Alterations in Regional Brain Neurotransmitters by Silymarin, a Natural Antioxidant Flavonoid Mixture, in BALB/c Mice

Marcin F. Osuchowski1,2, Victor J. Johnson1, Quanren He1 and Raghubir P. Sharma1

1Department of Physiology and Pharmacology, College of Veterinary Medicine, The University of Georgia, Athens, Georgia, USA; 2Department of Animal Anatomy, Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, Poland

Abstract

Silymarin, a natural antioxidant flavonoid mixture, exerts anti-inflammatory effects in the liver and hinders tumor formation. The effect of this flavonoid mixture on the central nervous system is unknown, although antioxidants are considered beneficial. Brain amines and metabolites were studied after a short-term silymarin treatment. BALB/c mice were intraperitoneally treated with 0, 10, 50, or 250 mg/kg of silymarin per day for 5 days. High-performance liquid chromatography coupled with electrochemical detection was performed to determine concentrations of norepinephrine (NE), dopamine (DA), dioxypheynalactic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT, serotonin) and 5-hydroxyindoleacetic acid (5-HIAA) in discrete brain regions. Analyses showed increased 5-HT levels in the cortex and increased DA and NE levels in the cerebellum in the highest dose group. Results indicated lack of general adverse effect on the brain amine metabolism and suggest that silymarin may have marginal serotonergic, dopaminergic, and noradrenergic effects.

Keywords: Brain biogenic amines, dopamine, flavonoids, mouse, neurotransmitters, norepinephrine, serotonin, Silybum marianum, silymarin.

Introduction

Silymarin is a mixture of naturally occurring polyphenolic flavonoid antioxidants found in the milk thistle, Silybum marianum (L.) Gaertn (Asteraceae). This annual or biennial plant grows principally in Europe and in limited range in some parts of United States. The highest concentration of bioactive flavonoids is present in the fruit portion of the plant but can also be extracted from its seeds and leaves. Betaine, trimethylglycine, and vital fatty acids have also been found in the plant's seed, presumably increasing the overall protective activity of crude extracts. These extracts have been used as a natural remedy in herbal medicine for centuries. Silymarin is composed of several flavonoids possessing pharmacological activity including silybin, isosilybin, silicristin, and silydianin. Silybin appears to be the most biologically active element of the blend (Silybum marianum monograph, 1991). However, the exact ratio of the silymarin constituents is not constant, as the flavonoid mixture is derived exclusively from natural sources.

Several studies have revealed that silymarin is a potent anti-inflammatory agent modulating the functions of different cells involved in this process (Middleton et al., 2000). This beneficial property led to the current use of silymarin in diseases of the liver and biliary tract (Jacobs et al., 1991). Evidence suggesting an antitumor effect of silymarin on malignant tumor formation, especially toward cancers derived from epithelial cells such as breast or prostate, has been also provided in recent years (Jiang et al., 2000).

The potent antioxidant capacity of flavonoids also may play a positive role in specific brain pathologies, as it has been shown that lipid peroxidases accumulate in damaged brain tissue. In spite of the wide use of silymarin as a peripherally active herbal remedy in modern medicine, there is a lack of information regarding possible side effects, adverse or beneficial, on the central nervous system.

The interaction between the nervous system and flavonoids has gained little attention, even in view of flavonoids to pass across the blood-brain barrier as lipid-soluble...
components. Existing data on possible adverse or beneficial effects of flavonoids is limited. Flavonoids can influence the metabolism of brain amines in human neuronal and neuroendocrine cell lines. Diosmetin has been shown to inhibit dopamine and serotonin uptake in control and differentiated neuroblastoma cells, and genistein hinders initiation of neuronal protein synthesis (Middleton et al., 2000). Baicalein \textit{[Scutellaria baicalensis Georgi (Labiatae)]} exerts potent inhibitory effects on nerve growth factor (NGF)-stimulated nerve fiber growth in pheochromocytoma PC12 cells (Middleton et al., 2000). In B 104 rat neuronal cells, reversible and dose-dependent apigenin-induced inhibition of proliferation was reported (Middleton et al., 2000).

The purpose of the current study was to examine the potential short-term effects of silymarin on neurotransmitters and metabolite levels in the discrete brain regions of BALB/c mice. We hypothesized that brain amine levels will be modified after a subacute exposure of animals to silymarin due to its possible entry into the central nervous system coupled with antioxidant properties.

\section*{Materials and Methods}

\subsection*{Animals}

Male, 7–8-week-old BALB/c mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and acclimated for 1 week prior to treatment at 21°C, 50% humidity, and 12 h light/dark cycle at the University of Georgia Animal Resources facility. Mice were maintained on commercial rodent chow and deionized water \textit{ad libitum}. Initial body weights ranged between 19 and 23.5 g. Protocols for animal use followed the Public Health Service Policy on Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee.

\subsection*{Chemicals}

Silymarin (product number: 254924) was purchased from Sigma-Aldrich Chemical Company Inc. (St. Louis, MO, USA). The mixture is composed of taxifolin (4%), silichristin (27.9%), silidianin (2.9%), silybin A (19.3%), silybin B (31.3%), isosilybin A (8.2%), and isosilybin B (2.3%), as determined by high-performance liquid chromatography (HPLC) 227-nm detection, and this is comparable to several commercial formulae described elsewhere (Campodonico et al., 2001).

\subsection*{Treatments}

Mice (six per group) were treated daily with intraperitoneal injection of phosphate buffered saline (PBS), vehicle, or 10, 50, and 250 mg/kg of silymarin suspended in PBS for the duration of 5 days. The employed route of exposure has previously been used to investigate the protective effects of silymarin in CCl\textsubscript{4}-induced liver damage (Letteron et al., 1990).

The protocol followed our recent experiments describing effects of silymarin on organ systems (He et al., 2002; Johnson et al., 2002). Food and water consumption and weight gain were recorded daily.

\subsection*{Sampling and HPLC assay}

Twenty-four hours following the last injection, mice were decapitated and brains were immediately dissected on ice into the following regions: cerebellum, medulla oblongata, hypothalamus, corpus striatum, midbrain, hippocampus, and cortex (Glowinski et al., 1966). Midbrain region included thalamus and subthalamic regions. Collected tissue samples were immediately placed in 4 volumes of ice-cold 0.05 M perchloric acid with 0.1% cysteine (Sigma) in tared vials and weighed. Samples were homogenized and centrifuged at 12,000 \texttimes g for 5 min at 4°C. Supernatants were filtered using ca 0.2-\mu filter (Poretics, Livermore, CA, USA) by centrifugation. The filtrate was stored at \textminus85°C until analysis.

Concentrations of norepinephrine (NE), dopamine (DA), dioxophenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT, serotonin), and 5-hydroxyindoleacetic acid (5-HIAA) in each extract were determined by HPLC coupled with electrochemical detection as previously described (Tsunoda et al., 2000). The analytical system was composed of Model L-ECD-6A electrochemical detector, Model LC-10 AD solvent delivery module, GT 104 degasser, Model SIL-10A automatic injector (Shimadzu, Columbia, MD, USA) and a Model CH-30 temperature controller with a column heater (Eppendorf, Madison, WI, USA). A reversed-phase Lichrosorb RP-18, 4 \times 125 mm column (EM Science, Gibbstown, NJ, USA) was employed for the assay. The signals were analyzed using a Chromatopac C-R501 integrator (Shimadzu). The mobile phase and chromatographic conditions were similar to those described previously (Mayer et al., 1983).

\subsection*{Statistics}

The mean concentration (\mu g/g wet tissue) of different neurotransmitters and metabolites in each brain region was calculated. Data were analyzed by single-factor analysis of variance (ANOVA) followed by Duncan multiple range test using the SAS software (SAS Institute, Cary, NC, USA).

\subsection*{Results}

There were no grossly apparent neurotoxic effects in any animal throughout silymarin treatment. Body weights showed no significant changes among treatments and the control groups. No differences were noted in the discrete brain region weights or absolute brain weights among animals in these groups (data not shown).

Intraperitoneal treatment with silymarin affected cortical (Fig. 1) and cerebellar (Fig. 2) brain amine concentrations.
in BALB/c mice. In cortex, 5-HT concentration was significantly increased by 17% at the highest (250 mg/kg) treatment group compared to control (Fig. 1). The concentrations of 5-HT and its metabolite (5-HIAA) in discrete brain regions are presented in Table 1. Cortical DA in the high-dose group (250 mg/kg) showed relatively higher levels compared to the control and low-dose group (10 mg/kg), although changes were not statistically significant (Table 2). A similar trend was noted for cortical and striatal NE (Table 3).

In cerebellum, alterations were observed in the levels of two major neurotransmitters: NE and DA (Fig. 2). Concentrations of NE in different brain regions are indicated in Table 3, whereas DA and its metabolites (DOPAC, HVA) are presented in Table 2. NE level was increased by 32%, whereas DA concentration was increased by 53% compared to the control in cerebellum at the highest dose (250 mg/kg). Levels of cerebellar DA metabolites (DOPAC, HVA) remained unaltered (Table 2).

Significant changes were also noted for DA in striatum and 5-HT in hippocampus: increased striatal DA (Table 2) and reduced hippocampal 5-HT levels (Table 1). However, these alterations were observed at the 250 mg/kg (for DA also at 50 mg/kg) dose level compared to the 10 mg/kg dose group, but not to the control group. No treatment-related differences occurred in concentrations of neurotransmitters and metabolites assessed in the medulla, hypothalamus, and midbrain regions (Tables 1, 2, and 3).

**Discussion**

Currently, silymarin is widely recognized as an effective medicine in liver and biliary tract pathologies (*Silybum marianum* monograph, 1991). Its hepatoprotective properties exhibit various pathways of action. Inhibition of lipid peroxidation and membrane stabilization, decreased glutathione depletion, and ability to scavenge and quench reactive oxygen species (ROS) are based on the strong antioxidant capacity of the flavonoids (Middleton et al., 2000). Silymarin also showed a protective role against deterioration of hepatic tissue by reducing the collagen level and decreasing elevated activity of serum and hepatic esterase in carbon tetrachloride (CCl₄)-induced cirrhosis in the rat (Middleton et al., 2000). In patients with different clinical forms of liver pathology, treatment with silymarin was shown to normalize total bilirubin levels, serum liver enzymes, and promote the liver tissue regeneration (*Silybum marianum* monograph, 1991).

Because there are no existing data on the influence of silymarin on the central nervous system, we investigated the possible influence of silymarin on biogenic amines in discrete brain regions in BALB/c mice. The presented results show that there were only marginal effects of short-term silymarin treatment at the doses employed on neurotransmitter levels in mouse brain. The highest dose of silymarin in this study corresponds to approximately 17.5 g daily in a 70-kg person, not considering the adjustment of the dose to surface area or...
Table 1. Concentrations of serotonin (5-hydroxytryptamine; 5-HT) and metabolite 5-hydroxyindoleacetic acid (5-HIAA) in discrete brain regions of mice treated with silymarin.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Silymarin (mg/kg)</th>
<th>Cerebellum</th>
<th>Medulla</th>
<th>Hypothalamus</th>
<th>Striatum</th>
<th>Midbrain</th>
<th>Hippocampus</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>Control</td>
<td>0.181 ± 0.034*</td>
<td>0.647 ± 0.044</td>
<td>0.255 ± 0.041</td>
<td>0.154 ± 0.024</td>
<td>0.713 ± 0.052</td>
<td>0.246 ± 0.024</td>
<td>0.424 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.194 ± 0.023</td>
<td>0.642 ± 0.030</td>
<td>0.251 ± 0.030</td>
<td>0.160 ± 0.027</td>
<td>0.789 ± 0.030</td>
<td>0.325 ± 0.031</td>
<td>0.436 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.203 ± 0.012</td>
<td>0.687 ± 0.033</td>
<td>0.228 ± 0.036</td>
<td>0.209 ± 0.017</td>
<td>0.831 ± 0.049</td>
<td>0.276 ± 0.033</td>
<td>0.468 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.186 ± 0.015</td>
<td>0.664 ± 0.016</td>
<td>0.254 ± 0.020</td>
<td>0.210 ± 0.021</td>
<td>0.626 ± 0.098</td>
<td>0.191 ± 0.035**</td>
<td>0.497 ± 0.025*</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>Control</td>
<td>0.069 ± 0.007</td>
<td>0.138 ± 0.004</td>
<td>0.070 ± 0.008</td>
<td>1.912 ± 0.274</td>
<td>0.161 ± 0.006</td>
<td>0.117 ± 0.010</td>
<td>0.104 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.066 ± 0.005</td>
<td>0.137 ± 0.002</td>
<td>0.074 ± 0.006</td>
<td>2.367 ± 0.465</td>
<td>0.166 ± 0.006</td>
<td>0.114 ± 0.007</td>
<td>0.114 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.063 ± 0.003</td>
<td>0.158 ± 0.009</td>
<td>0.078 ± 0.009</td>
<td>1.710 ± 0.071</td>
<td>0.173 ± 0.009</td>
<td>0.108 ± 0.010</td>
<td>0.113 ± 0.014</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.058 ± 0.004</td>
<td>0.136 ± 0.007</td>
<td>0.077 ± 0.006</td>
<td>2.007 ± 0.112</td>
<td>0.137 ± 0.007</td>
<td>0.149 ± 0.021</td>
<td>0.139 ± 0.005</td>
</tr>
</tbody>
</table>

*Mean ± SEM (n = 6).
*p < 0.05 compared with control group by Duncan multiple range test; **p < 0.05 compared with 10mg/kg group by Duncan multiple range test.

Table 2. Concentrations of dopamine (DA) and metabolites dixhydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in discrete brain regions of mice treated with silymarin.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Silymarin (mg/kg)</th>
<th>Cerebellum</th>
<th>Medulla</th>
<th>Hypothalamus</th>
<th>Striatum</th>
<th>Midbrain</th>
<th>Hippocampus</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>Control</td>
<td>0.137 ± 0.004*</td>
<td>0.107 ± 0.005</td>
<td>0.331 ± 0.022</td>
<td>5.879 ± 0.424</td>
<td>0.299 ± 0.010</td>
<td>0.266 ± 0.051</td>
<td>0.760 ± 0.029</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.152 ± 0.023</td>
<td>0.118 ± 0.006</td>
<td>0.343 ± 0.030</td>
<td>5.036 ± 0.454</td>
<td>0.312 ± 0.016</td>
<td>0.397 ± 0.099</td>
<td>0.661 ± 0.038</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.158 ± 0.014</td>
<td>0.128 ± 0.008</td>
<td>0.392 ± 0.051</td>
<td>6.842 ± 0.349**</td>
<td>0.298 ± 0.012</td>
<td>0.313 ± 0.115</td>
<td>0.857 ± 0.129</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.211 ± 0.014*</td>
<td>0.110 ± 0.004</td>
<td>0.379 ± 0.063</td>
<td>6.952 ± 0.569**</td>
<td>0.280 ± 0.015</td>
<td>0.340 ± 0.124</td>
<td>0.947 ± 0.157</td>
</tr>
<tr>
<td>DOPAC</td>
<td>Control</td>
<td>0.010 ± 0.002</td>
<td>0.026 ± 0.006</td>
<td>0.080 ± 0.060</td>
<td>0.876 ± 0.095</td>
<td>0.043 ± 0.006</td>
<td>0.056 ± 0.012</td>
<td>0.098 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.018 ± 0.002</td>
<td>0.048 ± 0.012</td>
<td>0.069 ± 0.006</td>
<td>0.708 ± 0.047</td>
<td>0.062 ± 0.008</td>
<td>0.073 ± 0.019</td>
<td>0.105 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.014 ± 0.001</td>
<td>0.038 ± 0.007</td>
<td>0.053 ± 0.015</td>
<td>0.702 ± 0.011</td>
<td>0.055 ± 0.009</td>
<td>0.066 ± 0.013</td>
<td>0.103 ± 0.017</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.018 ± 0.002</td>
<td>0.037 ± 0.004</td>
<td>0.045 ± 0.014</td>
<td>0.679 ± 0.038</td>
<td>0.039 ± 0.008</td>
<td>0.073 ± 0.022</td>
<td>0.107 ± 0.006</td>
</tr>
<tr>
<td>HVA</td>
<td>Control</td>
<td>0.018 ± 0.001</td>
<td>0.060 ± 0.015</td>
<td>0.084 ± 0.004</td>
<td>1.017 ± 0.074</td>
<td>0.246 ± 0.016</td>
<td>0.078 ± 0.016</td>
<td>0.193 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.021 ± 0.001</td>
<td>0.058 ± 0.017</td>
<td>0.102 ± 0.010</td>
<td>0.909 ± 0.080</td>
<td>0.254 ± 0.017</td>
<td>0.118 ± 0.024</td>
<td>0.183 ± 0.034</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.018 ± 0.002</td>
<td>0.080 ± 0.022</td>
<td>0.101 ± 0.012</td>
<td>0.885 ± 0.032</td>
<td>0.238 ± 0.012</td>
<td>0.096 ± 0.024</td>
<td>0.224 ± 0.015</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.028 ± 0.003</td>
<td>0.041 ± 0.006</td>
<td>0.126 ± 0.023</td>
<td>0.956 ± 0.071</td>
<td>0.230 ± 0.027</td>
<td>0.076 ± 0.017</td>
<td>0.235 ± 0.012</td>
</tr>
</tbody>
</table>

*Mean ± SEM (n = 6).
*p < 0.05 compared with control group by Duncan multiple range test; **p < 0.05 compared with 10 mg/kg group by Duncan multiple range test.
The specific dopamine response to silymarin treatment is interesting. A relatively high increase of DA (53%) was observed only in the cerebellum. The striatal DA and hippocampal 5-HT differences between the 250 and 10 mg/kg dose groups, but not from the control, are difficult to interpret. There may be a biphasic effect of silymarin, but this cannot be proven based on the current data.

Significantly increased cerebellar DA and NE were observed in the highest dose group. Previous studies showed the neuronal activity existing in the cerebellar region is implicated in different motor functions. In fact, a clear connection between cerebellum/striatum-related dopaminergic and noradrenergic pathways and the cerebellar region is implicated in motor functions. The role of cerebellum and striatum in motor skill acquisition in patients with Parkinson’s disease or cerebellar dysfunction has been shown earlier (Schugens et al., 2001). The importance of DA in the motor system is supported by the study of Mathangi et al. (2001), showing that chronic protein restriction in Wistar rats resulted in impaired motor coordination that correlated with a decrease of DA, HVA, and NE in corpus striatum and cerebellum. Moreover, cerebellar norepinephrine depletion was shown to result in impaired locomotor tasks (Watson & McElligott, 1984) or motor learning deficits in aged rats (Bickford, 1994). An increase in NE and DA concentrations noted in our study warrants further investigation of a possible relation between silymarin and locomotor activity via dopaminergic/noradrenergic pathways using higher doses of the flavonoid mixture employed in the current study.

Interestingly, evidence from in vitro studies using related flavonoid compounds indirectly supports the hypothesis of interaction between silymarin and certain neurotransmitters. Diosmetin inhibited 5-HT and DA uptake in all cell lines studied (Middleton et al., 2000). Similarly, a flavonoid-containing extract of Hypericum perforatum Linnaeus (Clusiaceae) has been shown to significantly inhibit uptake of NE and 5-HT in astrocytes (Neary & Bu, 1999). More importantly, it was reported that silymarin directly suppressed activity of monoamine oxidase (MAO) in the C6 astrocyte cell line (Mazzio et al., 1998).

Generally, the effects observed in our experiment were evident only in the highest dose group and with no apparent symptoms of neurobehavioral toxicity during the period of treatment. This suggests that overall effects of silymarin treatment on brain amines are minor and justifies the conclusion that silymarin shows no immediate adverse effects regarding brain amine homeostasis at the doses used and under the conditions employed in our experiment. However, the reported changes, although limited to discrete regions, may suggest a possible link between silymarin and serotonergic/dopaminergic/noradrenergic pathways. To confirm the specificity of this association and to investigate how silymarin may modulate these important neurotransmitter systems, further studies using a longer treatment regimen will be needed.
The concentration of several neurotransmitters, although significantly altered compared to the control group, remained still within physiological ranges. These observations suggest safety of silymarin use in terms of neurological functions. Specific behavioral parameters were not evaluated in the current study, therefore the importance of elevated levels of cortical 5-HT (along with observed increasing trend for DA), cerebellar DA and NE with cognition/general activation control and locomotor activity, are not apparent. It would be beneficial to expand further research in this direction; especially while employing the long-term oral treatment model to more closely emulate human exposures.

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References


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