Protective effect of young green barley leaf (*Hordeum vulgare* L.) on restraint stress-induced decrease in hippocampal brain-derived neurotrophic factor in mice

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ABSTRACT

Background: Many health experts support the hypothesis that stressful lifestyles are the leading cause of illness, like depression. Therefore, from the standpoint of preventive medicine, it is important to reduce stress. Young green barley leaves are a good natural source of vitamins and minerals, and their juice is widely consumed as a functional food for health reasons in Japan. This study investigated the protective effect of young green barley leaves for stress control. **Materials and Methods:** ICR outbred mice were exposed to 3-h sessions of restraint stress. Young green barley leaves (400 and 1,000 mg/kg) were administered orally 1 h before the sessions for 5 days. To analyze voluntary behavior, wheel-running activity was monitored during the dark period. Brain-derived neurotrophic factor (BDNF) messenger RNA (mRNA) expression in the whole hippocampus was measured by real-time quantitative polymerase chain reaction. **Results:** Restraint stress resulted in a significant decrease in voluntary wheel-running behavior, but this decrease was ameliorated by the administration of young green barley leaves. The leaves also enhanced the decreased levels of BDNF mRNA induced by restraint stress; in particular, a significant protective effect was shown in the exon IV variant as compared to vehicle control mice. **Conclusion:** The findings suggest that young green barley leaves have potent anti-stress properties, as evidenced by preventing decreases in the levels of voluntary wheel-running activity and hippocampal BDNF mRNA in response to restraint stress. Our findings support the possibility that supplementation with young green barley leaves might be beneficial for preventing stress-related psychiatric disorders like depression.

Key words: Behavioral study, brain-derived neurotrophic factor, functional food, hippocampus, preventive medicine

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INTRODUCTION

Many health experts support the hypothesis that a stressful lifestyle is the leading cause of illness, a typical example of which is depression.[1] Depression is a major global public health issue, both because of its relatively high lifetime prevalence (ranging from 2% to 15%) and because it is associated with substantial disability.[2]

The findings of many studies indicate that stress management by mindfulness-based stress reduction effectively reduces depressive episodes.[3] Therefore, from the standpoint of preventive medicine, it is important in the prevention of depression to reduce stress. From the perspective of self-medication specifically, we have been investigating functional foods known to have a positive effect on stress control.

Young green barley leaves are a good natural source of vitamins and minerals, and their juice is widely consumed as a functional food for health reasons in Japan. They are a rich source of the potent antioxidants saponarin and lutonarin[4] and also exhibit physiological activities, including hypolipidemic,[5] antidiabetic,[6] and anti-ulcer[7] effects, via its anti-oxidative action. In addition, we recently demonstrated that young green barley leaves have anti-depressive effects in mice during the forced swimming test.[8] Therefore, the aim of the present study was to

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investigate the protective effect of young green barley leaves for stress control.

It is well-known that glucocorticoid secretion is increased by activation of the hypothalamic–pituitary–adrenal (HPA) axis in response to stress.[9] Excessive glucocorticoid exposure over long periods impairs the function and integrity of the hippocampus, by causing neurotoxicity.[10,11] The progressive process of depression is suggested to be associated with hippocampal atrophy mediated by glucocorticoid neurotoxicity.[12] Brain-derived neurotrophic factor (BDNF), a small dimeric neuroprotective protein, is reported to play a critical role in the development and maintenance of the central and peripheral nervous systems as well as neuronal survival and proliferation in both animals[13,14] and humans.[15] Serum BDNF concentrations in patients untreated for major depression were found to be significantly lower than those in healthy control subjects.[16-18] In addition, in animals subjected to forced swimming and chronic restraint stress, BDNF messenger RNA (mRNA) expression levels in the hippocampus were significantly decreased.[19,20]

We recently reported a novel method for evaluating the influences of stress in mice.[21] Briefly, we evaluated the impact of stress on behavioral responses by suppressing wheel-running activity. We also assessed the influence of stress on neuroprotective agents in the brain by measuring BDNF levels in the hippocampus. Our findings suggested that the behavioral responsivity to restraint stress is associated with the production of hippocampal BDNF, and we showed that a tendency toward a sex difference in the stress response in mice is similar to the sex discrepancy in the prevalence of depression in humans.

In this study, we applied the same method as in our previous work to examine the effects of young green barley leaves as a potential functional food for stress control. First, we evaluated the protective effect of young green barley leaves on the decrease in locomotor activity in response to restraint stress in mice. Second, we measured mRNA expression levels of BDNF in the hippocampus to assess their neuroprotective potency in the increased corticosterone condition with a special focus on the splice variant exon IV.

MATERIALS AND METHODS

Animals

Six-week-old ICR outbred mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) and housed under controlled light (0700–1900 h) and temperature (24°C ± 1°C) conditions, with food and water available *ad libitum*. All experiments and procedures were approved by the Chiba University Institutional Animal Care and Use Committee.

Materials

Young green barley leaves (*Hordeum vulgare* L. var. *nudum* Hook), 20–35 cm in height, were supplied by JPD Co. Ltd. (Hyogo, Japan); the manufacturer collected the specimens in Oita Prefecture, Japan and extracted juice from the leaves to produce a dried powder, in accordance with the company's guide to Good Manufacturing Practice. RIZE® tablets were obtained from Mitsubishi Tanabe Pharma (Osaka, Japan), corticosterone was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and dexamethasone was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Young green barley leaves were dissolved in distilled water. Clotiazepam was extracted from RIZE® tablets with methanol and dissolved in distilled water containing 0.5% carboxymethyl cellulose, medium viscosity.

Experimental schedule

To examine the influence of young green barley leaves on locomotor activity, male mice were subjected to wheel-running seven times every other day from 1730 to 0930 h after 5 days of habituation [Figure 1a]. From day 1 to day 7, young green barley leaves (400 or 1,000 mg/kg), clotiazepam (10 mg/kg), or distilled water (vehicle) were administrated orally at 1000 h. To examine the impact of young green barley leaves on restraint stress, female mice were subjected to wheel-running eight times as mentioned above [Figure 2a]. From day 1 through day 5, mice were treated with each reagent at 1000 h. One hour after the oral administration, all mice except for the nonstressed control animals (Nil) were subjected to enforced restraint stress each day for 3 h from 1100 to 1400 h [Figure 2a].

Wheel-running behavior

Locomotor activity was evaluated by measuring voluntary wheel-running activity. The mice were housed with free access to a running wheel (wheel diameter 200 mm, cage size 220 mm × 90 mm × 80 mm; TK-48, Toyo-riko Co., Ltd., Tokyo, Japan) for 16 h (1730–0930 h) every other day during the training period (prior to day 0) and the experimental period (after day 0), and food and water were available *ad libitum*. Voluntary wheel-running activity, defined as the total number of wheel rotations, was recorded at 0930 h and is shown as a percentage compared to day-1.

Restraint stress

After six sessions of wheel-running training, female mice excluding those in the Nil group were subjected

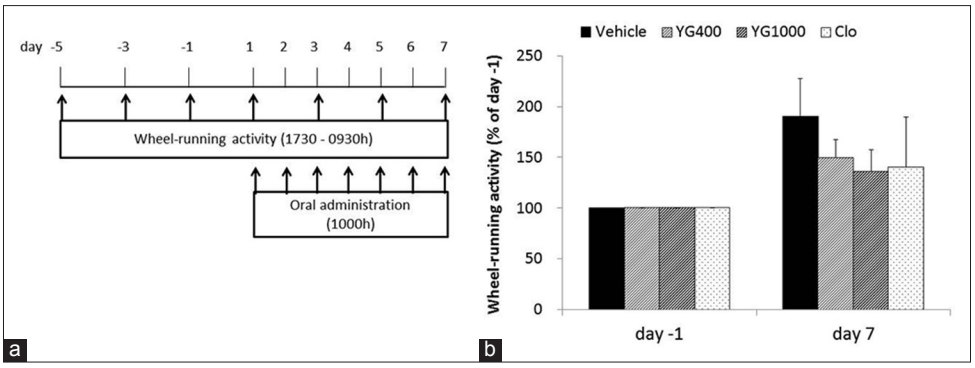

Figure 1: Experimental protocol for measuring wheel-running activity and treatment (a), and the effects of young green barley leaves on voluntary behavior measured by wheel-running activity in male mice (b). Wheel-running activity was evaluated by running distance measured as the total number of wheel rotations in 16 h (1730–0930 h). Results are expressed as mean ± standard error of the mean for n = 7–9 mice. Vehicle: Distilled water; YG400 and YG1000: Young green barley leaf extract at doses of 400 mg/kg and 1,000 mg/kg, respectively; Clo: Clotiazepam at a dose of 10 mg/kg

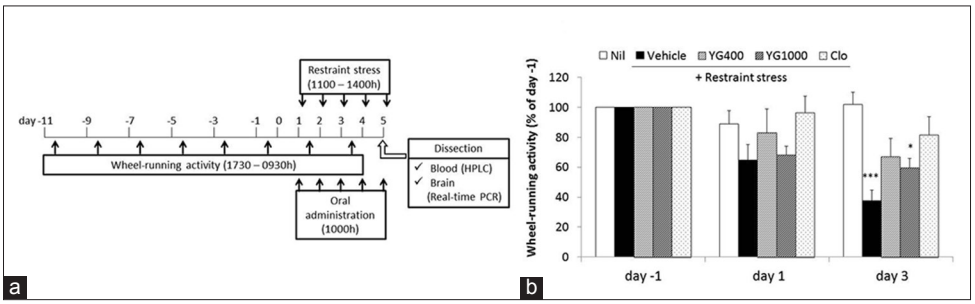

Figure 2: Experimental schedule for restraint stress measuring wheel-running activity and treatment administration (a), and the effects of young green barley leaves on voluntary behavior measured by wheel-running activity in female mice subjected to restraint stress (b). Wheel-running activity was evaluated by running distance measured as the total number of wheel rotations in 16 h (1730–0930 h). Results are expressed as mean ± standard error of the mean for n = 7–9 mice. *P < 0.05 and ***P < 0.001 versus nonstressed (Nil) group on day 3 (Tukey-Kramer test). Vehicle: Distilled water; YG400 and YG1000: Young green barley leaf extract at doses of 400 mg/kg and 1,000 mg/kg, respectively; Clo: Clotiazepam at a dose of 10 mg/kg. The mean wheel-running counts in each experimental group on day-1 were: Nil: 17474.6 ± 1765.0; Vehicle: 17325.0 ± 1200.2; YG400: 16233.5 ± 2204.1; YG1000: 18153.0 ± 1846.3; and Clo: 17154.1 ± 1565.3

to restraint. The animals were held in cylindrical plastic tubes (115 mm × 30 mm, with holes to allow access to fresh air) for 3 h (1100–1400 h) [Figure 2a].

Measurement of serum corticosterone

Blood was collected by retro-orbital bleeding immediately after the final restraint stress test, and centrifuged at 1,000 × g for 20 min. Serum was collected and stored at −80°C prior to analysis. Serum levels of corticosterone were determined by high-performance liquid chromatography (HPLC). Briefly, 20-μl aliquots of standards or samples were transferred to 1.5-ml Eppendorf centrifuge tubes. A 25-μl aliquot of internal standard solution (dexamethasone, 2 μg/ml final concentration) was added to the serum followed by 200 μl ethyl acetate, and briefly mixed on a vortex mixer. The mixture was centrifuged at 5,000 × g for 10 min at 4°C to remove precipitated proteins. An 80-μl aliquot of 0.05 M sodium hydroxide was added to the supernatant and mixed on a vortex mixer. The mixture was centrifuged at 5,000 × g for 5 min at 4°C. The supernatant was then transferred to a 1.5-ml Eppendorf centrifuge tube and evaporated to dryness using a centrifugal concentrator (DNA-mini, Heto, Denmark). Then, 25 μl HPLC mobile phase (35% acetonitrile/65% water) was added and transferred to a 250-μl injection vial. A 5-μl aliquot of the sample or standard solution in the injection vial was subjected to HPLC analysis. A Shiseido Nanospace SI-2 HPLC system (Shiseido Co. Ltd., Tokyo, Japan) was used to measure the concentration of corticosterone. A Unison UK-C-18 column (1.5 mm × 250 mm, 3 μm; Imtakt Corp., Kyoto, Japan) was used at 40°C with a flow rate of 100 μl/min. The mobile phase was 35% acetonitrile/65% water, and corticosterone was detected at a wavelength of 240 nm.

Messenger RNA expression for the glucocorticoid receptor, corticotropin-releasing hormone, and brain-derived neurotrophic factor in brain

Immediately after the final restraint stress test, mice were killed by decapitation and the hippocampus and hypothalamus were removed, according to the method of Hagihara *et al*.[22] Hippocampus and hypothalamus samples were homogenized in RNAzol®RT (Molecular Research Center, Inc., Cincinnati,

OH, USA), and centrifuged at 12,000 × *g* for 5 min at room temperature. The supernatant was collected, and the total RNA was extracted using RNAzol®RT reagent (Molecular Research Center, Inc.). Total RNA was quantified using an absorbance meter (Smart Spec™3000, BIO-RAD, Hercules, CA, USA). Complementary DNA was prepared from RNA by reverse transcription using a ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan) with a polymerase chain reaction (PCR) Thermal Cycler Dice® (Takara Bio Inc., Shiga, Japan). Real-time quantitative PCR was performed using a Step One TM Real-Time PCR system (Applied Biosystems Inc., Carlsbad, CA) with SYBR Premix Ex Taq (Tli RNaseH Plus), ROX plus (Takara Bio Inc.), for mouse BDNF, glucocorticoid receptor (GR), corticotropin-releasing hormone (CRH), and β-actin in accordance with the manufacturer's instructions (Takara Bio Inc.). Results are expressed as the mRNA level relative to β-actin mRNA as an internal control.

Statistical analysis

All data are presented as mean ± standard error of the mean Statistical significance was analyzed using the Tukey-Kramer test or Dunnett's method for multiple comparisons. Statistical differences in two groups were analyzed using Student's *t*-test or Aspin-Welch's test after an *F*-test. Differences at $P < 0.05$ were considered statistically significant. All statistical analyses were conducted using StatLight software (Yukms Co., Ltd., Tokyo, Japan).

RESULTS

Wheel-running behavior

To examine the influence of young green barley leaves on locomotor activity in mice, we measured wheel-running activity during the dark period. We found no influence of either young green barley leaves or clotiazepam on voluntary behavior in nonstressed mice [Figure 1b].

Next, we determined the effect of young green barley leaves on mice in response to restraint stress as described above. Restraint stress resulted in a decrease in voluntary wheel-running behavior day by day in vehicle control mice. Moreover, on day 3, wheel-running activity in the vehicle control mice was significantly decreased compared to the nonstressed mice. However, young green barley leaves (400 and 1,000 mg/kg) showed a protective effect on the decrease in wheel-running activity due to restraint stress. The positive control, clotiazepam, further increased wheel-running activity, also indicating an anti-stress effect [Figure 2b].

Serum corticosterone concentration

The serum glucocorticoid level was measured to investigate the effect of young green barley leaves on the HPA axis. Increased corticosterone levels are used as an index of the stress response in mice, analogous to cortisol in humans. Serum concentration of corticosterone in vehicle control mice increased significantly compared to the nonstressed mice after the final restraint stress test. Treatment with young green barley leaves or clotiazepam did not prevent the increase in serum corticosterone level, compared to vehicle treatment [Figure 3].

Expression of glucocorticoid receptor messenger RNA in the hippocampus and hypothalamus

Using quantitative PCR, we analyzed the effect of restraint stress in the presence or absence of young green barley leaves on the expression of GR mRNA. The expression of hippocampal GR mRNA in mice immediately after the restraint stress test was significantly decreased compared to the nonstressed mice. Treatment with young green barley leaves or clotiazepam did not alter this decrease in the levels of GR mRNA in the hippocampus [Figure 4a]. In addition, young green barley leaves had no effect on the levels of GR mRNA or CRH mRNA in the hypothalamus [Figure 4b and c].

Expression of brain-derived neurotrophic factor messenger RNA in the hippocampus

To investigate the impact of young green barley leaves on hippocampal BDNF, we analyzed the expression of total BDNF and BDNF exon I and exon IV mRNA in the hippocampus [Figure 5a-c]. BDNF mRNA levels in the vehicle control mice decreased significantly compared to levels in the nonstressed mice. The oral administration of young green barley leaves at the dose of 400 mg/kg weakened this restraint stress-induced decrease; in particular, there was a significant increase in BDNF exon IV as compared to the vehicle control mice. Young green barley leaves at 1,000 mg/kg did not show any effect on BDNF mRNA level. We observed

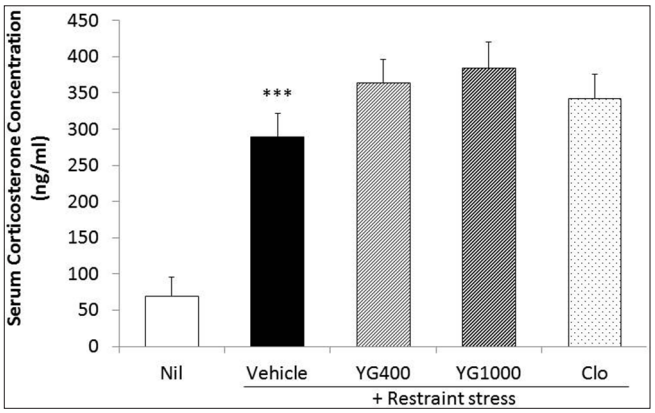

Figure 3: Effects of young green barley leaves on serum corticosterone concentration. Results are expressed as mean ± standard error of the mean for $n = 7–9$ mice. ***$P < 0.001$ versus nonstressed (Nil) group (Student's *t*-test). Vehicle: Distilled water; YG400 and YG1000: Young green barley leaf extract at doses of 400 mg/kg and 1,000 mg/kg, respectively; Clo: Clotiazepam at a dose of 10 mg/kg

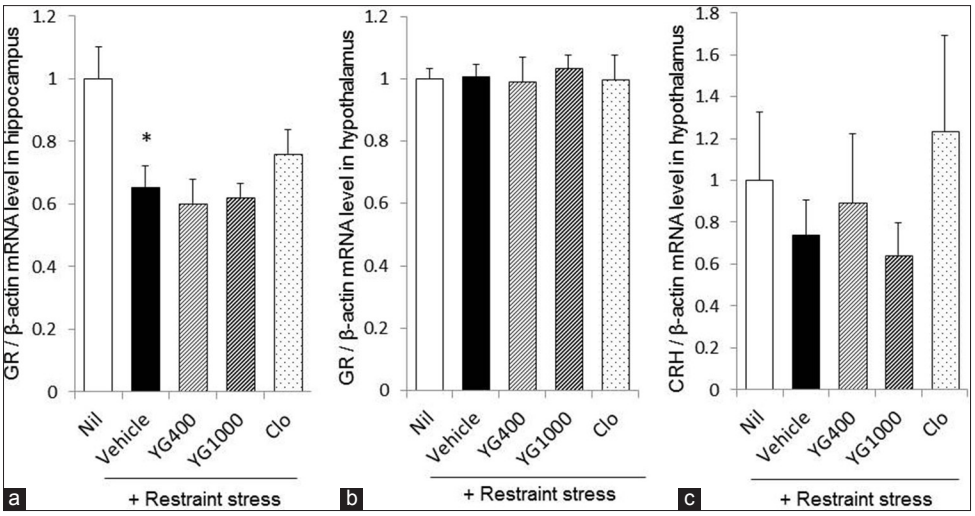

Figure 4: Effects of young green barley leaf extract on glucocorticoid receptor messenger RNA expression in the hippocampus (a) and hypothalamus (b) and corticotropin-releasing hormone expression in the hypothalamus (c) measured by real-time polymerase chain reaction. Results are expressed as mean ± standard error of the mean for $n = 7–9$ mice. *$P < 0.05$ versus nonstressed (Nil) group (Student's *t*-test). Vehicle: Distilled water; YG400 and YG1000: Young green barley leaf extract at doses of 400 mg/kg and 1,000 mg/kg, respectively; Clo: Clotiazepam at a dose of 10 mg/kg

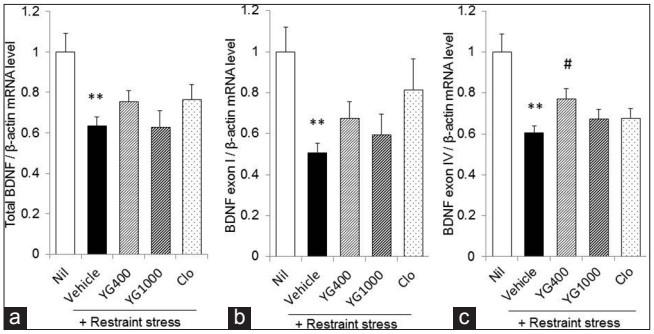

Figure 5: Effects of young green barley leaf extract on total brain-derived neurotrophic factor (BDNF) (a), BDNF exon I (b), and BDNF exon IV messenger RNA expression (c) in the hippocampus evaluated by real-time polymerase chain reaction. Results are expressed as mean ± standard error of the mean for $n = 7–9$ mice. **$P < 0.01$ versus nonstressed (Nil) group (Aspin Welch's *t*-test). #$P < 0.05$ versus vehicle group (Dunnett's test). Vehicle: Distilled water; YG400 and YG1000: Young green barley leaf extract at doses of 400 mg/kg and 1,000 mg/kg, respectively; Clo: Clotiazepam at a dose of 10 mg/kg

a moderate protective effect of clotiazepam on the decreased levels of total BDNF and BDNF exon I mRNA [Figure 5a and b].

DISCUSSION

Restraint stress, a severe stressor, is a commonly used method of inducing psychological stress in rodents.[23,24] This animal model is useful for understanding the pathophysiology of stress-induced behavioral alterations. In our previous study, we demonstrated that restraint stress significantly decreases locomotor activity in murine wheel-running behavior.[21] In the present study, we first demonstrated that young green barley leaves enhanced the development of adaptation/resistance of mice in the restraint stress test evaluated by wheel-running behavior. It is known that young green barley leaves do not contain central nervous system stimulants such as caffeine and ephedrine. In our study, young green barley leaves did not have any influence on locomotor activity in untreated mice. Therefore, these leaves might be a functional ingredient with an anti-stressor effect.

Glucocorticoid secretion is increased by activation of the HPA axis in response to stress.[9] That is, stress stimulates hypothalamic CRH release, which leads to pituitary adrenocorticotropic hormone (ACTH) secretion, resulting in elevated levels of glucocorticoids from the adrenal cortex. The elevation in basal corticosteroids exerts negative feedback regulation on the HPA axis. Excessive stress is associated with insensitivity to the negative feedback regulation of ACTH secretion by glucocorticoids, leading to over-activation of the HPA axis. Therefore, we focused on the GR in the hippocampus and hypothalamus and CRH in the hypothalamus. Excessive release of glucocorticoids impairs the function and integrity of the hippocampus, a brain region with high levels of GR, by causing neurotoxicity.[10,11] In the present study, we showed the mRNA levels of GR significantly decreased in the hippocampus following stress loading; however, young green barley leaves showed no impact on these levels. In addition, young green barley leaves had no effect on the levels of GR mRNA or CRH mRNA in the hypothalamus. Therefore, we conclude that young green barley leaves have less effect on the HPA axis.

We also tried to investigate the anti-stressor effect of young green barley leaves using this murine restraint stress model. We first demonstrated the protective effect of young green barley leaves on the restraint stress-induced decrease in locomotor activity. Second, we showed that young green barley leaves alleviated the decreased levels of hippocampal BDNF in restraint-stressed mice. It has been reported that high plasma glucocorticoid levels caused by stress induce hippocampal damage via the GR. Furthermore, exogenous corticosterone, without interfering with the endocrine stress response, induces a reduction in the hippocampal BDNF mRNA level in rats.[25] The present findings suggest that the decreased level of BDNF mRNA is caused by increased levels of serum corticosterone induced by restraint stress. The mouse BDNF gene has at least eight (I–VIII) noncoding exons and one coding exon (exon IX).[26] It was reported that a single bout of restraint stress decreased total BDNF (exon IX) and BDNF exon I and IV, but had different effects on BDNF exon I (increase) and IV/IX (decrease), in the rat hippocampus with chronic exposure (10 days).[27] In the present study, 5 days of restraint stress significantly decreased both exon I and IV levels in the vehicle group. These results suggest that restraint stress in this study can be classified as acute stress. However, the decreased levels of wheel-running activity in the vehicle control mice were much greater on day 3 than on day 1. These findings suggest that chronic stress might be induced by continuing the restraint stress test. Our study was designed from the standpoint of the preventive medicine to gather evidence on functional foods for stress prevention. Therefore, the degree of stress in our model appears to be suitable for investigating the therapeutic effect of young green barley leaves on "sub-health" (health immediately before disease onset).

Brain-derived neurotrophic factor exon IV is the most commonly studied variant, and its expression changes have been associated with behavioral responses following antidepressant treatment in animal models of depression.[28,29] Moreover, treatment with antidepressants leads to an up-regulation of BDNF transcript IV levels in the prefrontal cortex of depressive subjects.[30] The significant ameliorating effect of young green barley leaves on the decreased BDNF exon IV mRNA levels would indicate the possibility of a preventive effect on depression in "sub-healthy" subjects.

In this study, we assessed locomotor activity using running wheel behavior as an indicator of behavioral responses following restraint stress. However, it was reported that the wheel running induced an increase in BDNF concentration in the hippocampus in mice.[31] First, we demonstrated that young green barley leaves have no effect on voluntary exercise in untreated healthy mice. In addition, young green barley leaves showed a protective effect on the decrease in wheel-running behavior in response to restraint stress. Baj *et al*. concluded that the increase in BDNF in response to exercise (for 28 consecutive days) was accounted for by the exon VI variant.[31] In contrast, we demonstrated that young green barley leaves increased the BDNF exon IV variant, and our exercise protocol was 2 alternate days. Based on these findings, the protective effects of young green barley leaves on voluntary exercise against stress could be concluded, as supported by the protective effect on the decrease in BDNF in the hippocampus. Further studies are needed to confirm this link.

Polyphenols, including flavonoids, are well-known antioxidants. Recently, in a critical review of antioxidants, neuroprotective properties in the central nervous system of blueberry polyphenols were reported.[32] Young green barley leaves also contain the flavonoids saponarin and lutonarin which have potent antioxidant activities. These flavonoids are thought to be responsible for the biological activities of young green barley leaves.[33] However, further studies are warranted to clarify the active components of young green barley leaves with regard to the anti-stress activity seen in our study. To the best of our knowledge, this is the first study to report the anti-stress properties of young green barley leaves in relation to the protective effect against the hippocampal decrease in BDNF levels in response to restraint stress in mice. Further work is necessary to investigate the molecular signaling pathways that promote the protective effect of these leaves on hippocampal BDNF levels.

Young green barley leaves have potent anti-stress properties, as evidenced by preventing the decrease in levels of voluntary wheel-running activity and hippocampal BDNF mRNA in response to restraint stress. Supplementation with young green barley leaves might, therefore, be beneficial to prevent stress-related psychiatric disorders like depression.

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