

## *Folium mori* Increases Cell Proliferation and Neuropeptide Y Expression in Dentate Gyrus of Streptozotocin-Induced Diabetic Rats

Hong KIM,<sup>a,b</sup> Mi-Hyeon JANG,<sup>a</sup> Min-Chul SHIN,<sup>a</sup> Hyun-Kyung CHANG,<sup>a</sup> Taek-Hyun LEE,<sup>a</sup> Baek-Vin LIM,<sup>a</sup> Chang-Young JUNG,<sup>c</sup> Choong-Yeol LEE,<sup>c</sup> Ee-Hwa KIM,<sup>d</sup> and Chang-Ju KIM<sup>\*,a</sup>

<sup>a</sup>Department of Physiology, College of Medicine, Kyung Hee University; #1 Hoigi-dong, Dongdaemoon-gu, Seoul 130–701, South Korea; <sup>b</sup>Research Institute of Sports Science, Korea University; #5–1 Anam-dong, Sungbuk-gu, Seoul 136–701, South Korea; <sup>c</sup>Department of Physiology, College of Oriental Medicine, Kyungwon University; #65 Bogjung-dong, Sujung-gu, Sungnam 461–701, South Korea; and <sup>d</sup>Department of Meridianology, College of Oriental Medicine, Semyung University; #21–1 Shinwol-dong; Jechon 390–711, South Korea.

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The possibility has been raised that *Folium mori* is clinically effective for the treatment and prevention of diabetes. In the present study, the effects of *Folium mori* on cell proliferation and expression of neuropeptide Y (NPY) in the dentate gyrus of rats with streptozotocin (STZ)-induced diabetes were investigated by 5-bromo-2'-deoxyuridine (BrdU) immunohistochemistry and NPY immunohistochemistry. In rats with STZ-induced diabetes, cell proliferation and NPY expression in the dentate gyrus were suppressed, and treatment with *Folium mori* was shown to increase new cell formation and NPY expression in the dentate gyrus in both normal rats and those with STZ-induced diabetes. In light of previous studies, this result appears to indicate that increased expression of NPY in the dentate gyrus induced by treatment with *Folium mori* is associated with the observed effect of *Folium mori* extract on cell proliferation. Based on the present results, it is suggested that *Folium mori* treatment may aid in the recovery from the central nervous system complications of diabetes mellitus.

**Key words** *Folium mori*; diabetes; cell proliferation; neuropeptide Y; dentate gyrus

Diabetes mellitus is a common metabolic disorder associated with numerous secondary complications in humans. It has been demonstrated that diabetes results in subtle cerebral disorders<sup>1)</sup> including alterations in neurotransmission, electrophysiological abnormalities, and structural changes.<sup>2–4)</sup> Furthermore, increasing evidence shows that diabetes may be associated with deficits in learning and memory.<sup>5)</sup> Ryan reported that learning and memory capacities are reduced in Type 1 diabetes mellitus patients.<sup>6)</sup> Impairments in cognition, particularly in tasks involving verbal memory or complex information processing was also reported in patients with type 2 diabetes mellitus.<sup>7,8)</sup> In addition, it has been demonstrated that streptozotocin (STZ)-induced diabetes significantly reduces the number of proliferating cells in the dentate gyrus of rats.<sup>9)</sup>

New cell birth and neurogenesis have been demonstrated in the dentate gyrus of several adult mammals including humans.<sup>10)</sup> Neurogenesis in the dentate gyrus of the hippocampus has been associated with learning and memory formation.<sup>11–13)</sup> Previous studies have shown that several factors such as enriched environments, learning, seizure, *N*-methyl-D-aspartate (NMDA) receptor antagonists, serotonin, and physical exercise, and ischemia enhance the proliferation of granular cell precursors and/or neurogenesis in the dentate gyrus<sup>11,12,14–18)</sup> while adrenal steroids, opioid peptides, and stress inhibit it.<sup>15,19,20)</sup>

In recent years, it has been reported that neuropeptide Y (NPY) is also a factor which is associated with enhanced cell proliferation in the dentate gyrus of rats.<sup>21)</sup> NPY is a 36-amino acid neuropeptide that is expressed in the central and peripheral nervous systems during development and adulthood. It is known to regulate feeding behavior, gastrointestinal activity, and central cardiovascular functions and to influence seizure threshold and alcohol intake.<sup>22–24)</sup> Recently, it has been demonstrated that NPY promotes the proliferation

of basal cells in the olfactory epithelium of rats,<sup>21)</sup> and it has been shown that increases in cell proliferation are closely related to increases in NPY expression in the dentate gyrus in STZ-induced diabetic rats.<sup>25)</sup>

The various parts of the mulberry tree (*Morus alba* L.) have been applied in the clinical treatment of various diseases in Oriental medicine. Recent evidence shows that the leaves and shoots from the mulberry tree possess several medicinal properties including hypoglycemic, hypotensive, and diuretic effects.<sup>26–28)</sup> In addition, it has been demonstrated that *Folium mori* is clinically effective in the treatment and prevention of diabetes.<sup>29)</sup> However, no study has been made to date on the effect of *Folium mori* treatment on the relationship between cell proliferation and NPY expression in the dentate gyrus of diabetes mellitus. The effects of *Folium mori* on cell proliferation and NPY expression in the dentate gyrus of STZ-induced diabetic rats were thus investigated *via* immunohistochemistry in the present study.

### MATERIALS AND METHODS

**Animals and Treatments** Male Sprague–Dawley rats weighing 200 ± 10 g (6 weeks of age) were used for the experiment. Each animal was housed at a controlled temperature (20 ± 2 °C) and was maintained under 12-h of light and 12-h of dark cycles (lights on from 07:00 to 19:00), with food and water available *ad libitum*. The experimental procedures were performed in accordance with the animal care guidelines of the US National Institute of Health and the Korean Academy of Medical Sciences. Animals were divided into six groups: the control group; the 10 mg/kg *Folium mori*-treated group; the 100 mg/kg *Folium mori*-treated group; the STZ-induced diabetic group; the STZ-induced diabetic and 10 mg/kg *Folium mori*-treated group; and the STZ-induced diabetic and 100 mg/kg *Folium mori*-treated group (*n* = 5 in

\* To whom correspondence should be addressed. e-mail: changju@khu.ac.kr

each group).

The *Folium mori* used in this experiment was obtained from Kyung Dong Market (Seoul, Korea). After washing, *Folium mori* was immersed in cold water for 12 h. To obtain the aqueous extract, 200 g of *Folium mori* was added to distilled water, heat-extracted at 80°C, concentrated using a rotary evaporator, and lyophilized. The resulting powder, weighing 22 g, was diluted with saline. After filtering through a 0.45- $\mu$ m syringe filter, animals in the *Folium mori*-treated groups received the extract at the respective doses intraperitoneally for 3 consecutive days, and those in the control group received equivalent amounts of saline once a day for the same duration of time.

To induce diabetes in the experimental animals, a single intraperitoneal injection of STZ (50 mg/kg in saline; Sigma Chemical Co., St. Louis, MO, U.S.A.) was given to each animal in the STZ-induced diabetic groups. Blood glucose levels were determined 2 d after the STZ injection using a blood glucose analyzer (Arkay, Kyoto, Japan). Only the animals with blood glucose levels of 300 mg/dl or higher were used in this study. Starting on the second day after STZ administration, 5-bromo-2'-deoxyuridine (BrdU; 50 mg/kg i.p. in saline; Sigma Chemical) was given to all animals once a day for 3 consecutive days.

**Tissue Preparation** Animals were weighed and overdosed with Zoletil 50<sup>®</sup> (10 mg/kg, i.p.; Vibac Laboratories, Carros, France). After a complete lack of response was observed, the rats were transcardially perfused with phosphate-buffered saline (PBS) 50 mM and then with 4% paraformaldehyde in phosphate buffer (PB) 100 mM at pH 7.4. The brains were dissected, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Serial coronal sections 40  $\mu$ m thick were made using a freezing microtome (Leica, Nussloch, Germany).

**BrdU Immunohistochemistry** For visualization of cell birth in the dentate gyrus, BrdU-specific immunohistochemistry was performed.<sup>17</sup> In brief, sections were first pretreated by incubation in 50% formamide-2 $\times$  standard saline citrate at 65°C for 2 h, denaturing in 2 N HCl at 37°C for 30 min, and rinsing twice in sodium borate (pH 8.5) 100 mM. After the pretreatment, the sections were incubated overnight at room temperature with BrdU-specific mouse monoclonal antibody (1:600) (Boehringer Mannheim, Mannheim, Germany). The sections were washed three times with PBS and incubated for 1 h with a biotinylated mouse secondary antibody (1:100) (Vector Laboratories, Burlingame, CA, U.S.A.). The sections were then incubated for additional 1 h with avidin-biotin-horseradish peroxidase complex (1:100) (Vector Laboratories). For staining, the sections were incubated in a solution consisting of 0.02% 3,3'-diaminobenzidine containing nickel chloride (40 mg/ml) (Nickel-DAB) and 0.03% hydrogen peroxide in 50 mM Tris-HCl (pH 7.6) for 5 min. Finally, the sections were mounted onto gelatinized glass slides. The slides were air-dried overnight at room temperature, and cover slides were mounted using Permount<sup>®</sup>.

**NPY Immunohistochemistry** For visualization of NPY expression,<sup>25</sup> free-floating tissue sections were washed twice in PBS 50 mM and were then permeabilized in 0.2% Triton X-100 for 30 min. After washing twice with PBS, sections

were incubated overnight with rabbit anti-NPY antibody (Di-aSorin, Stillwater, MN, U.S.A.) at a dilution of 1:4000. Sections were washed twice in PBS and incubated for 1 h with biotinylated anti-rabbit antibody (1:200). Bound secondary antibody was then amplified using the Vector Elite ABC<sup>®</sup> kit (Vector Laboratories). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.05% diaminobenzidine. The sections were mounted onto gelatinized glass slides, air dried, and cover slides were mounted using Permount<sup>®</sup>.

**Data Analysis** The area of the granular layer of the dentate gyrus was measured using an image analyzer (Multiscan, Fullerton, CA, U.S.A.). The mean number of cells positive for BrdU or NPY was counted hemilaterally and expressed as number of cells per mm<sup>2</sup> of the cross-sectional area of the granular layer of the dentate gyrus. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's post-hoc analysis, and results are expressed as mean  $\pm$  standard error mean (S.E.M.). Differences were considered significant at  $p < 0.05$ .

## RESULTS

**Effect of *Folium mori* on Cell Proliferation in the Hippocampal Dentate Gyrus** Photomicrographs of BrdU-positive cells in the dentate gyrus of the control group are presented in Fig. 1. The mean number of BrdU-positive cells in the dentate gyrus was 140.52  $\pm$  9.28/mm<sup>2</sup> in the control group, 157.60  $\pm$  8.96/mm<sup>2</sup> in the 10 mg/kg *Folium mori*-treated group, 193.32  $\pm$  8.84/mm<sup>2</sup> in the 100 mg/kg *Folium mori*-treated group, 105.32  $\pm$  7.28/mm<sup>2</sup> in the STZ-induced diabetic group, 113.88  $\pm$  7.60/mm<sup>2</sup> in the STZ-induced diabetic and 10 mg/kg *Folium mori*-treated group, and 140.28  $\pm$  10.20/mm<sup>2</sup> in the STZ-induced diabetic and 100 mg/kg *Folium mori*-treated group. Cell proliferation in the dentate gyrus was suppressed in rats with STZ-induced diabetes, and treatment with *Folium mori* was shown to increase new cell formation in the dentate gyrus in both normal rats and those with STZ-induced diabetes (Fig. 2).

**Effect of *Folium mori* on NPY Expression in the Hippocampal Dentate Gyrus** Photomicrographs of NPY-positive cells in the dentate gyrus of the control group are presented in Fig. 3. The mean number of NPY-positive cells in the dentate gyrus was 96.28  $\pm$  4.20/mm<sup>2</sup> in the control group, 113.72  $\pm$  2.92/mm<sup>2</sup> in the 10 mg/kg *Folium mori*-treated group, 147.32  $\pm$  8.84/mm<sup>2</sup> in the 100 mg/kg *Folium mori*-treated group, 74.00  $\pm$  4.72/mm<sup>2</sup> in the STZ-induced diabetic group, 93.72  $\pm$  3.20/mm<sup>2</sup> in the STZ-induced diabetic and 10 mg/kg *Folium mori*-treated group, and 110.00  $\pm$  6.84/mm<sup>2</sup> in the STZ-induced diabetic and 100 mg/kg *Folium mori*-treated group.

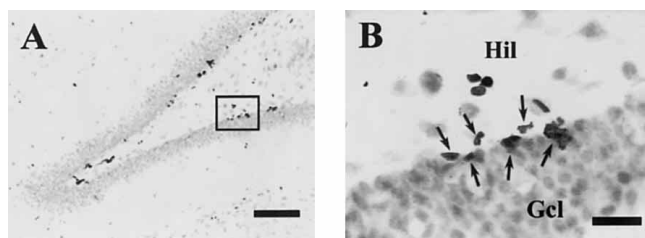


Fig. 1. Photomicrographs of 5-Bromo-2'-deoxyuridine (BrdU)-Positive Cells in the Dentate Gyrus of the Control Group

Sections were stained for BrdU (black). Hil, hilus; Gcl, granular cell layer. Scale bar represents 100  $\mu$ m (A) and 25  $\mu$ m (B).

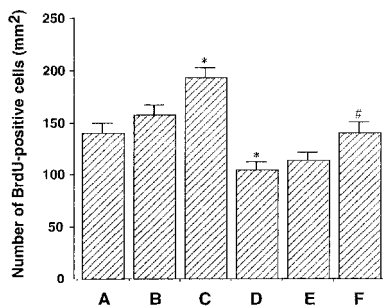


Fig. 2. Number of 5-Bromo-2'-deoxyuridine (BrdU)-Positive Cells in the Dentate Gyrus of the Hippocampus in Each Group

(A) Control group; (B) 10 mg/kg *Folium mori*-treated group; (C) 100 mg/kg *Folium mori*-treated group; (D) streptozotocin (STZ)-induced diabetic group; (E) STZ-induced diabetic and 10 mg/kg *Folium mori*-treated group; (F) STZ-induced diabetic and 100 mg/kg *Folium mori*-treated group. Values are expressed as mean  $\pm$  S.E.M. \* $p$ <0.05 compared with the control group; # $p$ <0.05 compared with the STZ-induced diabetic group.

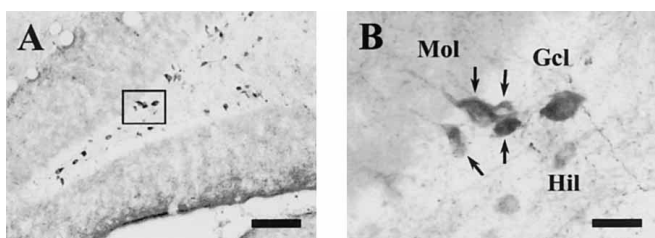


Fig. 3. Photomicrographs of Neuropeptide Y (NPY)-Positive Cells in the Dentate Gyrus of the Control Group

Sections were stained for NPY (black). Mol, molecular layer; Hil, hilus; Gcl, granular cell layer. Scale bar represents 100  $\mu$ m (A) and 25  $\mu$ m (B).

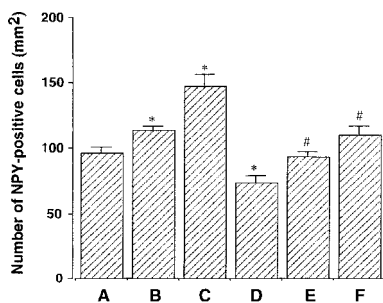


Fig. 4. Number of Neuropeptide Y (NPY)-Positive Cells in the Dentate Gyrus of the Hippocampus in Each Group

(A) Control group; (B) 10 mg/kg *Folium mori*-treated group; (C) 100 mg/kg *Folium mori*-treated group; (D) streptozotocin (STZ)-induced diabetic group; (E) STZ-induced diabetic and 10 mg/kg *Folium mori*-treated group; (F) STZ-induced diabetic and 100 mg/kg *Folium mori*-treated group. Values are represented as mean  $\pm$  S.E.M. \* $p$ <0.05 compared with the control group; # $p$ <0.05 compared with the STZ-induced diabetic group.

mm<sup>2</sup> in the STZ-induced diabetic and 100 mg/kg *Folium mori*-treated group. NPY expression in the dentate gyrus was suppressed in rats with STZ-induced diabetes, and treatment with *Folium mori* increased NPY expression in the dentate gyrus in both normal rats and those with STZ-induced diabetes (Fig. 4).

## DISCUSSION

Jackson-Guilford *et al.*<sup>9)</sup> demonstrated that STZ-induced diabetic rats undergo a significant reduction in the number of proliferating cells in the dentate gyrus. Suppression of cell

proliferation in the dentate gyrus of diabetic rats was also observed in the present study. Over the years, the possibilities of prevention and/or treatment of various diseases using medicinal herbs have received considerable attention. It has been reported that mulberry leaf extract has a potent antihyperglycemic effect in mice with streptozotocin-induced diabetes.<sup>29)</sup> Iizuka *et al.*<sup>30)</sup> reported that *Folium mori* increases insulin sensitivity and curbs insulin resistance. In the present study, treatment with *Folium mori* was shown to increase the number of BrdU-positive cells in the dentate gyrus in normal rats and those with STZ-induced diabetes.

Although the precise mechanism of cell proliferation is unknown, other studies have suggested that cell proliferation could be mediated by NPY among other factors. Hansel *et al.*<sup>21)</sup> reported that NPY increases the number of neuronal precursor cells undergoing cell division and thus produces more olfactory neurons and that the action of NPY in increasing neuronal precursor proliferation is mediated by protein kinase C (PKC), indicating an upstream PKC-dependent activation of ERK1/2. Close correlation of NPY expression with cell proliferation in STZ-induced diabetic rats has been reported in other studies.<sup>25)</sup>

In the present study, treatment with *Folium mori* in normal rats and those with STZ-induced diabetes was shown to enhance NPY expression in the dentate gyrus. This result indicates that the increase in the expression of NPY in the dentate gyrus induced by treatment with *Folium mori* may be associated with the observed effect of *Folium mori* extract on cell proliferation. Based on the present results, it can be suggested that *Folium mori* treatment may aid in the recovery from the central nervous system complications of diabetes mellitus by enhancing cell proliferation in the dentate gyrus via augmented NPY expression.

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