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Effects of Anthocyanins on Psychological Stress-Induced Oxidative Stress and Neurotransmitter Status

M. Mamunur Rahman,[†] Takashi Ichiyanagi,[‡] Tadazumi Komiyama,[‡] Shinji Sato,[†] and Tetsuya Konishi*,[†]

Faculty of Applied Life Sciences, Faculty of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, 265-1 Higashijima, Niigata 956-8603, Japan

There is strong evidence that oxidative stress participates in the etiology of neurodegenerative diseases such as Parkinson's, and Alzheimer's diseases. Moreover, emotional stress effects in the central nervous system play a vital role in homeostasis. The protective effect of anthocyanins on the cerebral oxidative stress was studied using the whiskers cut model. In mice, such treatment causes psychological or emotional distress leading to oxidative stress in tissues. To investigate the in vivo antioxidant activity of anthocyanins, an extract of *Vaccinium myrtillis* L., an anthocyanin mixture, was orally administered (100 mg/kg of body weight.) to mice for 7 days, and then psychological stress was assessed by cutting off their whiskers. Whisker removal increased both protein carbonyl formation and lipid peroxidation in the brain, heart, kidney, and liver. Further, the levels of oxidative markers showed regional differences in the brain. Concomitantly, dopamine neurotransmitter levels were altered in both the midbrain and the brain cortex. Orally administered anthocyanins were also active in the brain, suppressing stress-induced cerebral oxidative stress and dopamine abnormalities in distressed mice. These effects of anthocyanin treatment suggest their possible usefulness for the treatment of cerebral disorders related to oxidative stress.

KEYWORDS: Anthocyanin; antioxidant activity; psychological stress; dopamine

INTRODUCTION

Currently, emotional stress has attracted attention for its significant role in cancer pathogenesis and is associated with increased oxidant production and oxidative tissue damage (*1–4*). There is strong evidence that oxidative stress participates in the etiology of neurodegenerative diseases, such as Parkinson's, and Alzheimer's diseases (*5, 6*). Moreover, emotional stress affects the central nervous system, which plays a vital role in the way an organism monitors internal and surrounding conditions through dopaminergic neurons (*7, 8*). Disruption of dopaminergic transmission affects behavioral activity and is related to the ability to "feel" the environment and make decisions on the basis of sensations, which change the emotional status of the individual; that is, survival through the attribution of incentive salience to significant environmental stimuli and contextual reward/avoidance learning (*9*). This unique ability has established dopamine (DA) as the principal neurotransmitter of motivated action, in the sense of physical and psychological movement associated with "pleasure" or away from "pain" (*10, 11*). Reactive oxygen species are also known to cause lipid peroxidation and protein carbonyl formation (*12*). Oxidative modifications of different intracellular proteins, including key enzymes and structural proteins, have been demonstrated to lead to the neurofibrillary degeneration of neurons in the Alzheimer's diseased brain (*13, 14*).

Recent epidemiological studies have shown that diets rich in fruits and vegetables are associated with a reduction in the risk of life style-related diseases. Anthocyanins (ACNs), the reddish-blue pigments present in a variety of plant tissues, are a widespread source of naturally occurring colorants of foods in the form of fruits, vegetables, and red wine, as examples (*15, 16*). Dietary intake has been estimated at up to 200 mg/day, which is higher than that of other flavonoids, such as quercetin (*17*). Several studies have indicated the potential antioxidant property of ACNs (*18–20*). ACNs have also been reported to have many physiological effects, such as vision improvement (*21*) and anticancer (*22*) and anti-inflammatory (*23*) activities. Recently, neuroprotective effects of ACNs were also reported (*24–26*).

Several animal stress models of various stressors, such as immobilization, burn shock, and cold-restraint, have been developed, and all cause oxidative damage of lipid, protein, and DNA in tissues (*27–29*). However, almost all of them are accompanied by physical abuse in addition to psychological or emotional stressors. Here, the simple cut whiskers model was used to cause psychological or emotional distress in mice,

* To whom correspondence should be addressed. Tel: +81-250-25-5126. Fax: +81-250-25-5127. E-mail: konishi@nupals.ac.jp.
[†] Faculty of Applied Life Sciences.
[‡] Faculty of Pharmaceutical Sciences.

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leading to oxidative stress in tissues that we have developed previously as a model of "Mibyo" (a subhealthy condition) (*4*). Whiskers play a critical role as a locomotive sensor in mice, and thus, sensory input is directly connected to motor neurons controlling their locomotive activity (*30*). Therefore, the removal of whiskers affected their locomotive behavior, causing anxiety leading to hyperlocomotion. The oxidative stress associated with psychological or emotional stress is an appropriate target for assessing the preventive potentiality of dietary supplements and functional foods against diseases.

In the present study, to assess the in vivo antioxidant activity of anthocyanins, an anthocyanin mixture, *Vaccinium myrtillis* L., was orally administered (100 mg/kg) to mice for 7 days, and then the mice were subjected to psychological stress by whisker cutting. The effects on tissue oxidative stress, especially on brain oxidative stress and DA status, were examined to evaluate the protective effect of anthocyanins on radical-mediated physiopathological conditions in the brain leading to aging, Alzheimer's diseases, arthritis, and pulmonary diseases among other conditions.

MATERIALS AND METHODS

Chemicals. Antidinitrophenyl (DNP) IgG developed in rabbit, 2,4-dinitrophenylhydrazine (DNPH), isoproterenol hydrochloride, dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were obtained from Sigma Co., Ltd., U.S.A. Antimouse rabbit IgG conjugated to horseradish peroxidase (HRP) was obtained from Zymed, U.S.A. 3,3′,5,5′-Tetramethylbenzidine (TMB) was obtained from Bio-Rad Laboratory, USA. Perchloric acid and EDTA were obtained from Cica. Citric acid monohydrate, trisodium citrate dehydrate, sodium 1-octanesulfonate, bovine serum albumin, bicinchoninic acid (BCA), streptomysin sulfate, and all other reagents were purchased from Wako Pure Chemical Industries Co. Ltd., Japan.

Bilberon 25 (powdered extract of bilberry (*V. myrtillus* L., bilberry)) was provided by Tokiwa Phytochemicals Co., Ltd. It contains 15 types of anthocyanins, and the net anthocyanin content was 38% (w/w) (*37*).

Animal Treatment. Male DDY 6-week-old mice were purchased from SLC Inc. (Japan). The mice were divided into four groups (control, control + ACN, stress, stress + ACN, $n = 9$ for each group) and habituated in a cage for a week at 24 °C with a 12-h dark/light cycle (light cycle starting from 7 a.m. and ending at 7 p.m.) under free access to a normal composite diet (KIC Laboratory MR Stock Co. Ltd., Japan) and water. To induce the oxidative stress by psychological stress, each mouse was seized by hand, and the whiskers around the nose and mouth were completely cut off with scissors without the use of anesthesia. For the control group, the same treatment was administered without cutting the whiskers. After the removal of whiskers, the mice were kept separately in a small square cage with free access to food and water. Powdered bilberon-25 (a mixture of ACNs) was dissolved in 0.1% citric acid solution and orally administered to the mice with a syringe once a day for seven days before stress was induced by whisker removal. The dose corresponded to 100 mg of original dry bilberon-25 per kg of body weight per day. For the control group, the same volume of 0.1% citric acid was given instead of the anthocyanin samples.

The mice were quickly sacrificed by breaking their necks just after anesthetizing them with diethyl ether at 12 h after the removal of whiskers (whiskers were removed at 9 p.m.). Tissues (brain, liver, kidney, and heart) were removed and rinsed in ice-cold physiological saline for biochemical assays immediately after sacrificing. After quick excision, the entire brain was dissected into three parts (brain cortex, midbrain, and cerebellum) using a modified method of Glowinski et al. (*31*). All samples were stored at −80 °C until use. All animal experiments were carried out under the guidance of the NUPALS Animal Regulation Code.

Protein Carbonyl Measurement. Tissues were suspended in ice-cold 0.05 M phosphate buffer containing 1.15% (w/v) KCl (1 g wet tissue per 7 mL), and homogenized in ice using an Ultra Turrax T8 homogenizer. The protein carbonyl contents were measured by ELISA (*32*). A standard curve was prepared using oxidized bovine serum albumin (BSA) obtained by oxidation of BSA with Cu^{2+}/H_2O_2 (300 μM per 5 mM). The carbonyl content of the oxidized BSA standard was determined using the colorimetric method reported previously (*33*). Tissue homogenates were centrifuged at 4000 rpm for 10 min to remove any debris, and the supernatants were incubated with 1% streptomycin sulfate for 15 min. The supernatant was used to determine the protein content by the BCA method, with BSA as the standard (*34*). The protein concentration was adjusted to 500 μg/mL with PBS, and samples were then reacted with 10 mM DNPH in 2 N HCL in darkness at room temperature for 1 h. The protein was precipitated with 20% trichloric acid, after which the precipitate was solubilized in PBST and the protein concentration was determined again. A standard ELISA curve was prepared for oxidized BSA that was diluted sequentially with intact BSA at a defined ratio (0−40%). Aliquots (100 μL) of test samples and standards (2 μg as protein) were placed in 96-well plastic plates and incubated overnight at 4 °C. The plates were then washed with PBS containing 0.1% Tween 20 (PBST) and incubated with blocking buffer (1% BSA in PBST) for 5 h at 4 °C. Samples incubated for additional 4 h with a primary antibody (anti-DNP rabbit IgG; Sigma) at 37 °C were washed with PBST, then reacted with a secondary antibody (antimouse rabbit IgG HRP conjugate) for 1 h. Peroxidase reactions were performed with the addition of TMB for 1 h and stopped with the addition of 0.18 M H_2SO_4. Absorbance was measured at 450 nm using a Bio-Rad model 550 micro plate reader.

Measurement of Thiobarbituric Acid Reactive Substance (TBARS). Tissues were suspended in ice-cold 0.2 M MES buffer containing 1.15% (w/v) KCl (1 g wet tissue per 7 mL) and homogenized in ice using an Ultra Turrax T8 homogenizer. Tissue homogenates were centrifuged at 4000 rpm for 10 min to remove any debris. The supernatant protein concentration was adjusted (10 mg/ml for liver, kidney and heart; 3.33 mg/mL for brain samples) with MES. The following were added to 0.2-mL aliquots of samples and incubated at 95 °C for 1 h: 1.5 mL of 20% acetic acid (pH = 3.5), 0.2 mL of 15% SDS, 1.5 mL of 0.8% 2-thiobarbituric acid, and 0.5 mL of MES. TBARS as an index of lipid peroxidation was measured spectrophotometrically (532 nm) after extraction into a butanol/pyridine mixture (vortexed for 1 min and centrifuged at 1200*g* for 15 min) using 1,1,3,3-tetraethoxypropane (Sigma) as a standard, as reported previously (*35*). The levels of lipid peroxides were expressed in terms of nanomoles of TBARS per gram of wet tissue.

Determination of DA, DOPAC, and HVA Levels in Different Parts of the Brain. The brain regional concentrations of DA, DOPAC, and HVA were determined by a modification of the high-performance liquid chromatographic (HPLC) assay of Murai et al. (*36*) using isoproterenol (Wako) as the internal standard. The brain samples (0.05 g) were put into glass test tubes and homogenized with a Polytron homogenizer (PT 10-35, Kinematica, Switzerland) at 15 000 rpm for 10 s in 500 μL of ice-cold 0.1 M perchloric acid containing 10 μM 2Na−EDTA and isoproterenol (100 ng). After centrifugation at 4000 rpm for 10 min at 4 °C, the clear supernatants were filtered through a 0.45-μm filter (disposable syringe filter, dismic-3 cP cellulose acetate, Advantec, Tokyo, Japan), and 5 μL of the filtrate was loaded onto a reversed-phase HPLC column (Supelcosil LC-18-DB, Supelco). The solvent delivery system (L-5000 LC controller and 655A-11 pump, Hitachi, Tokyo, Japan) was equipped with an electrochemical detector (ECD-100, Eicom, Kyoto, Japan) operating at +0.45 V versus a Ag−AgCl reference electrode, an autosampler (AS-8010, Tosoh, Tokyo, Japan), and a chromatointegrator (D-2500, Hitachi). A guard column (Supelcosil LC-18-DB, Supelco) was placed between the autosampler and the analytical colum. The mobile phase was 0.01 M citrate buffer (pH 4.4)−MeOH (85:15 v/v) containing 10 μM 2Na−EDTA and 0.5 mM sodium 1-octanesulfonate, and the flow rate was 0.5 mL/min. Retention times were 11, 21, 24, and 33 min for DOPAC, HVA, DA, and the internal standard, respectively. To test the linearity of the calibration curve, various amounts of DA, DOPAC, and HVA, ranging from 25 to 1000 ng concentrations, were prepared. Linear relationships were obtained, and the regression lines were $y = 0.2094x + 0.2222$ ($R^2 = 0.9922$), $y = 0.1196x + 0.1157$ ($R^2 = 0.9915$), and $y = 0.0417x$

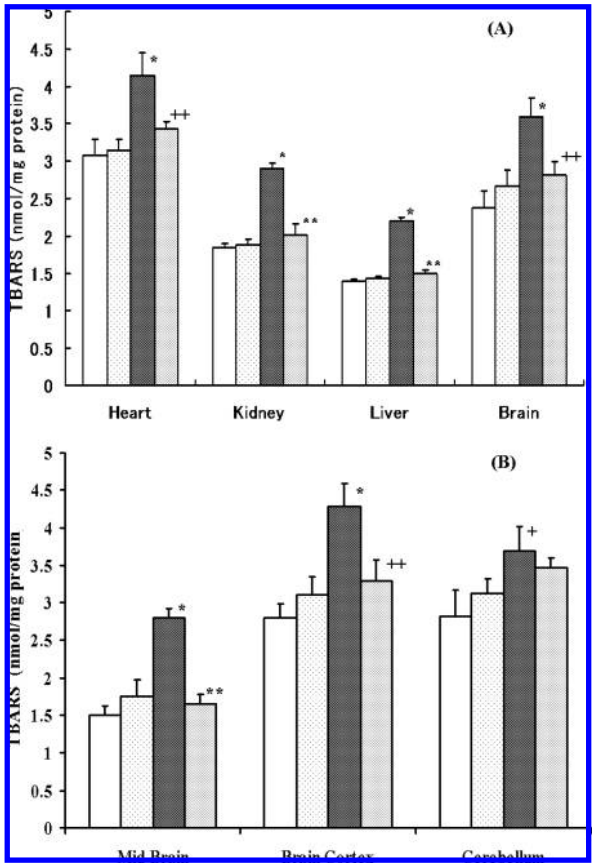


Figure 1. Preventive effects of ACN on TBARS formation in (**A**) various tissues, and in (**B**) specific brain regions (midbrain, brain cortex, cerebellum) in mice stressed by whisker cutting. ACN was orally administered once a day for 7 days before whisker removal. At 12 h after whisker removal, tissues were removed and homogenized, and the levels of TBARS formation were determined. Data represent means ± SEM (n = 9 mice). *, $p < 0.01$; +, $p < 0.05$ versus untreated control mice. **, $p < 0.01$; +2, $p < 0.05$ versus stressed mice. Control, control + ACN, stress, stress + ACN.

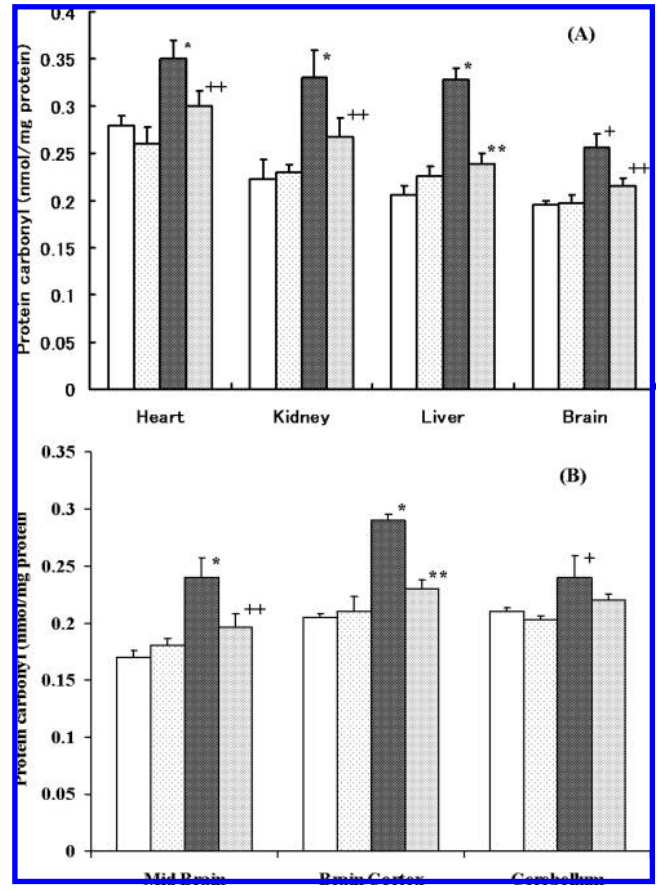


Figure 2. Preventive effects of ACN on protein carbonyl formation in (**A**) tissues and (**B**) specific brain regions (midbrain, brain cortex, cerebellum) in whisker-cut stressed mice. ACN was orally administered once a day for 7 days before whisker removal. At 12 h after whisker removal, tissues were removed to determine protein carbonyl formation. Data represent means ± SEM (n = 9 mice). *, $p < 0.01$; +, $p < 0.05$ versus untreated control mice. **, $p < 0.01$; +2, $p < 0.05$ versus stressed mice. Control, control + ACN, stress, stress + ACN.

− 0.0171 (R^2 = 0.9946) for DA, DOPAC, and HVA, respectively, where y is the peak-area ratio and x is the amount of DA, DOPAC, or HVA. The coefficient of variation was 3% or less in these concentration ranges on the calibration curve. Detection limits of sensitivity to these substrates (DA, DOPAC, and HVA) based on a signal-to-noise ratio of 3 were determined by injection of diluted standard solutions. The detection limits of DA, DOPAC, and HVA were all about 2.5 ng respectively, under this condition.

Statistical analysis. Statistical analysis was carried out using paired and unpaired Student t tests. The results are expressed as means ± SEM ($n = 3 \times 3$) and were considered statistically significant when $p \leq 0.05$.

RESULTS

Tissue Oxidative Stress Induced by Whisker Removal in Mice. Tissue oxidative injuries were measured to confirm whether oxidative stress occurs under the psychologically distressed condition. Cellular oxidative markers for lipid (TBARS) and protein (carbonyl formation) were significantly increased in tissues or organs. The TBARS levels were increased by about 22, 35, and 30% in heart, kidney, and liver, respectively, indicating the occurrence of psychological depression-induced oxidative stress (**Figure 1A**). Further, regional differences in TBARS levels were observed in the brain, such as by about 40, 35, and 15% in the midbrain, cortex, and cerebellum, respectively (**Figure 1B**). However, anthocyanin-treated mice showed less lipid peroxidation in all of these tissues, indicating that ACN is an active antioxidant in vivo. The TBARS levels were reduced by 13, 25, 27, 35, 25, and 8% in the heart, kidney, liver, midbrain, brain cortex and cerebellum, respectively, as compared with those in distressed mice (**Figure 1**).

The same tendency was observed for protein carbonyls measured as a marker of protein oxidation by ELISA. Whisker removal significantly increased protein carbonyl levels: by ∼20, 30, and 35% in heart, kidney and liver (**Figure 2A**) and by 25, 28, and 9% in the midbrain, brain cortex, and cerebellum, respectively (**Figure 2B**), as compared with the controls. Orally administered ACN reduced protein oxidation markedly in the kidney, liver, midbrain, and brain cortex (**Figure 2**). The protein carbonyl levels in the tissues of ACN-administered mice were maintained at almost the same levels as those in the tissues of the control mice; the same was true of TBARS levels.

Effect of ACN on DA, DOPAC, and HVA Levels. The levels of DA and its metabolites, DOPAC and HVA, were measured in the midbrain, brain cortex, and cerebellum. The DA level was decreased by around 35% in the midbrain, whereas it was increased by around 40% in the brain cortex. Both DA metabolites, DOPAC and HVA, showed the same trend as DA. In ACN-treated mice, the stress-induced shift of DA in the brain was almost completely suppressed, and the DA levels were the same as those in the control brain regions,

indicating that preadministration of ACN increased the stress resistance potential (**Figure 3A**; 3**B**). Compared with other brain regions, the cerebellum showed about 20 times lower concentrations of DA, but the level was increased by 40% after stress exposure (**Figure 3C**). In ACN-treated mice, the DA levels were fairly well maintained in the midbrain, brain cortex, and cerebellum (**Figure 3**).

DISCUSSION

Bilberry extract (bilberon-25) is rich in anthocyanosides. Over 15 different anthocyanosides have been found in bilberon-25 (*37*). We previously reported the potent antioxidant activity of 15 anthocyanins present in bilberry extract toward superoxide, AAPH, the hydroxyl radical, and peroxynitrite (*18, 38*). Moreover, we clearly showed that the antioxidant activity of bilberon-25 is almost dependent on the anthocyanins contained (*18*). We also suggested that the oxidative stress is associated with a subhealthy condition, "Mibyo" (*4*). The condition was defined in ancient oriental medicines as neither ill nor completely healthy, and the treatment for this condition was recognized to be more important than treating the end-point disease (*39*). This idea seems quite important in current preventive medicine to treat complex lifestyle- and age-related diseases.

Whisker removal induced oxidative stress in tissues so that the levels of both protein carbonyl and TBARS formation were significantly increased (**Figure 1**, **2**), as seen in other stress models (*27–29*). Since physical abuse contributes less in this model than in other stress models, such as immobilization (*27*), electric shock (*28*), and water-immersion (*29*), the oxidative stress observed here is mainly due to the psychological or emotional stress induced by whisker removal. The levels of DA in different brain regions were significantly altered (**Figure 3**), indicating the occurrence of neuromodulation in this stress model. Several neurotransmitters are directly related to mental health (*40, 41*). A decline in the levels of DA in certain brain regions can cause a loss of neurocognitive functions, including memory, attention, problem resolution, movement, emotional response, and emotional capacity to feel pleasure or pain (*40, 42*).

Currently, ACN is attracting much attention as a dietary antioxidant supplement because of its high antioxidant property (*43*). The present study has revealed that orally administered ACN markedly suppressed stress-induced oxidative stress in the tissues of distressed mice (**Figure 1**, **2**). The extent of suppression of lipid peroxidation and protein carbonyl formation were varied in different tissues and brain regions, but the tendency was quite similar for both biomarkers in tissues. Therefore, a radical scavenging property of ACN might play a critical role in preventing tissue oxidative stress, as suggested elsewhere (*44*). Although ACN suppressed both oxidative stress markers in the brain, especially in the midbrain and brain cortex, it is not yet conclusive that orally administered ACN itself reached the brain through the blood−brain barrier to function as an antioxidant. The tissue existing form in the brain was not determined. In our previous studies, we showed that methylated metabolites are the major species in liver and kidney (*45, 46*). Thus, further studies are necessary to show whether the methylated ACN is associated with observed neuroprotection in the present study, although some studies have suggested that ACN can enter the brain through the blood brain barrier and have a protective effect (*24, 47*).

The protective effect of ACNs against neurotoxicity was also evaluated in terms of the levels of brain DA and its metabolites, as determined by HPLC. Mouse brains were divided into three parts. The midbrain containing the hippocampus and striatum

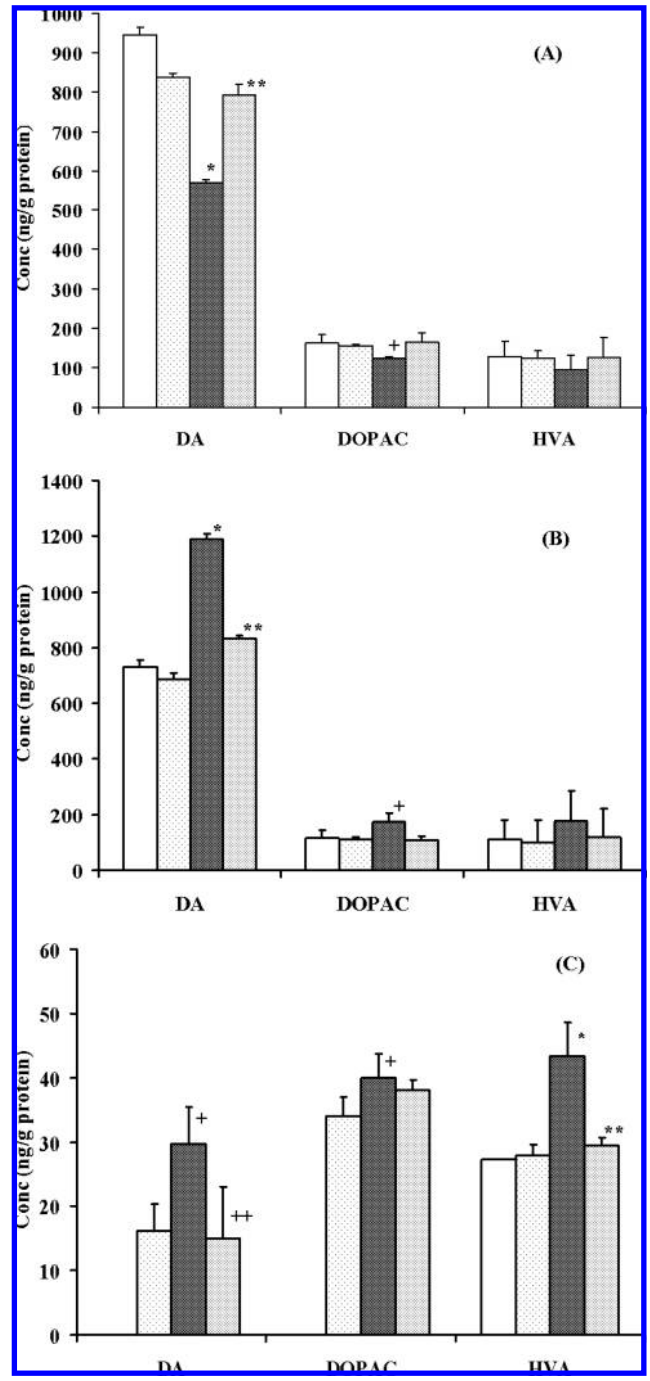
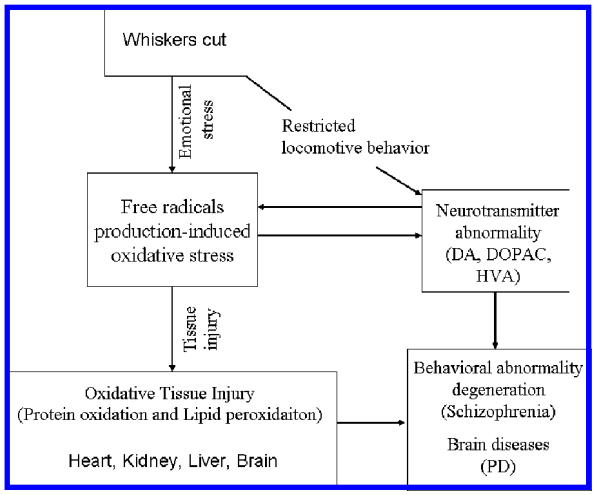


Figure 3. Preventive effects of ACN on neurotransmitter disorders in the (**A**) midbrain, (**B**) brain cortex, and (**C**) cerebellum in the mice stressed by whisker cutting. ACN was orally administered once a day for 7 days before whisker removal. At 12 h after whisker removal, tissues were removed to determine the levels of DA, DOPAC, and HVA by HPLC. Data represent means ± SEM ($n = 9$ mice). *, $p < 0.01$; +, $p < 0.05$ versus untreated control mice. **, $p < 0.01$; +2, $p < 0.05$ versus stressed mice. Control, control + ACN, stress, stress + ACN.

showed a significant reduction in the levels of DA and its metabolites, DOPAC and HVA, after stress exposure (**Figure 3A**), but this change was almost completely protected by ACN. This finding is quite interesting because DA functions as a neurotransmitter in several neural pathways in the brain. Among these, mesolimbic and nigrostriatal pathways are linked in the midbrain and striatum. A decrease in dopamine levels causes a decline in neurocognitive functions, especially memory, attention, and problem-resolution. However, recent research has

Scheme 1. Tissue Oxidative Stress Induced by Psychological or Emotional Stress



pointed out that the dopamine pathway plays a role when incentive salience is involved, rather than euphoric mood states. A marked reduction in DA function in the nigrostriatal region of the brain is one of the main pathological features of Parkinson's disease; however, the symptoms of the disease typically do not show up until 80−90% of dopamine function has been lost (*42, 48*).

The results of the present study suggest that psychological stress-induced oxidative stress is a causative factor reducing the number of dopaminergic neurons and that ACN might be a protective antioxidant for degenerative pathogenesis. On the other hand, the levels of DA and its metabolites were drastically increased in the frontal lobe containing the brain cortex when the mice were distressed in the present study, by around 40%, as compared with control mice (**Figure 3B**). This enhanced DA excretion may be reasonable on the basis of the following discussion. In the frontal lobes, DA controls the flow of information from other areas of the brain. The mesocortical pathway is a neural pathway that connects to the cortex and is involved in motivation, schizophrenia, avolition, and alogia. An increase in DA levels in this region causes pathological and behavioral disorders, such as delusions and auditory hallucinations, and these are typically regarded as manifestations of psychosis. Despite the appearance of blunted affect, recent studies have indicated that there is often a normal or even heightened level of emotionality in schizophrenia patients, especially in response to stressful events (*49, 50*).

In the cerebellum, we detected a very small amount of DA as compared with other brain regions, but the change was very clear in the stress model (**Figure 3C**). This region of the brain plays an important role in sensory perception and motor control. It contains more than 50% of all neurons in the brain, but it represents only 10% of the total brain volume. Increasing DA levels in this region might accelerate motor skills and movement. In our previous study, we found that psychologically stressed mice showed increased automotive activity as compared with the control mice. Further, it was revealed that orally administered Shengmai San, a TCM formula, was active in the brain and suppressed stress-induced cerebral oxidative stress in distressed mice (*4*). Considering all of these pieces of information together, we summarized the process of oxidative stress induced by whisker removal in **Scheme 1**. It was thus suggested that ACN is possibly useful for the protection and treatment of neurodegenerative diseases associated with oxidative stress, such as Alzheimer's and Parkinson's diseases.

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