperscript{7}. Hippocampus is one of the important brain regions implicated in the symptoms of depression\textsuperscript{8}. Reduction in the hippocampal volume is observed in the depressive patients\textsuperscript{9}, Hippocampal neurogenesis is decreased by stress and increased by antidepressants\textsuperscript{10}. Serotonin, N-methyl-D-aspartate receptor antagonists, and physical exercise\textsuperscript{11} facilitate new formation in the hippocampal dentate gyrus. Clinically, depression is treated with antidepressants, however, not all patients respond to the medication, and sometimes antidepressants cause unwanted side effects\textsuperscript{12}.

Acupuncture has long been practiced for treating various disease conditions, Acupuncture has therapeutic effects on depression, anxiety, and substance abuse\textsuperscript{13}. Some acupoints are closely related to brain function. In particular, Zusanli acupoint (ST36) is major acupoint that improves memory function and facilitates cell proliferation in the hippocampal gyrus dentatus\textsuperscript{14}. Acupuncture enhanced hippocampal neuronal cell proliferation following stress\textsuperscript{15}. In the ischemic model and in the streptozotocin-induced diabetic model, acupuncture at Zusanli acupoint also raised cell proliferation in the hippocampal gyrus dentatus\textsuperscript{14}.

Although many studies have attempted to explain the effects of acupuncture, the effect of acupuncture on maternal separation-induced depression is not proved. In this study, the effect of acupuncture on depression in relation with cell proliferation in the hippocampal gyrus dentatus was investigated using maternal-separated rat pups.

Materials and Methods

1. Treatments and animals

Female and male Sprague–Dawley rat pups were chosen to be used in this experiment. The experimental procedures were performed according to the animal care guidelines of the Korean Academy of Medical Sciences, and the National Institutes of Health. The rat pups were maintained under controlled temperature environment of 20±2°C and the lighting (07:00–19:00 hours) conditions. Water and food were made accessible ad libitum. The delivery day was designated postnatal 0 day. On the postnatal 14th day, the rat pups from six dams were grouped into one of the five groups: the maternal care group, the maternal separation group, the maternal separation and non-acupoint-acupunctured group, the maternal separation and Zusanli-acupunctured group, and the maternal separation and fluoxetine-treated group (n=8 in each group). The rat pups that belong in the maternal separation groups were kept individually while the rat pups that belong in the maternal care group were kept with their respective mothers under standard conditions. The maternal separation procedures began on the postnatal 14th day. 50 mg/kg 5-bromo-2'-deoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO, USA) were injected subcutaneously into all the rat pups, once a day at 1 hour prior to starting acupuncture from post-
natal 28th day to postnatal 37th day.

0.3 mm diameter of acupuncture needles were used for acupuncture stimulation. For the rat pups that belong in the maternal separation and non-acupoint-acupunctured group, were lightly immobilized using hands to minimize stress and the acupuncture needles were inserted 3 mm in depth at both side of hip. The needles were twisted at the speed of twice a second for 30 sec and removed immediately afterwards. For the rat pups that belong in the maternal separation and Zusanli-acupunctured group, the same manipulation was applied to Zusanli acupoint, near the knee joint of hind limb 2 mm lateral to the anterior tubercle of the tibia. The rat pups that belong in the maternal care group and in the maternal separation group were also lightly immobilized with the same method for 30 sec, and then returned to their cages. Acupuncture was performed from postnatal 28th day to postnatal 37th day. The rat pups that belong in the maternal separation and fluoxetine-treated group were injected subcutaneously with 5 mg/kg fluoxetine hydrochloride (Tocris, Bristol, UK), once a day for the same period of time.

3. Tissue preparation

To begin the sacrificial process, Zoletil 50® (10 mg/kg i.p.; Vibac Laboratories, Carros, France) was used to anesthetized animals fully. After observing a complete lack of response, 50 mM phosphate-buffered saline (PBS) was used to perfuse the rats pups transcardially, and then, it was fixed with freshly prepared 500 mM phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde. Rat pups' brains were removed and placed in the same fixative overnight, after that, it was transferred into a 30% sucrose solution for cryoprotection. A freezing microtome (Leica, Nussloch, Germany) was used to obtain serial coronal section of 40 μm thickness.

4. Immunofluorescence for 5-HT synthesis and TPH expression

Immunofluorescence was carried out to evaluate the TPH-positive cells and the 5-HT-positive in the dorsal raphe as previously described method. An average of 10 sections within the dorsal raphe region spanning from bregma -7.30 mm to -8.00 mm was obtained from each brain. To start the procedure, the sections were incubated in PBS for 10 minutes and it was washed 3 times in the same buffer. After that, Free-floating sections were incubated in 3% hydrogen peroxide for 30 minutes. Next, blocking solution (1% bovine serum albumin and 10% goat serum for 5-HT or horse serum for TPH in 0.05 M PBS) was used to incubate the sections overnight. The sections were next incubated for 90 minutes with FITC anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and CY3 anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA). Gelatin-coated glass slides were...
used to mount the sections, and fluorescent mounting medium (DakoCytomation, Carpinteria, CA, USA) was used to mount the coverslips. Confocal laser-scanning microscopy with LSM 510 META (Carl Zeiss, Oberkochen, Germany) was used to capture the slides of the fluorescent images.

5. Immunohistochemistry for BrdU

BrdU immunohistochemistry was performed according to a previously described method\(^{14}\) to detect newly generated cells in the gyrus dentatus. First, the brain sections were permeabilized by incubation in 0.5% Triton X-100 in PBS for 20 minutes, then pretreated in 50% formamide-2×standard saline citrate (SSC) at 65°C for 2 hours, denaturated in 2 N Hydrogen chloride at 37°C for 30 minutes, after that, it was washed two times in 100 mM sodium borate (pH 8.5). Thereafter, incubation of the sections was processed overnight at 4°C with mouse monoclonal anti-BrdU antibody (1:600; Roche, Mannheim, Germany). Then, the sections were rinsed 3 times with PBS and incubated for 90 minutes with the biotinylated mouse secondary antibody (1:200; Vector Laboratories). Then, incubation of the sections were processed with avidin-peroxidase complex (1:100; Vector Laboratories). For visualization, incubation of the sections were processed in 50 mM Tris-HCl (pH 7.6) containing 0.02% diaminobenzidine (DAB), 40 ng/mL NiCl\(_2\) and 0.03% H\(_2\)O\(_2\) for 5 minutes. After BrdU-specific staining, a mouse monoclonal anti-neuronal nucleic antibody (1:300; Chemicon International, Temecula, CA, USA) was used to perform counter-staining on the same section. The sections were rinsed 3 times with PBS and incubate for 1 hr with a biotinylated anti-mouse secondary antibody. For staining, incubation of the section was processed in a reaction mixture containing 0.02% DAB and 0.03% H\(_2\)O\(_2\) for 5 minutes. Finally, the sections were mounted onto gelatin-coated slides. The slides were air dried overnight at room, and coverslips were mounted with Permount\(^{16}\) (Olympus, Tokyo, Japan). Under a light microscope, the numbers of BrdU-positive cells in the gyrus dentatus was counted hemilaterally, and they were expressed as the number of cells per square mm in the gyrus dentatus. The area of the gyrus dentatus was measured with the Image-Pro\(^{17}\) Plus image analysis system (Media Cyberbetics Inc., Silver Spring, MD, USA).

6. Data analysis

SPSS by the one-way analysis of variance (ANOVA) was used to evaluate difference among the groups, followed by Duncan’s post-hoc test. The mean±standard error of the mean was used to express all the values. Statistically significant differences were established at p<0.05.

Results

1. Effects of acupuncture on activity in the open fields test

Figure 1 shows the activity score of the open field. The activity score was 71.42±11.05 in the maternal care group, 32.42±5.71 in the maternal separation group, 32.57±7.90 in the maternal separation and non-acupoint-acupuncture treated group, 70.75±10.60 in the maternal separation and Zusanli-acupuncture treated group and 57.75±4.92 in the maternal separation and fluoxetine-treated group. The results present that the activity was decreased by maternal separation. In contrast, acupuncture at Zusanli overcome maternal separation-induced hypoactivity. Acupuncture at the non-acupoint exerted no significant effect on activity in the maternal separated rat pups.
Effect of Acupuncture on Depression and Cell Proliferation in Hippocampal Gyrus Dentatus of Maternal-separated Rat Pups

2. Effects of acupuncture on the number of 5-HT-positive cells in the dorsal raphe

Photomicrographs of 5-HT-positive cells in the dorsal raphe are illustrated in Figure 2. The number of 5-HT-positive cells in the dorsal raphe was 582.25±34.40/mm² in the maternal care group, 349.09±13.70/section in the maternal separation group, 394.09±13.70/mm² in the maternal separation and non-acupoint-acupunctured group, 632.37±22.62/mm² in the maternal separation and Zusanli-acupunctured group and 560.92±29.10/mm² in the maternal separation and fluoxetine-treated group. The results present that 5-HT synthesis in the dorsal raphe was decreased by maternal separation. In contrast, acupuncture at Zusanli alleviated maternal separation-induced decrease of 5-HT synthesis. Acupuncture at the non-acupoint exerted no significant effect on 5-HT synthesis in the maternal separated rat pups.

3. Effects of acupuncture on the number of TPH-positive cells in the dorsal raphe

Photomicrographs of TPH-positive cells in the dorsal raphe are illustrated in Figure 3. The number of TPH-positive cells in the dorsal raphe was 592.98±50.12/mm² in the maternal care group, 471.05±16.96/mm² in the maternal separation group, 420.09±22.97/mm² in the maternal separation and non-acupoint-acupunctured group, 617.10±25.71/mm² in the maternal separation and Zusanli-acupunctured group and 558.24±28.40/mm² in the maternal separation and fluoxetine-treated group. The results present that TPH expression in the dorsal raphe was decreased by maternal separation. In contrast, acupuncture at Zusanli alleviated maternal separation-induced decrease of TPH expression in the dorsal raphe. Acupuncture at the non-acupoint exerted no significant effect on TPH expression in the maternal separated rat pups.
4. Effects of acupuncture on the number of BrdU-positive cells in the hippocampal gyrus dentatus

Photomicrographs of BrdU-positive cells in the hippocampal gyrus dentatus are illustrated in Figure 4. The number of BrdU-positive cells in the hippocampal gyrus dentatus was 142.07±13.87/mm² in the maternal care group, 72.26±7.34/mm² in the maternal separation group, 60.53±5.69/mm² in the maternal separation and non-acupoint-acupunctured group, 136.79±5.81/mm² in the maternal separation and Zusanli-acupunctured group and 101.15±4.56/mm² in the maternal separation and fluoxetine-treated group.

The results present that cell proliferation in the hippocampal gyrus dentatus was decreased by maternal separation. In contrast, acupuncture at Zusanli alleviated maternal separation-induced decrease of cell proliferation. Acupuncture at the non-acupoint exerted no significant effect on cell proliferation in the maternal separated rat pups.

Discussion

The lack of a mother-infant relationship is known to exert an influence on neonatal development and to increase the vulnerability of the offspring to certain neuropsychiatric disorders. Maternal deprivation causes cell
death in the infant rat brain. In this study, the rat pups were separated from their mothers from postnatal 14th day. In order to assess the influence of maternal separation on the activity of rat pups, open field test was performed. In this study, maternal separated rat pups showed a decreased activity on the open field test. However, acupuncture at Zusanli for 10 days overcame maternal separation-induced hypoactivity. Maternal separation caused decrease of locomotion activity. Depression is primarily characterized by a lowering of mood and an inhibition on both mental and physical activity. Many studies suggested that acupuncture improves depression-like behavior. In this study, anti-depression effect of acupuncture was verified by the findings that acupuncture at Zusanli significantly increased locomotor activity in the maternal separation rat pups.

Maternal separation during infancy may influence serotonergic neuronal growth and development. Serotonergic system in the brain is influenced by various stressful stimuli and reduced activity in the brain serotonergic system is implicated in the pathophysiology of depression. In order to confirm whether maternal separation induced depression in this study, we measured 5-HT and TPH expression in the dorsal raphe. In this study, 5-HT level in the dorsal raphe of rat pups was suppressed by maternal separation, but acupuncture at Zusanli increased the synthesis of 5-HT. This tendency was supported by Yang et al. showing that 5-HT synthesis in the dorsal raphe was lower in the depression model than that in the normal rats. Reduction in TPH expression leads to a rapid decrease in 5-HT release, indicating that changes TPH activity can influence 5-HT synaptic activity. In this study, TPH expression in the dorsal raphe of rat pups was decreased by maternal separation, and acupuncture at Zusanli also increased TPH expression. Acupuncture is known to activate descending serotonergic systems originating in the brainstem and acupuncture at Zusanli was effective in the treatment of stress-related physical and mental disorders. The present results suggest that acupuncture at Zusanli restored serotonin content in the maternal-separated rat pups.

5-HT is also known to modulate neurogenesis in rat brains. Inhibition of 5-HT synthesis significantly decreased the number of newly generated cells in the subventricular zone and the subgranular zone. Therefore, the acupuncture-induced increase in 5-HT synthesis may contribute to increased cell proliferation, in this study. The present results demonstrate that maternal separation induced depressive state through decreasing cell proliferation in the hippocampal gyrus dentatus and suppressing 5-HT synthesis in the dorsal raphe of rat pups. However, since this study only observed the effects of acupuncture at Zusanli on cell proliferation in the hippocampal gyrus dentatus associated with mechanism of depression, continuous research will be needed to further investigate the effects of other type of depression and the developmental mechanism of depression.

Conclusion

We investigated the effect of acupuncture on depres-
Acupuncture at Zusanli acupoint shows that
1. Acupuncture at Zusanli overcame maternal separation-induced hypoactivity.
2. Acupuncture at Zusanli alleviated maternal separation-induced decrease of 5-HT synthesis.
3. Acupuncture at Zusanli alleviated maternal separation-induced decrease of TPH expression in the dorsal raphe.
4. Acupuncture at Zusanli alleviated maternal separation-induced decrease of cell proliferation.

Acupuncture at Zusanli ameliorated depressive state through increasing cell proliferation and enhancing 5-HT synthesis. The present results show that acupuncture might be used as the therapeutic strategy for depression patients.

References