Evaluation of Spirulina Supplementation on Intermittent Binge Ethanol - Induced Neurotoxicity in Dentate Gyrus of Rats

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Summary. Spirulina is a widely used nutritional supplement which is rich in antioxidants and proteins. Studies have shown that intermittent binge-like ethanol consumption during adolescent period caused neuronal damage in specific parts of the brain, including the dentate gyrus. It has been suggested that antioxidant therapy may provide some level of protection against neurotoxicity of ethanol at cellular level. The purpose of this study was to examine the preventive effects of spirulina supplementation on ethanol-induced neurotoxicity in the dentate gyrus of adolescent rats. Male Sprague-Dawley rats were given ethanol (10 g/kg/day, intermittent binge model), or spirulina platensis (1000 mg/kg/day) or both from postnatal day 30 for two weeks duration. The cerebral hemispheres were processed for routine histological staining and immunohistochemistry with anti-GFAP antibody. Ethanol-treated group showed significant deficit in the numbers of granule cells and hilar neurons of the dentate gyrus when compared to the control group. Spirulina supplementation failed to provide protection against ethanol-induced neuronal loss. Spirulina supplementation also failed to alter increased expression of GFAP immunoreactivity induced by ethanol exposure. In conclusion, these findings indicate that spirulina supplementation is not effective in reducing the ethanol-induced neurotoxicity in the dentate gyrus of adolescent rats.

Industrial Relevance. Spirulina is one of the widely used nutritional supplements particularly in Asian population. Being a strong antioxidant, spirulina has been shown to have many therapeutic effects in human. However, the question of whether spirulina supplementation is able to mitigate the effect of ethanol neurotoxicity is largely unknown. Therefore, the study was undertaken to investigate the possibility that spirulina supplementation is able to provide some protection against ethanol-induced neurotoxicity in a rat model.

Keywords. Spirulina; Antioxidants; Ethanol; Dentate gyrus; GFAP

INTRODUCTION

Spirulina is a blue-green alga (cyanobacterium) that has been consumed as food since ancient times. Nowadays, spirulina is widely marketed as a food supplement as it is believed to have many therapeutic benefits. It is also being incorporated into various food products and beverages to enhance their nutritional values (Chu et al. 2010; Deng and Chow 2010). There have been many investigations on the therapeutic effects of spirulina. Spirulina supplementation has been reported to help prevent or manage hypercholesterolemia, certain inflammatory diseases, allergies, cancers, viral infections, cardiovascular diseases, diabetes and other metabolic disorders. Many of the health benefits of spirulina are believed to be due to its high anti-oxidative activities (Mani et al. 2008; Belay 2002).

A number of animal investigations have shown that spirulina has some protective effects against environmental toxicants, chemical or drug-induced oxidative stress and toxicity (Deng and Chow 2010). For instance, spirulina has been shown to provide protection against oxidative-stress induced by lead acetate and gentamicin (Ponce-Canchihuaman et al. 2010; Karadeniz et al. 2008) in certain organs such as liver and kidney. Moreover, spirulina has been shown to provide central neuroprotective effect against ischemic injury and against oxidative-stress induced by various chemicals and drugs (Chamorro et al. 2006; Thaakur and Sravanthi 2010; Wang et al. 2005).

Ethanol is well known for its neurotoxicity effects. Animal studies showed that episodic ethanol intoxication or binge type drinking could result in brain injury and neurodegeneration in certain parts of the brain. Binge ethanol administration has been shown to produce neurodegeneration by inducing necrosis in corticolumbic regions including the hippocampus as well as by inhibiting adult neurogenesis in rats ( Nixon and Crews 2002; Crews et al., 2000). The effects of ethanol bingeing could be more damaging during adolescent period compared to adult (Crews et al. 2000; Crews et al. 2004). Crews et al., (2000) showed that 4 days of binge alcohol consumption affected the frontal cortical olfactory and anterior portions of the piriform and perirhinal cortices in the adolescent rats, but not the adult. Pascual et al., (2007) demonstrated that exposure to intermittent binge ethanol over 14 days period in adolescent rats caused inflammatory brain damage and neuronal death in the neocortex, hippocampus and cerebellum.
Oxidative stress has been proposed for causing neurodegeneration in ethanol neurotoxicity (Crews and Nixon 2009). Several studies have evaluated the ability of antioxidants to mitigate ethanol-induced neurotoxicity. For instance, administration of vitamin C and vitamin E, both of which are powerful antioxidants, were found to ameliorate ethanol-induced oxidative stress and neurotoxicity in the treated animals (Peng et al. 2005; Heaton et al. 2000). As far as we know, there is little information available regarding the effectiveness of spirulina, another good antioxidant, in alleviating the toxic effects of ethanol in the brain. Therefore, the present study was designed to examine the potential therapeutic effect of *Spirulina platensis* as alternative antioxidant source in protecting the brain against the neurotoxic effects of ethanol.

**MATERIALS AND METHODS**

**Animals and treatments.** Thirty two male Sprague Dawley rats were obtained from Animal Laboratory Research Unit of the Universiti Sains Malaysia. The animals were housed in plastic cages with free access to food and water. The use of the animals and various experimental protocols performed in the present study were approved by the animal ethic committee of this university (approval number 2008/38/120).

The animals were randomly divided into 4 groups: group 1, control (C); group 2, spirulina treated group (Sp); group 3, ethanol treated group (Eth) and group 4, spirulina plus ethanol treated group (Sp+Eth). A pilot study was conducted to determine the appropriate dosage of *Spirulina platensis* to be administered. The dose of 1000 mg/kg was chosen as the rats could not tolerate higher doses of spirulina. Rats in group 3 (Eth) and group 4 (Sp+Eth) were administered ethanol (40% v/v) via oral gavage at a dose of 10 g/kg/day. The ethanol regime used in the present study follows the intermittent alcohol binge model used by Pascual et al., (2007) where ethanol was administered for two consecutive days followed with a gap of two days without ethanol for a total of two weeks, resulting in eight ethanol doses administered to each rat. In addition to ethanol, rats in group 4 (Sp+Eth) also received spirulina at a dose of 1000 mg/kg on daily basis via oral gavage. Spirulina was given one hour before ethanol administration. Rats in group 2 (Sp) received daily administration of spirulina at the same dose as rats in group 4. Rats in the control group received appropriate amount of normal saline via oral gavage. *Spirulina platensis* used in the present study was obtained from a local distributer (Eiken Pte Ltd) which has been certified by various international agencies.

Twenty-four hours after the last dose of the different treatments, animals were deeply anesthetized and immediately perfused via cardiac puncture with physiological saline followed by 4% paraformaldehyde. Following perfusion, each brain was carefully removed from the cranial cavity. The cerebrum was separated from the hindbrain, divided into two halves and then stored in the same fixative at 4 °C until tissue processing. The cerebral hemispheres were blocked in coronal plane and embedded in paraffin wax using automatic tissue processing machine (Thermo Shandon). During sectioning, every tenth and its adjacent section of the tissue blocks from approximately bregma -1.4 to -1.7 mm were systematically sampled at 5 μm thickness for routine histological staining and GFAP immunohistochemistry.

**Morphometric Measurement of Dentate Gyrus.** Morphometric measurements were carried out using a computer installed with Image-Pro® Plus software (Media Cybernetics) and a digital video camera attached to a light microscope (Nikon Eclipse E600). These connections allow the digitized image to be viewed on a high-resolution computer monitor. In the study, three variables were measured: granule cell density, granule cell layer thickness and neuron density in the hilar region in the dorsal part of the dentate gyrus. Neuronal density estimation was derived from the method used by Miki et al. (1997) with some modifications (Manoonkitiwongsa et al. 2001). To standardize measurements, only neurons exhibiting well-stained nuclei were counted under high-power 40x objective and 20x objectives for granule cell and hilar neurons.
Spirulina and Ethanol Neurotoxicity

respectively (Figure 4). Neuronal counting was performed using manual tagging tool provided in the software. Random selection of area to be counted was done using point grid system superimposed on the reference space. The mean number of granule cell and hilar neuron across the tissue sections were averaged and expressed as number of cell per mm². Besides the neuronal density, the thickness of granule cell layer of the dentate gyrus was also measured at ten different locations for each section using the Image-Pro® Plus software (Media Cybernetics).

**GFAP Immunohistochemistry.** Standard immunohistochemical methods were used to stain the tissue sections using the GFAP antibody (Ramos-Vara et al. 2008). Briefly, the sections were deparaffinized with xylene, rehydrated and brought to water. The sections were treated with 3% hydrogen peroxide for 15 min and then exposed to microwave oven heat for ten minutes. The sections were incubated with monoclonal mouse anti-GFAP (DAKO) for two hours, universal biotinylated secondary Ig G antibody (DAKO LSAB⁺ kit) for one hour and then avidin-biotin peroxidase complex solution (DAKO LASB⁺ kit) for 30 minutes. The reaction was visualized using 3,3-diaminobenzene tetrahydrochloride (DAB). The sections were counterstained with Harris hematoxylin, dehydrated, cleared in xylene, mounted with DPX and coverslipped. Negative control was performed by omitting incubation with the primary antibody. The quantitative analysis of GFAP immunohistochemistry was based on the measurement of the total number of immunoreactive cells per squared area of measuring field. Briefly, images of the granule cell layer viewed under x 40 objectives were snapped at random using the Image-Pro® Plus software. The number of GFAP immunoreactive cell was counted in an area field of 2150 µm². The data were then averaged and expressed as the number of GFAP immunoreactive cell per mm².

**RESULTS**

**Body and Brain weight.** The body weight gain was calculated by subtracting the initial body weight from the final body weight measurement. The ANOVA revealed that there were significant differences in body weight gain among the four experimental groups. Post hoc analysis indicated that rats exposed ethanol gained significantly less weight compared to the other three groups. In other words, this indicated that rats supplemented with spirulina gained more body weight compared to the rats treated with ethanol alone. Identical analysis on the brain weight failed to show any statistical difference between all treatment groups. The mean body weight gain and brain weight for all experimental groups are shown in Table 1.

**GFAP Immunohistochemistry.** Immunohistochemical staining of astrocytes using GFAP antibody showed a significant increase in GFAP immunoreactivity after ethanol-treatment in comparison to the control. However, spirulina supplementation (Sp+Eth) failed to alter this increase in GFAP immunoreactivity when compared to ethanol-treated (Eth) group (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>C</th>
<th>Sp</th>
<th>Eth</th>
<th>Sp+Eth</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>44.4 ± 1.9</td>
<td>41.7 ± 2.1</td>
<td>19.3 ± 1.7*</td>
<td>34.6 ± 2.3*</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>1.64 ± 0.03</td>
<td>1.66 ± 0.02</td>
<td>1.60 ± 0.03</td>
<td>1.61 ± 0.04</td>
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The values are presented as mean ± SEM
* p < 0.001 between ethanol and ethanol+spirulina treated groups in comparison to the control. C: control; Sp: spirulina; Eth: ethanol.

**Morphometry of Dentate Gyrus.** Statistical analysis showed a significant reduction in the granule cell density in the dentate gyrus following the ethanol treatment in comparison with the control. Rats treated with ethanol had about 18% less number of granule cell (per mm²) as compared to the control (Figure 2). Similar result was obtained with regard to neuronal density in the hilus of the dentate gyrus. Ethanol treatment significantly reduced neuronal density of the hilar region as compared to the control (Figure 3). However, *Spirulina platensis* supplementation failed to prevent these ethanol-induced neuronal losses as demonstrated by the insignificant difference in neuronal density between the ethanol treated (Eth) and spirulina plus ethanol treated (Sp+Eth) group (Figure 2 and Figure 3). Analysis of granule cell thickness also failed to find any significant differences between all treatment groups (Table 2).

**GFAP Immunohistochemistry.** Immunohistochemical staining of astrocytes using GFAP antibody showed a significant increase in GFAP immunoreactivity after ethanol-treatment in comparison to the control. However, spirulina supplementation (Sp+Eth) failed to alter this increase in GFAP immunoreactivity when compared to ethanol-treated (Eth) group (Table 2).
Table 2. Granule cell layer thickness and GFAP immunoreactive astrocytes in the dentate gyrus in various treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>C</th>
<th>Sp</th>
<th>Eth</th>
<th>Sp+Eth</th>
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<tr>
<td>Granule layer thickness (µm)</td>
<td>62.98 ± 2.91</td>
<td>64.92 ± 2.24</td>
<td>60.53 ± 2.51</td>
<td>60.28 ± 2.69</td>
</tr>
<tr>
<td>GFAP immunoreactive astrocyte/mm²</td>
<td>283.4 ± 17.6</td>
<td>275.3 ± 15.0</td>
<td>393.1 ± 12.3*</td>
<td>387.1 ± 14.0*</td>
</tr>
</tbody>
</table>

The values are presented as mean ± SEM
* p < 0.001 between ethanol and ethanol+spirulina treated groups in comparison to the control. C: control; Sp: spirulina; Eth: ethanol.
DISCUSSION

There have been numerous studies investigating the role of antioxidant in protecting our body from ethanol-induced toxicity. Although this is not a new concept, it has wide implication since antioxidant is found in many organic substances and oxidative stress is thought as one of the important mechanisms causing alcohol-induced toxicity (Crews and Nixon 2009). Dietary antioxidant therapy such as spirulina supplementation is a potential way to help alleviate the deleterious effects of the ethanol in everyday life and this notion is supported by several animal studies. Various antioxidant containing substances such as vitamin E, vitamin C, β-carotene, flavonoids and polyphenols have been shown to be effective in protecting the brain against the toxic effects of ethanol (Peng et al. 2005; Heaton et al. 2000; Mitchell et al. 1999; Shirpoor et al. 2009; Altura and Gebrewold 1996). However, the evidence is not conclusive and the idea is still controversial (Kane et al. 2008). Several other investigators revealed that exogenous antioxidant therapy was not quite effective to counteract the effects of ethanol-induced neurotoxicity (Edwards et al. 2002; Tran et al. 2005; Grisel and Chen 2005; Pierce et al. 2006).

Spirulina supplementation has been shown to offer significant protection against a number of environmental toxicants, chemical or drug-induced oxidative stress and inflammation. For instance, spirulina has been shown to be effective in protecting the kidney against the nephrotoxic effects of gentamicin (Karadeniz et al. 2008) and cyclosporine (Khan et al. 2006). Similarly, spirulina partially reversed the dopamine-depleting effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and completely blocked the oxidative stress induced by MPTP (Chamorro et al. 2006). Moreover, spirulina was found to be effective in increasing the endogenous antioxidant levels and reducing lipid peroxidation activity in the liver after exposure to cadmium (Karadeniz et al. 2009). While these data suggested that spirulina intake significantly relieved or completely prevented oxidative stress or inflammatory reactions associated with those toxic compounds, the data on the neuroprotection of spirulina is still limited, particularly whether spirulina supplementation can protect neurons against ethanol-induced death.

The present study shows that Spirulina platensis is not effective in protecting the dentate gyrus against toxicity induced by the ethanol. More importantly, the present study also shows spirulina supplementation alone does not have any toxic effects on the dentate gyrus. Nevertheless, the present study confirms the previous findings that intermittent binge ethanol intake during adolescent period significantly disrupts neuronal development, in this case, the granule cell and hilar neurons of the dentate gyrus (Pascual et al. 2007).

The daily dose of spirulina used in the present study (1 g/kg/day) was approximately seven times higher (on a per kilogram basis) than typical amount of supplements recommended for human intake (up to 140 mg/kg/day, assuming average weight of 60 kg). Clinical studies indicated that spirulina at doses of 1 to 2 g per day were effective in managing hyperlipidemia in various subgroup of volunteers (Deng and Chow 2010). In animal studies, spirulina at a dose range of 300 mg/kg/day to 1 g/kg/day have been shown to be effective in reducing the toxicity in various organs caused by heavy metals and pharmaceutical drugs (Karadeniz et al. 2008; Khan et al. 2006; Karadeniz et al. 2009).

The results of the present study are parallel with the findings by other studies but using different types of antioxidant (Edwards et al. 2002; Tran et al. 2005 Grisel and Chen 2005). For instance, Tran et al. (2005) showed that vitamin E supplementation was not effective in protecting the cerebellar neurons from the toxic effects of ethanol despite following the exact protocols of an earlier study by Heaton et al., (2000) who showed that the vitamin E was effective in protecting the neurons. Another powerful antioxidant, melatonin has also been shown to be useless in protecting the Purkinje cells in developing cerebellum against ethanol-induced neuronal death (Edwards et al. 2002). Similar result was obtained using N-
acetylcysteine, clinically useful and powerful antioxidant that works by increasing the natural cellular antioxidant capacity. N-acetylcysteine supplementation did not ameliorate the loss of neurons from a single ethanol exposure (Pierce et al. 2006).

CONCLUSION
Ethanol exposure during adolescent period results in neuronal loss in the dentate gyrus as shown by previous researchers. However, concurrent Spirulina platensis supplementation failed to provide effective protection against ethanol-induced neuronal loss.

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REFERENCES


