Neuroprotective effect of *Silybum marianum* in brain regions after experimental ochratoxicosis

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*Silybum marianum* L. (*SM*) was investigated for its possible protective effect against ochratoxin A-induced (OTA) toxicity in mice brain. OTA instigates oxidative changes leading to reactive oxygen/nitrogen species (ROS/RNS) overproduction. SM oral administration prevents the physiological abnormalities and improves the oxidative stress parameters in the cerebral cortex (CX) and cerebellum (CB). Moreover, analysis showed that SM administration decreased ROS production and DNA genotoxicity in the brain regions, even in the OTA group. The significantly increased SOD and CAT activities in SM and SM + OTA groups confirm the positive neuroprotective effect of SM on the cellular antioxidant system. The results suggested that SM protects against OTA-induced brain oxidative disorders and other abnormalities.

**Keywords:** SM, OTA, CB, CX, oxidative stress

**INTRODUCTION**

Ochratoxin, a mycotoxin produced by various toxigenic fungal species (*Aspergillus ochraceus* and *Penicillium verrucosum*), as a secondary metabolite, is a common food contaminant [1, 2] with a long half-life. In relation to the three isofoms (ochratoxin A, B, and C), ochratoxin A (OTA) is the most potent toxin [1]. OTA has been implicated in hepatotoxicity, teratotoxicity, immunotoxicity, enzymuria, and neurotoxicity [3, 4]. The chronic and acute mechanisms of OTA-induced neurotoxicity are still unknown. Hayes et al. [5] and Wangikar et al. [6] suggest that the deleterious OTA effects on neural tissue are expressed in its accumulation in the adrenal medulla, substantia nigra, striatum, cortex, and hippocampus. Other studies, suggested the OTA involvement in mitochondrial damage, inhibition of protein synthesis, single-stranded DNA breaks, and oxidative stress (OS) damage [2, 8]. OTA crosses the blood-brain barrier (BBB), accumulates in the central nervous system, and induces neuronal apoptosis [9-11]. Sava et al. [12] commented that acute OTA administration produced higher levels of reactive oxygen/nitrogen species (ROS/RNS) in six brain regions and induced redox malformations. Acute OTA exposure also suggests depletion of striatal dopamine and associated metabolites, as well as decreased tyrosine hydroxylase immunoreactivity [12] in the brain. Bhat et al. [13] concluded that OTA treatment alters various biological pathways mobilized by OS and increases ROS/RNS productions. Chronic, low-dose OTA exposure increases lipid peroxidation, causes Parkinsonism in mouse models [12, 14] and simultaneously involved in neurodegenerative disorders [15].

Plant antioxidants are possible reducers of the progression of neurotoxic/ neurodegenerative diseases in animal models [16-17]. *Silybum marianum* L. (*SM*) as a potent milk thistle extract has possessed antioxidant, cyto-protective, anti-inflammatory, and anticancer properties [18]. The flavonolignant and polyphenolic constituents due to the ability to antioxidant-scavenging ROS/RNS, inhibit lipid peroxidation [18, 19], and act in protein synthesis [20]. The SM ability to cross the blood-brain barrier and improve psychomotor and cognitive impairment in animal models defines the extract as a possible neuroprotective agent [17, 21].

The main objectives of the study were to elucidate the neuroprotective effect of *SM* by determining: 1) the *SM* and OTA pharmacokinetics; 2) the *SM* protective effects on OS parameters and enzymatic cerebral defensive system in mouse brain regions after OTA-induced neurotoxicity.
EXPERIMENTAL

2-3 mg/g OTA isolated from Aspergillus Ochraceus strain (Isolate D2306; 80°C / 1 h) was described previously [22]. The SM powder and other reagents were purchased from Sigma Aldrich, USA.

Experimental design

The animal protocol was approved by the Directive 2010/63/EU and by the Ethical Committee for Animals and Trakia University, Stara Zagora, Bulgaria (131/ 6000-0333/ 09.12.2015).

Male, BALB/c mice (n = 24; 36 ± 2.0 g, 7-weeks old; 7 days acclimatized) were used. Neurotoxicity was induced chronically using OTA administration at 1.32 mg/kg body weight (b.w.), orally for 28 days (~18.5 mg OTA/ kg). The mice were divided into 4 groups (n=6) as follows: (1) Controls with basic diet; (2) SM group - administered orally with SM (200 mg/kg b.w.) every day for 28 days; (3) The OTA group- mice were fed with OTA (ED50:1.32 mg/kg) per day, orally given for 28 days; (4) SM + OTA group, the mice were given both SM extract (200 mg/kg per day for 28 days, orally) and OTA (1.32 mg/kg per day for 28 days, orally, 2 hrs after SM administration). The defined quantities of SM and OTA were mixed with virgin olive oil (Mikroo, Bulgaria (131/2015).)

Additionally, the physiological status and changes in spontaneous behavior were monitored on the 7th, 14th and 28 day until euthanasia.

Brain regions isolation

The mice were weighed, and observed for changes in spontaneous behavior daily until euthanasia (50 mg/kg Nembutal, i.p.), on day - 29. The both brains sections: cerebral cortex (CX) and cerebellum (CB) were separated, fixed in cold PBS (pH = 7.5) under ice (−4°C), homogenized and estimated for OS injuries.

Biodistribution

100 mg of CX and CB regions were sonicated in cold PBS (10% w/v), centrifuged (2000×g 15 min, 4°C) and evaluated directly for SM and OTA biodistribution, by X-band EPR spectrometer (Bruker), by method previously described [23].

ROS production

100 mg CX and CB regions were added to 900 μl (50 mM) N-t-butyl-alpha-phenylnitron (PBN) dissolved in dimethyl sulfoxide (DMSO) (2:1 w/v), centrifuged (4000 rpm, 10 min, 4°C) and studied according to Shi et al. [24].

The biodistribution and ROS production studies were performed with an Electron Paramagnetic Spectroscopy (X-Band EPR, Bruker, Germany) analyzer, in fivefold spectral measurement in: 3503 – 3515 G center field, 6.42-20.00 mW microwave power, 5 – 10 G modulation, 5 scans per sample, and the results are presented in arbitrary units (a.u.).

Oxidative DNA damage and enzymatic defense

The 8-hydroxydeoxyguanosine (8-OHdG), superoxide dismutase (SOD) and catalase (CAT) measurements were carried out using CellBiolabs Ins - ELISA kits (Germany), following manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., two-way ANOVA. EPR processing was performed using Bruker Win-EPR and Sim-foinia Software. The results were expressed as means ± standard error, mean (SEM, n = 6). The differences between groups were analyzed by Student's t-test, and p < 0.05 was considered as significant.

RESULTS AND DISCUSSION

SM possesses protective effects on delayed neuronal cell death in the rat hippocampus [18], on dopaminergic neuron [21], on prophylactic capabilities in acetaminophen-induced injuries on CX [25].

The present study reports the SM-neuroprotective efficacy against OTA-induced neurotoxicity in brain regions, is in terms of OS lesions. OTA neurotoxic potential has been observed in Neuro-2a cells in a dose-dependent manner [13] and its known that induce changes in the cells might be mediated by OS pathways, producing ROS/ RNS oxidants, which was effectively reversed by natural antioxidants [13]. In contrast, Kaur et al. [26] reported that SM effectively reducing dopaminergic neurons damages against induced neurotoxicity in brains, acting as ROS/ RNS scavenger. The chronic OTA exposure registered weak depression, weakness, and spontaneous locomotor activity, well expressed on 19th day (Table 1).

Notably, SM (p < 0.003) significantly restored food consumption to animals receiving OTA, vs. OTA group. This was evident after the first 19 days when food intake was reduced >17%. In contrast, no changes in physiological status and behavior in the SM group and SM + OTA were detected.
Table 1. SM and OTA results of the mean body weight values (BW) and behavior in experimental ochratoxicosis in male BALB/c mice. In group ± SEM (n = 6); (0): none, (1): mild, (2): moderate.

<table>
<thead>
<tr>
<th>Treatment (n = 6)</th>
<th>BW(g)</th>
<th>BW(g)</th>
<th>Weak depression</th>
<th>Weakness locomotor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>19th day</td>
<td>28th day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>204.7 ± 1.4</td>
<td>258.2 ± 4.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OTA</td>
<td>205.9 ± 0.8</td>
<td>230.2 ± 3.8</td>
<td>++</td>
<td><code>62</code></td>
</tr>
<tr>
<td>SM + OTA</td>
<td>208.1 ± 1.03</td>
<td>241.2 ± 2.51</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Controls</td>
<td>207.7 ± 3.45</td>
<td>251.8 ± 4.12</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Several evidences indicated the flavonoid-containing SM as potent immune-modulatory and anti-inflammatory action and suppression of oxidative immune-toxicity [26] against mycotoxins. In addition, SM probably acts as a potent cognitive enhancer and neuro-inflammatory without affecting cerebellar neurons, regulates oxidative stress oxidation and improves memory processes [26, 27]. Favorable changes in BW, food intake, and normalizing of appetite were observed in SM and SM + OTA combination.

Although SM is a large molecule for absorption by simple diffusion, Bosch-Barrera et al. comment on notable improvements in central nervous system and brain metastases in patients receiving an oral nutraceutical product containing SM [28]. The SM and OTA biodistribution in brain regions, were investigated and are presented on Fig. 1.

![Figure 1. EPR analyses of SM and OTA biodistribution in brain regions.](image)

At the 29th day, the EPR results showed highest SM and OTA localization in CB, followed by the CX. A relatively almost 2:3-fold OTA accumulation found in the CB and CX, compared to SM localization in the same regions, was a prerequisite for a high OTA neurotoxicity in the brain regions. Increased OTA accumulation found in the CB and CX is consistent with the findings of other investigators [14]. Sava et al. [14] reported that OTA, as an inhibitor of mitochondrial oxidative metabolism, is stably delayed in the CB, but despite the highest OTA accumulation, this area remains the least regionally vulnerable. Moreover, OTA exposure contributed to impaired hippocampal neurogenesis in vivo, leading to cognitive deficits and depression also observed in our groups [29].

Deficient repair processes and the uneven distribution of oxidative DNA damage across brain regions caused by endo- and exogenous factors have been associated with many neurodegenerative diseases [14, 29]. Our report on the brain region effects of OTA, were focused on DNA damages, the augmented ROS generation (including HOO●, HO2●, and OH●), and on SM cerebral OS and enzymatic protection, alone and in combination [30]. According to the data in Table 2, significantly higher DNA damages (p < 0.05) and ROS levels (p < 0.001) and significantly lower SOD (p < 0.001) and CAT levels (p < 0.001) were detected in the CB vs CX regions, after OTA treatment. SM administration resulted in a significant decrease in oxidative DNA damages and ROS concentrations, a significant restoration in enzymatic protection, and decrees in OS injuries, in both regions.

Acute/ chronic OTA administration previously has been reported to cause OS cascades in mouse brain, evidenced by significant increases in ROS/ RNS, lipid peroxidation and oxidative DNA malformations across 6 brain regions [14, 29]. Our findings suggest that chronic OTA administration provoke strong inflammatory responses in CB and CX, as well as increase in the DNA breaks, ROS/ RNS accumulation and depletes CB/CX enzymatic defense. In addition, Aktas and Sevimli [31] comment that SM has protective properties against oxidative insults by potentially modulating the ●O2-, HOO●, and OH● formation, along with protein oxidation products in the cortices of the elderly rodent brain. In another experiment, SM increased the enzyme activities witch neutralized lipid peroxidation and normalized ROS production [32].
In the OTA toxicity, inhibited ROS/ lipid peroxidation and restarting of SOD and CAT antioxidants in the neurons of the cerebellum and cerebral cortex after SM protection are signs of brain tissues reduction and antioxidant protective mechanism. In consistent to our results, SM has been reported to act as an antioxidant, to stimulate respiratory activity, and inhibit ROS/ lipid peroxidation in brain mitochondria by increasing the concentrations of endogenous antioxidant enzymes [31, 33]. Moreover, SM is able to alleviate cognitive impairment and through redox modulatory reactions to improve cellular antioxidant status, which protects the cerebellum better vs. cerebral cortex in SM, and in SM + OTA treated rodents [27].

CONCLUSION

In conclusion, our results indicate a neuroprotective effect of SM on OTA-induced brain toxicity. The protective properties of SM are strongly related to its antioxidant potential against OS and could find application as protector against OTA-induced experimental ochratoxicosis.

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