

Original Article

Effects of Prenatal *Cnidium officinale* Makino Treatment on Spatial Memory and Neurogenesis in the Hippocampus of Rat Pups Born from Maternal Rats Exposed to Noise Stress during Pregnancy

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During the prenatal period, the development of the individual is influenced by a host of environmental factors. Exposure to noise stress during pregnancy was determined to result in the retardation of growth, a reduction in neurogenesis, and an impairment of spatial learning ability in the rat pups. In the present study, we have attempted to characterize the effects of prenatal treatment with *Cnidium officinale* Makino on spatial memory and neurogenesis in the hippocampus of rat pups born from maternal rats exposed to noise stress during pregnancy. Prenatal treatment with *Cnidium officinale* Makino was shown to increase neurogenesis and enhanced spatial learning ability in rat pups born from maternal rats exposed to noise stress. In this study, we have determined that prenatal treatment with *Cnidium officinale* Makino can stimulate spatial development and neurogenesis in the brain of the fetuses exposed to prenatal stresses.

Key Words : Noise, Neurogenesis, Hippocampus

Introduction

It has been well documented in a host of animal studies that the development of an individual is beset by a complex set of environmental influences during the prenatal period. Prenatal stresses, in particular, are known to significantly influence brain development and behavior in the affected fetuses¹⁾. Considerable experimental and clinical evidence has been collected, and appear to indicate that prenatal stresses occurring during pregnancy may induce

the retardation of growth as well as impairments in the proliferation and differentiation of neurons²⁾, resulting in deficits in cognitive function as well as the neurological and behavioral abnormalities of offspring³⁾. A host of neurological abnormalities and cognitive impairments appear to be related to the formation of hippocampus⁴⁾.

The hippocampus, which begins to form in the gestation stage and continues to be formed until the postnatal period, is a brain region which is known to be vitally involved in both learning ability and memory capacity²⁾. Hippocampal neurogenesis appears to be influenced by several environmental factors and stimuli^{5,8)}. It has been well documented that prenatal stresses, including noise and restraint during pregnancy, suppress the formation of hippocampal granule cells in many different mammalian species^{9,10)}.

Cnidium officinale Makino is believed to exert

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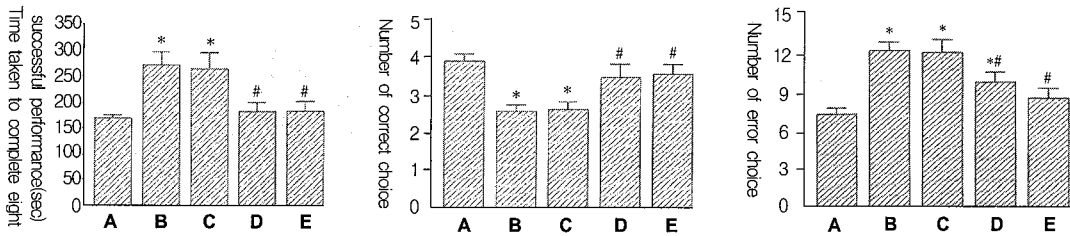


Fig. 1. The influence of prenatal noise-stress and *Cnidium officinale* Makino treatment on spatial memory of rat pups

The data are presented as the mean±SEM.

* represents P<0.05 compared to the control group.

represents P<0.05 compared to the noise-stress applied group.

A : control group

B : noise-stress applied group

C : noise-stress applied with 50 mg/kg *Cnidium officinale* Makino treated group

D : noise-stress applied with 100 mg/kg *Cnidium officinale* Makino treated group

E : noise-stress applied with 200 mg/kg *Cnidium officinale* Makino treated group (n=15 in each group)

Upper : The time required to complete eight successful performances.

Middle : The number of correct choice made prior to the occurrence of the first error.

Lower : The number of error made prior to eight successful performances.

a regulatory effect on blood circulation, and is also known to possess anti-inflammatory properties¹¹. This natural product is one of the primary herbs used by Oriental medical practitioners to treat pain, inflammation, and menstrual disturbance and to reduce blood congestion¹². Recently, it has been demonstrated that *Cnidium officinale* Makino can prevent or decrease ischemic cerebral damage¹³, and an herbal formulation containing *Cnidium officinale* Makino has been shown to significantly reverse the reference and working memory impairment associated with exposure to scopolamine¹⁴.

In this study, the effects of *Cnidium officinale* Makino on spatial memory and neurogenesis in the hippocampus of rat pups born from maternal rats exposed to noise stress during pregnancy were investigated.

Materials and Methods

1. Animals and Treatments

The experimental procedures were performed

in accordance with the guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences. Male Sprague-Dawley rats (250±10g, 12 weeks old) and female Sprague-Dawley rats (180±10g, 8 weeks old) were used in this study. The female rats (n=30) were allowed to mate with male rats (n=30) during a 24 h period. After mating, the female rats were individually housed in plastic home cages at a controlled temperature (20±2°C) and a light/dark cycle of 12 h of light and 12 h of darkness (lights on 07:00 am-off at 07:00 pm). Food and water were made available ad libitum. After pregnancy had been confirmed 14 days after mating, the female rats (n=20, pregnancy rate=67%) were divided into five groups: the control group, the noise stress applied group, the noise stress applied with 50 mg/kg *Cnidium officinale* Makino treated group, the noise stress applied with 100 mg/kg *Cnidium officinale* Makino treated group and the noise stress applied with 200 mg/kg *Cnidium officinale* Makino treated group (n=4 in each group).

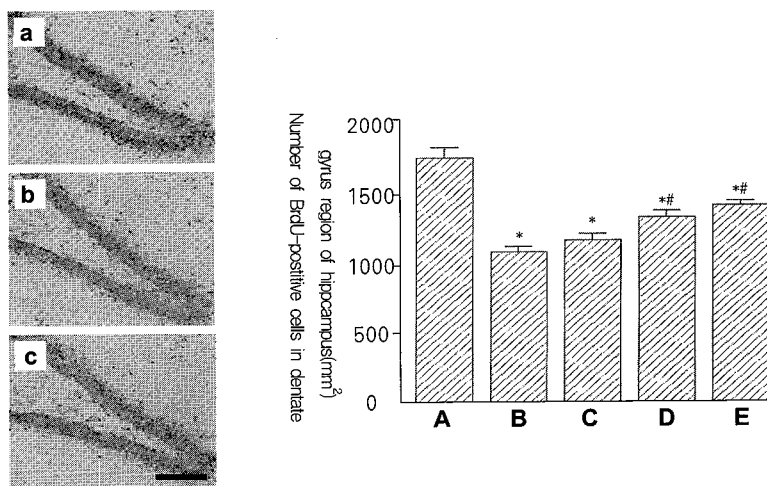


Fig. 2. The influence of prenatal noise-stress and *Cnidium officinale* Makino treatment on the neurogenesis in the hippocampal dentate gyrus of rat pups

Upper : Photomicrographs of 5-bromo-2'-deoxyuridine (BrdU)-positive cells in the dentate gyrus of hippocampus. The sections were stained for BrdU (black) and neuronal nuclei (NeuN; brown). The scale bar represents 500 μ m.

a : control group b : noise-stress applied group
 c : noise-stress applied with 200 mg/kg *Cnidium officinale* Makino treated group

Lower : The mean number of BrdU-positive cells in the dentate gyrus of the hippocampus. The data are presented as the mean \pm SEM.

* represents $P < 0.05$ compared to the control group.

represents $P < 0.05$ compared to the noise-stress applied group.

A : control group B : noise-stress applied group

C : noise-stress applied with 50 mg/kg *Cnidium officinale* Makino treated group

D : noise-stress applied with 100 mg/kg *Cnidium officinale* Makino treated group

E : noise-stress applied with 200 mg/kg *Cnidium officinale* Makino treated group (n=15 in each group)

Between the 15th day of pregnancy until delivery, all of the rats received subcutaneous injections containing 100 mg/kg of 5bromo2'deoxyuridine (BrdU: Sigma Chemical Co., St. Louis, MO, USA) once per day, 30 min before the beginning of the experiments. The rats in the noise stress applied groups were exposed to 95 decibels of sound generated by a supersonic sound machine for 1 h once a day, for 1 week, from the 15th day of pregnancy until delivery. The rats in the control group were not exposed to noise. After birth, the offspring were left with their mothers and remained undisturbed. The spatial learning capacities of the rat pups were then tested 21

days after birth (n of rat pups=15 in each group). The rat pups were immediately sacrificed upon the completion of the spatial learning test.

The *Cnidium officinale* Makino used in this experiment was obtained from the Oriental Medical Hospital, Kyung Won University (Seoul, Korea). After washing, *Cnidium officinale* Makino was immersed in cold water for 12 h. In order to obtain the aqueous extract of *Cnidium officinale* Makino, 200g of *Cnidium officinale* Makino was added to distilled water, heat extracted at 80 $^{\circ}$ C, concentrated using a rotary evaporator and then lyophilized. The resulting powder, weighing 25g, was then diluted with saline. The animals in

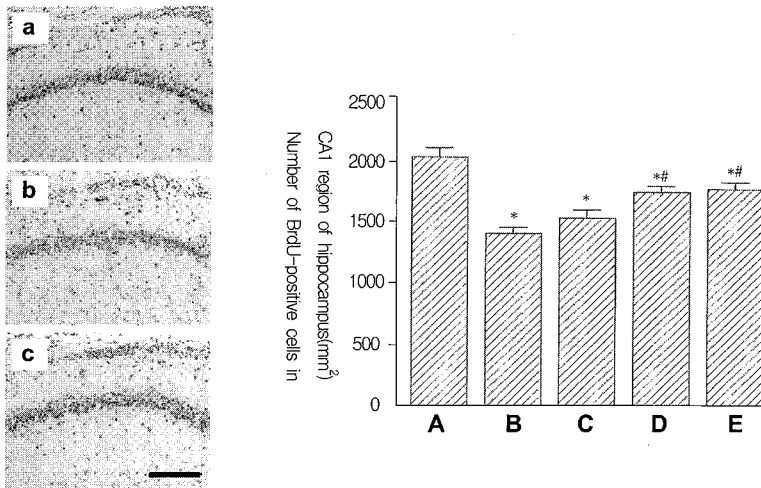


Fig. 3. The influence of prenatal noise-stress and *Cnidium officinale* Makino treatment on the neurogenesis in the hippocampal CA1 region of rat pups

Upper : Photomicrographs of 5-bromo-2'-deoxyuridine (BrdU)-positive cells in the CA1 region of the hippocampus. The sections were stained for BrdU (black) and neuronal nuclei (NeuN; brown). The scale bar represents 500 μ m.

a : control group b : noise-stress applied group
c : noise-stress applied with 200 mg/kg *Cnidium officinale* Makino treated group

Lower : The mean number of BrdU-positive cells in the CA1 region of the hippocampus. The data are presented as the mean \pm SEM.

* represents P<0.05 compared to the control group.
represents P<0.05 compared to the noise-stress applied group.

A : control group B : noise-stress applied group
C : noise-stress applied with 50 mg/kg *Cnidium officinale* Makino treated group
D : noise-stress applied with 100 mg/kg *Cnidium officinale* Makino treated group
E : noise-stress applied with 200 mg/kg *Cnidium officinale* Makino treated group (n=15 in each group)

the *Cnidium officinale* Makino treated groups were treated with subcutaneous injections of *Cnidium officinale* Makino extract at the appropriate respective doses. Those animals in the control group received an equivalent amount of saline subcutaneously, once a day for the same duration.

2. Radial Arm Maze Test

The spatial learning ability of the rat pups was assessed using a radial arm maze apparatus as Olton's method¹⁵⁾. The radial arm maze apparatus consisted of a central octagonal plate (30cm in diameter) and eight radiating arms (50cm in length and 10cm in width). The apparatus was

placed 1 meter above the floor. A small receptacle filled with water (3cm in diameter and 1cm in depth) was located at the end of the arms. The rat pups were trained on the maze for three sessions prior to the spatial learning test. The rat pups, deprived of water for 24 h, were permitted to search for water and to drink for 10 min. At 21 days after birth, we conducted the spatial learning ability test. The time spent seeking for water at the end of the arms was recorded. The test was terminated after the rat found water in all eight arms or over 10 min elapsed. Reentering to a previously visited arm was scored as an error and we also counted the number of correct choices

made before the first error.

3. Tissue Preparation

For brain tissue preparation, the rat pups were fully anesthetized with Zoletil 50 □ (10 mg/kg, i.p.; Vibac Laboratories, Carros, France), transcardially perfused with 50mM phosphate buffered saline (PBS), and fixed with a freshly prepared solution consisting of 4% paraformaldehyde (PFA) in 100mM phosphate buffer (PB, pH 7.4). The brains were then removed, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Coronal sections of 40 μm thickness were made using a freezing microtome (Leica, Nussloch, Germany).

4. BrdU Immunohistochemistry

For the detection of newly generated cells in the dentate gyrus, BrdU incorporation, which is commonly used as an indicator of DNA synthesis, was assessed via immunohistochemistry as per Takamiya's method¹⁶. In brief, the sections were permeabilized by incubating with 0.5% Triton X100 in PBS for 20 min, treated with 50% formamide 2 x standard saline citrate (SSC) at 6 5°C for 2 h, denaturated in 2 N HCl at 37°C for 30 min, and rinsed twice in 100mM sodium borate (pH 8.5). Subsequently, the sections were incubated overnight at 4°C with BrdU-specific mouse monoclonal antibody (1:600; Roche, Mannheim, Germany). The sections were then washed three times with PBS and incubated for 1 h with a biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Then, the sections were incubated for another 1 h with avidin peroxidase complex (1:100; Vector Laboratories). For the visualization of BrdU, the sections were incubated with 50mM TrisHCl (pH 7.6) containing 0.02% 3,3'-diaminobenzidine containing nickel chloride (40 mg/ml) (nickelDAB)

and 0.03% hydrogen peroxide for 5 min.

After the BrdU-specific staining, counterstaining was performed on the same sections using a mouse antineuronal nuclei (NeuN) antibody (1:300; Chemicon International, Temecula, CA, USA). The sections were washed three times with PBS, incubated for 1 h with a biotinylated antimouse secondary antibody, and processed with VECTASTAIN □ ABC Kit (1:100; Vector Laboratorie). For staining, the sections were reacted with 0.02% DAB and 0.03% hydrogen peroxide in 50mM TrisHCl (pH 7.6) for 5 min and the sections were finally mounted onto gelatin-coated slides. The slides were air dried overnight at room temperature, and coverslips were mounted using Permount □ (Fisher Scientific Int.).

5. Data Analysis

The area in the selected region of the hippocampus was measured using ImagePro Plus software (Media Cybernetics, Silver Spring, MD, USA). The number of BrdU-positive cells in the selected regions of the hippocampus was counted hemilaterally. The data were expressed as the number of cells per mm² in the regions of the hippocampus. Statistical analysis was performed using one-way ANOVA followed by Duncan posthoc test. The results are presented as the mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

1. Body Weight of Rat Pups

Two weeks after birth, the body weight in the control group was 26.41±0.63g, the noise stress applied group 21.32±0.45g, the noise stress applied with 50 mg/kg *Cnidium officinale* Makino treated group 22.97±0.34g, the noise stress applied with 100 mg/kg *Cnidium officinale* Makino treated group 25.98±0.85g, and the noise stress applied

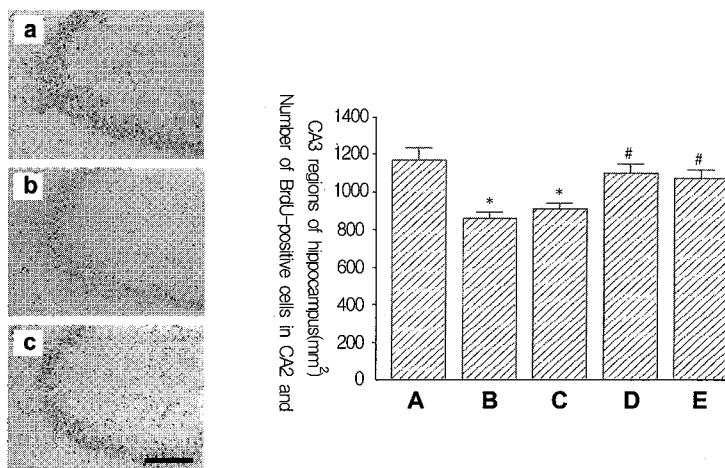


Fig. 4. The influence of prenatal noise-stress and *Cnidium officinale* Makino treatment on the neurogenesis in the hippocampal CA2 and CA3 regions of rat pups

Upper : Photomicrographs of 5-bromo-2'-deoxyuridine (BrdU)-positive cells in the CA2 and CA3 regions of the hippocampus. The sections were stained for BrdU (black) and neuronal nuclei (NeuN; brown). The scale bar represents 500 μ m.

a : control group b : noise-stress applied group

c : noise-stress applied with 200 mg/kg *Cnidium officinale* Makino treated group

Lower : The mean number of BrdU-positive cells in the CA2 and CA3 regions of the hippocampus. The data are presented as the mean \pm SEM.

* represents $P < 0.05$ compared to the control group.

represents $P < 0.05$ compared to the noise-stress applied group.

A : control group B : noise-stress applied group

C : noise-stress applied with 50 mg/kg *Cnidium officinale* Makino treated group

D : noise-stress applied with 100 mg/kg *Cnidium officinale* Makino treated group

E : noise-stress applied with 200 mg/kg *Cnidium officinale* Makino treated group (n=15 in each group)

with 200 mg/kg *Cnidium officinale* Makino treated group 26.32 \pm 0.12g (Table I).

Table 1. Body Weight of Rat Pups

Group	Body weight of rat pups (g)
A	26.41 \pm 0.63
B	21.32 \pm 0.45
C	22.97 \pm 0.34
D	25.98 \pm 0.85
E	26.32 \pm 0.12

A : control group

B : noise-stress applied group

C : noise-stress applied with 50 mg/kg *Cnidium officinale* Makino treated group

D : noise-stress applied with 100 mg/kg *Cnidium officinale* Makino treated group

E : noise-stress applied with 200 mg/kg *Cnidium officinale* Makino treated group (n=15 in each group)

We detected a significant retardation of body weight gain in the noise stress applied group. By way of contrast, treatment with *Cnidium officinale* Makino resulted in a restoration of body weight near to the control group.

2. Influence of Prenatal *Cnidium officinale* Makino Treatment on Spatial Memory of Rat Pups

The rats in the control group completed eight successful performances in 170.50 \pm 5.72 sec, the noise stress applied group in 271.33 \pm 24.48 sec, the noise stress applied with 50 mg/kg *Cnidium officinale* Makino treated group in 263.57 \pm 31.35 sec, the noise stress applied with 100 mg/kg *Cnidium officinale* Makino treated group in

183.50±16.73 sec, and the noise stress applied with 200 mg/kg *Cnidium officinale* Makino treated group completed in 184.66±17.81 sec.

The number of correct choices before the first error was 4.00±0.18 in the control group, 2.66±0.15 in the noise stress applied group, 2.71±0.19 in the noise stress applied with 50 mg/kg *Cnidium officinale* Makino treated group, 3.58±0.35 in the noise stress applied with 100 mg/kg *Cnidium officinale* Makino treated group, and 3.66±0.28 in the noise stress applied with 200 mg/kg *Cnidium officinale* Makino treated group.

The number of errors made before eight successful performances was 7.50±0.40 in the control group, 12.50±0.68 in the noise stress applied group, 12.35±1.05 in the noise stress applied with 50 mg/kg *Cnidium officinale* Makino treated group, 10.00±0.79 in the noise stress applied with 100 mg/kg *Cnidium officinale* Makino treated group, and 8.75±0.77 in the noise stress applied with 200 mg/kg *Cnidium officinale* Makino treated group (Fig. 1).

Our data show that the rat pups born from the maternal rats exposed to noise stress during pregnancy were associated with a lower number of correct choices and a higher number of errors compared to the control rat pups. By way of contrast, *Cnidium officinale* Makino treatment was determined to have shortened the time required for the rat pups to find the water at the end of all eight of the radial arms.

3. Influence of Prenatal *Cnidium officinale* Makino Treatment on Hippocampal Neurogenesis of Rat Pups

Fig. 2 shows BrdU-positive cells in the hippocampal dentate gyrus. The number of BrdU-positive cells in the hippocampal dentate gyrus was 1779.50±68.43/mm² in the control group,

1102.50±35.30/mm² in the noise stress applied group, 1190.62±42.76/mm² in the noise stress applied with 50 mg/kg *Cnidium officinale* Makino treated group, 1361.00±41.46/mm² in the noise stress applied with 100 mg/kg *Cnidium officinale* Makino treated group, and 1442.73±33.01/mm² in the noise stress applied with 200 mg/kg *Cnidium officinale* Makino treated group.

Fig. 3 shows BrdU-positive cells in the hippocampal CA1 region. The number of BrdU-positive cells in the hippocampal CA1 region was 2059.73±73.44/mm² in the control group, 1428.06±41.77/mm² in the noise stress applied group, 1549.66±62.93/mm² in the noise stress applied with 50 mg/kg *Cnidium officinale* Makino treated group, 1767.88±45.83/mm² in the noise stress applied with 100 mg/kg *Cnidium officinale* Makino treated group, and 1794.20±54.78/mm² in the noise stress applied with 200 mg/kg *Cnidium officinale* Makino treated group.

Fig. 4 shows BrdU-positive cells in the hippocampal CA2 and CA3 regions. The number of BrdU-positive cells in the hippocampal CA2 and CA3 regions was 1172.00±61.71/mm² in the control group, 864.75±31.20/mm² in the noise stress applied group, 915.80±23.98/mm² in the noise stress applied with 50 mg/kg *Cnidium officinale* Makino treated group, 1104.77±43.49/mm² in the noise stress applied with 100 mg/kg *Cnidium officinale* Makino treated group, and 1078.90±41.32/mm² in the noise stress applied with 200 mg/kg *Cnidium officinale* Makino treated group.

Our data clearly indicate that the rat pups born from the maternal rats exposed to noise stress during pregnancy exhibited significantly reduced neurogenesis in their dentate gyrus and CA (*cornu ammonis*) regions. However, *Cnidium officinale* Makino treatment was associated with an improvement of neurogenesis in both the

dentate gyrus and the CA regions.

Discussion

The formation of the dentate gyrus of the hippocampus is a process which occurs throughout the gestational period, continuing into the postnatal period²⁾. The later stage of dentate gyrus development is particularly sensitive to both environmental and experience-related structural changes²⁾. Stressful experiences occurring during the development period may exert long-term effects on hippocampal functions, and may result in exhibition of a variety of psychosomatic problems, including mental retardation or developmental disorders^{2,10,17)}. Recently, a variety of prenatal stresses have been shown to reduce the density of pyramidal neurons and nitric oxide producing neurons, reduce the total volume of hippocampus, and induce synaptic loss within the hippocampus^{1,18)}. Reduced cell proliferation in the dentate gyrus has been observed in pups born from the maternal animals exposed to restraint and noise stresses during pregnancy^{9,10,19)}.

It is well known that hippocampal formation plays a pivotal role in learning ability and memory capability, and the generation of new neurons in the hippocampus appears to be vital for the maintenance of normal learning and memory process²⁰⁾. Increased neurogenesis in the dentate gyrus of rats has been shown to improve learning ability⁶⁾. In contrast, prenatal stresses which reduce cell proliferation impair both learning and memory functions¹⁰⁾. Recently, it has been determined that deleterious environmental conditions which occur in early life exert profound effects on neurogenesis in the dentate gyrus and these alterations are generally associated with impaired performances on spatial memory

tasks¹⁰⁾. Offspring born from rats exposed to stresses during pregnancy tend to exhibit lower ability with regard to water maze learning and discrimination learning than control rats²¹⁾.

The findings of our study also indicate that neurogenesis was decreased in the hippocampus of rat pups born from maternal rats exposed to noise stress during the late gestational stage (Fig. 2, 3, 4). The loss of cells in the hippocampus also impairs spatial learning and spatial memory capability (Fig. 1). This impairment of memory functions may be principally attributable to the suppression of new cell formation in the hippocampus of the rat pups.

The root of *Cnidium officinale* Makino has traditionally been used as a medicinal herb. It is a perennial plant, a member of the *Umbelliferae* family¹³⁾. It has been utilized in the treatment of female genital inflammatory disorders including menstrual irregularity, dysmenorrhea, amenorrhea and especially in the treatment of headaches²²⁾. Considerable interest has begun to be focused on this medicinal plant, due to its purported anti-inflammatory and analgesic effects²³⁾, and antitumor and antimetastatic properties^{24,25)}. It has been demonstrated that the ethyl acetate soluble fraction of *Cnidium officinale* Makino can inhibit neuronal cell death via the reduction of excessive nitric oxide (NO) generation in lipopolysaccharide (LPS)-treated rat hippocampal slice cultures²⁶⁾. Recently, *TokiShakuyakuSan*, an herbal formulation which contains *Cnidium officinale* Makino, has been shown to significantly reverse the reference and working memory impairment associated with exposure to scopolamine¹⁴⁾. This formulation is also employed in the treatment of neuronal dysfunctions including Alzheimer's disease, senile dementia, memory loss, and a host of other cognitive disorders²⁷⁾. Furthermore, Baek

et al. have demonstrated that *Cnidium officinale* Makino can play a role in protecting against focal cerebral ischemia by regulating extracellular levels of excitatory and inhibiting against neurotoxic and oxidative damage in cortical neurons¹³⁾. Therefore, we investigated the effects of *Cnidium officinale* Makino on neurogenesis in the hippocampus of rat pups born from maternal rats exposed to noise stress during pregnancy.

According to this study, prenatal *Cnidium officinale* Makino treatment can prevent the reduced neurogenesis in the hippocampus of rat pups born from maternal rats exposed to noise stress (Fig. 2, 3, 4), and also can prevent spatial learning impairment in these rats (Fig. 1). These results means that it can be successfully utilized to improve the development of spatial learning ability in fetuses exposed to certain prenatal stresses, which may be due to its cholinergic activating, circulation improving effects²⁷⁾ and protecting effects on cerebral ischemia¹³⁾.

Conclusion

Exposure to noise stress during pregnancy induced growth retardation, reduced neurogenesis and impaired spatial learning ability in rat pups. By way of contrast, prenatal treatment with *Cnidium officinale* Makino can prevent the reduced neurogenesis and impaired spatial learning ability of rat pups born from maternal rats exposed to noise stress during pregnancy.

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