Effect of naturally mouldy wheat or fungi administration on metallothioneins level in brain tissues of rats

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OBJECTIVE: The aim of this study is to determine level of metallothioneins (MTs) in brain tissues of rats administered by feed mixtures with different content of mouldy wheat or fungi.

DESIGN: Selected male laboratory rats of Wistar albino at age of 28 days were used in our experiments. The rats were administered by feed mixtures with different content of vitamins, naturally mouldy wheat or fungi for 28 days. At the very end of the experiment, the animals were putted to death and brains were sampled. MT level was determined by differential pulse voltammetry Brdicka reaction.

RESULTS: We found that MTs’ level in brain tissues from rats administered by standard feed mixtures was significantly higher compared to the level of MTs in rats supplemented by vitamins. Further we studied the effect of supplementation of naturally mouldy wheat on MTs level in rats. In mouldy wheat we detected the presence of following fungi species: Mucor spp., Absidia spp., Penicillium spp., Aspergillus spp. and Fusarium spp. Moreover we also identified and quantified following mycotoxins – deoxynivalenol, zearalenone, T2-toxin and aflatoxins. Level of MTs determined in rats treated with 33 or 66% of mouldy wheat was significantly lower compared to control ones. On the other hand rats treated with 100% of mouldy wheat had less MTs but not significantly. Supplementation of vitamins to rats fed by mouldy wheat had adverse effect on MTs level compared to rats with no other supplementation by vitamins. Moreover vitamins supplementation has no effect on MTs level in brain tissues of rats treated or non-treated with Ganoderma lucidum L.

CONCLUSION: Both mycotoxins and vitamins have considerable effect on level of MTs in brain tissues. It can be assumed that the administered substances markedly influence redox metabolism, which could negatively influence numerous biochemical pathways including those closely related with MTs.
INTRODUCTION

Metallothioneins (MTs) are a group of low molecular mass (from units to ten of kDa) single-chain proteins. Four major isoforms (MT-1 through MT-4) have been identified in mammals (Miles et al. 2000). They are found in cytoplasm, lysosomes, mitochondria and nuclei of cells. MT-1 and 2 have ubiquitous tissue distribution particularly in liver, pancreas, intestine, and kidney, whereas MT-3 is found in brain and MT-4 in skin (Davis & Cousins, 2000). Protection against metal toxicity is ensured mainly by MT-1 and MT-2, although MT-3 plays a role in Zn homeostasis in neurons (Davis & Cousins, 2000; Sato & Kondoh, 2002).

The neuroprotective function of MT-1 and MT-2 appears to be important. MTs-knockout mice show significantly enhanced brain tissue destruction, neuronal cell death, and clinical symptoms after cortical injury when compared with wild-type controls (Carrasco et al. 2000; Penkowa et al. 1999). MT-1 overexpression after brain injury stimulates the astrogial responses including the expression of anti-inflammatory cytokines, growth factors, neurotrophins and their receptors, reduced inflammatory responses of macrophages and lymphocytes including significantly decreased levels of proinflammatory cytokines, matrix metalloproteinases, and reactive oxygen species (Campagne et al. 1999; Penkowa et al. 2005). In addition, MTs enhance cell cycle progression, mitosis and cell survival, while neuronal apoptosis is inhibited (Penkowa, 2006). A receptor was identified, which mediates MTs transport into neurons - megalin. MTs stimulate regeneration of axons. This regeneration is dependent on megalin-mediated MT uptake (Chung et al. 2008). In addition low intracellular levels of MT-3 detected by real time polymerase chain reaction, immunohistochemistry and western blotting were found in temporal cortex of brains of patients suffering from Alzheimer’s disease. These observations support the conclusion that loss of MT’s protective effects lead to an exacerbation of pathogenic processes (Yu et al. 2001).

Based on the previously mentioned facts MTs have presumable protective effect on brain tissues. Therefore, it can be assumed that these proteins can be associated with some pathways protecting brain tissues against xenobiotics. Due to many adverse mycotoxins’ effects including mutagenic, carcinogenic, teratogenic, goitrogenic, nephrogenic and oestrogenic (Cigic & Prosen, 2009; Foroud & Eudes, 2009; Marangi & Riley, 2007; Pfohl-Leszkowicz & Manderville, 2007; Stoev, 2008; Yazar & Omurtagy, 2008), it is not surprising that the affecting of nervous systems by these substances have been studied (Banczerowski et al. 2008; Girish et al. 2008; Chaudhari et al. 2009; Islam et al. 2007; Leung et al. 2006; Sam et al. 2006; Yegani et al. 2006; Zhang et al. 2009; Zurich et al. 2005). From very recently published papers, it seems that ochratoxin A can induce apoptosis in neuronal cells (Zhang et al. 2009). Moreover, Zurich earlier investigated the effect of this mycotoxin on astrocytes and observed an unusual activity of these cells due to the presence of ochratoxin A (Zurich et al. 2005). Interesting results of Islam et al. shows on mycotoxins as neurotoxins (Islam et al. 2007). In spite of the intensive research, the relation between MT level and administered mycotoxins has not been studied yet.

To detect MTs various analytical techniques including spectrometry, liquid chromatography, capillary electrophoresis and electrochemistry (Kizek et al. 2001; Kukacka et al. 2006; Szpunar, 2005) can be employed, whereas differential pulse voltammetry (DPV) Brdicka reaction belongs to the most sensitive ones (Eckslhenger et al. 2009; Petlova et al. 2006). The aim of this paper is to determine level of MTs in brain tissues of rats as a good model for studying of the effects of xenobiotics on nervous system of animals (Ek et al. 2007; Hodek et al. 2006; Cheng et al. 2008; Nusier et al. 2007; Qu et al. 2008). The rats were administered by feed mixtures with different level of mouldy wheat or fungi producing mycotoxins.

MATERIAL AND METHODS

**Chemicals.** Rabbit liver MT (MW 7143 g/mol), containing 5.9% Cd, 0.5% Zn and both MT-1 and MT-2, Co(NH3)6Cl3 and other chemicals used were purchased from Sigma Aldrich (St. Louis, USA) unless noted otherwise. The stock standard solutions of MT (10 μg ml⁻¹) was prepared with ACS water (Sigma-Aldrich, USA) and stored in the dark at –20 °C. Working standard solutions were prepared daily by the dilution of the stock solutions with ACS water. The pH value measured using WTW inoLab pH Meter (Weilheim, Germany). All nutrients for animals were purchased from Mikoř Čebín (Czech Republic). Deionised water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic O2 (Aqua Osmotic, Czech Republic) and then it was subsequently purified using Millipore RQ (Millipore Corp., USA, 18 MΩ) – MiliQ water.

**Animals.** Selected male laboratory rats of Wistar albino at age of 28 days were used in our experiments. Experimental animals were kept in vivariums with controlled temperature (23 ± 1°C) and photoperiod (12 hours day:12 hours night with maximal intensity 200 μE.m⁻².s⁻¹). Rats were stabled in plastic cages with slotted floor. Tempered feed mixtures and drinking water were accessible ad libitum. Twenty eight days old experiment animals were divided into 10 groups (7 male rats per group). We used feed mixtures with different content of vitamins, naturally mouldy wheat or fungi (Tab. 1). The rats were administered by these mixtures for 28 days. In the very end of the experiment, the animals were putted to death and brains were sampled.

**Fungi identification and quantification in mouldy wheat.** Mouldy wheat (app. 20 g) was shaking in 180 ml distilled water for 15 min. The suspension was 10 times
diluted with water. Then, ten times diluted suspension (1 ml) was introduced onto Petri dish with cultivation medium (Chloramphenicol Glucose Agar, Biokar Diagnostics, France). Fungi were cultivated for 125 hours at 25 °C. Detection of fungi species was performed microscopically. Total number of fungi was 2 × 10^6 CFU/g (colony forming unit per a gram of mouldy wheat).

Preparation of biological samples. An animal tissue (app. 0.2 g) was transferred into a test tube and then deep froze by liquid nitrogen to disrupt cells. The frozen tissues were mixed with extraction buffer (100 mM potassium phosphate, pH 8.7) to a final volume of 1 ml and homogenised using hand-operated homogenizer ULTRA-TURRAX T8 (IKA, Germany) placed in an ice bath for 3 min at 25,000 rpm. The homogenate was centrifuged at 10,000 g for 15 min and at 4°C (Eppendorf 5402, USA). The processed tissues samples were prepared by heat treatment. Briefly, the sample was kept at 99 °C in a thermomixer (Eppendorf 5430, USA) for 15 min. with occasional stirring, and then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C, 15,000 g for 30 min. (Eppendorf 5402, USA). Heat treatment effectively denatures and removes high molecular weight proteins out from samples (Erk et al. 2002).

Electrochemical determination of MTs. Electrochemical measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm² was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary electrode. GPES 4.9 supplied by EcoChemie was employed for treatment of the measured data. The Brdicka supporting electrolyte containing 1 mM Co(NH₃)₆Cl₃ and 1 M ammonia buffer (NH₃(aq) + NH₄Cl, pH = 9.6) was used and changed per one analysis. DPV parameters were as follows: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, E_ads = open circuit, time of adsorption: 240 s. All experiments were carried out at 4 °C (Julabo F25, Germany).

Automated spectrometric measurements. Spectrometric measurements were carried out with an automated chemical analyser BS-200 (Mindray, China). Reagents and samples were placed on cooled sample holder (4 °C) and automatically pipetted directly into plastic cuvettes heated at 37°C. The mixture was consequently stirred. The washing steps by distilled water (MiliQ water) were done in the midst of the pipetting. Apparatus was controlled by software BS-200 (Mindray).

Statistical analyses. Data were processed using MICROSOFT EXCEL* (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (S.D.) unless otherwise noted. Statistical significances of the differences between MT levels in brain tissues were determined using STATISTICA.CZ by one way ANOVA test (particularly Scheffe test), which was applied for means comparison. Differences with p < 0.05 were considered significant.

RESULTS AND DISCUSSION

Biochemical and haematological parameters

Research in the field of the effects of nutrients on biochemical pathways is still topical. In this study we aimed our attention at investigation of influence of feed mixtures with different content of mouldy wheat or fungi on level of MTs in rats’ brain tissues. Primarily, we determined basic biochemical markers and performed haematological examination of the experimental animals. The results obtained are summarized in Tab. 2 and Tab. 3 as means from seven independent measurements. Rats treated with mouldy wheat had slightly higher levels of AST and ALT activities in blood serum.
The highest determined value was 4.8 µkat/l. Moreover we observed increase in bilirubin and urea. If we evaluated haematological parameters, we did not observe any changes.

Detection of brain metallothionein MT-3

The experimental animals were putted to death at the end of the experiment and their brains were sampled. The brain tissues were subsequently prepared according to protocol in “Material and Methods” section and analyzed using DPV Brdicka reaction. We have shown previously that this method is suitable for precise and sensitive detection of MTs (Adam et al. 2008; Adam et al. 2007; Fabrik et al. 2008; Krizkova et al. 2008; Petrlova et al. 2006). Typical DP voltammograms of tissue extracts are shown in Fig. 1. We can observe three signals corresponding to complex of reaction between free -SH moieties of MTs and cobalt(III) n supporting electrolyte (Erk et al. 2002; Petrlova et al. 2006). For quantification of MTs signal called Cat2 was taken, because it is proportional to concentration of the protein (Petrlova et al. 2006).

Effect mouldy wheat on content of MTs in brain tissues.

Levels of MTs quantified in brain tissues are shown in Fig. 2. We found that the level of MTs in brain tissues from rats administered by standard feed mixtures was significantly higher compared to the level of MTs in rats supplemented by vitamins. Vitamins have diverse biochemical functions including function as hormones, mediators of cell signalling, antioxidant and others. The antioxidant role of these substances probably decreased risk of oxidative damaging of brain tissue and therefore related with decrease in MTs level, which could serve as scavenger of reactive oxygen species (Fabrik et al. 2008; Krizkova et al. 2009).

Table 2. Blood count of experimental rats.

<table>
<thead>
<tr>
<th>Activity (µkat/l)</th>
<th>ALP</th>
<th>AST</th>
<th>ALT</th>
<th>Bilirubin</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.3</td>
<td>1.9</td>
<td>1.1</td>
<td>1.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Control-Vit</td>
<td>4.2</td>
<td>1.9</td>
<td>1.5</td>
<td>1.3</td>
<td>4.6</td>
</tr>
<tr>
<td>*MW 33</td>
<td>4.9</td>
<td>2.1</td>
<td>1.8</td>
<td>1.4</td>
<td>4.8</td>
</tr>
<tr>
<td>*MW 66</td>
<td>4.8</td>
<td>1.9</td>
<td>1.9</td>
<td>1.7</td>
<td>5.1</td>
</tr>
<tr>
<td>*MW 100</td>
<td>4.3</td>
<td>2.1</td>
<td>1.3</td>
<td>1.6</td>
<td>5.5</td>
</tr>
<tr>
<td>**MW 33-Vit</td>
<td>4.6</td>
<td>2.3</td>
<td>1.7</td>
<td>1.5</td>
<td>5.4</td>
</tr>
<tr>
<td>**MW 66-Vit</td>
<td>4.5</td>
<td>1.8</td>
<td>1.4</td>
<td>1.5</td>
<td>5.2</td>
</tr>
<tr>
<td>**MW 100-Vit</td>
<td>5.3</td>
<td>4.8</td>
<td>1.6</td>
<td>1.6</td>
<td>5.2</td>
</tr>
</tbody>
</table>

* ... Rats administered by 33, 66 or 100 % of mouldy wheat.
** ... Rats administered by 33, 66 or 100 % of mouldy wheat and supplemented by vitamins.

ALP – Alkaline phosphatase; AST – Aspartate transaminase; ALT – Alanine transaminase

Table 3. Haematological parameters of experimental rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Leu (10⁹/l)</th>
<th>Ery (10¹²/l)</th>
<th>Trombo (10⁹/l)</th>
<th>Hb (g/l)</th>
<th>Ht (l/l)</th>
<th>MVC (fl)</th>
<th>MCH (g/l)</th>
<th>MCHC (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.2</td>
<td>7.3</td>
<td>970.2</td>
<td>141.8</td>
<td>0.45</td>
<td>59.8</td>
<td>18.8</td>
<td>314.8</td>
</tr>
<tr>
<td>Control-Vit</td>
<td>9.2</td>
<td>7.3</td>
<td>850.8</td>
<td>138.7</td>
<td>0.44</td>
<td>60.1</td>
<td>18.9</td>
<td>314.4</td>
</tr>
<tr>
<td>*MW 33</td>
<td>6.0</td>
<td>7.0</td>
<td>963.5</td>
<td>138.1</td>
<td>0.43</td>
<td>62.2</td>
<td>19.7</td>
<td>318.1</td>
</tr>
<tr>
<td>*MW 66</td>
<td>8.3</td>
<td>7.1</td>
<td>790.0</td>
<td>136.9</td>
<td>0.42</td>
<td>60.6</td>
<td>19.5</td>
<td>321.0</td>
</tr>
<tr>
<td>*MW 100</td>
<td>6.6</td>
<td>7.9</td>
<td>955.8</td>
<td>157.3</td>
<td>0.48</td>
<td>61.2</td>
<td>20.1</td>
<td>328.6</td>
</tr>
<tr>
<td>**MW 33-Vit</td>
<td>7.7</td>
<td>7.9</td>
<td>945.6</td>
<td>153.0</td>
<td>0.44</td>
<td>59.4</td>
<td>19.5</td>
<td>328.2</td>
</tr>
<tr>
<td>**MW 66-Vit</td>
<td>9.5</td>
<td>7.2</td>
<td>756.4</td>
<td>142.9</td>
<td>0.44</td>
<td>61.5</td>
<td>19.9</td>
<td>324.2</td>
</tr>
<tr>
<td>**MW 100-Vit</td>
<td>8.6</td>
<td>7.2</td>
<td>970.7</td>
<td>147.3</td>
<td>0.45</td>
<td>63.6</td>
<td>20.6</td>
<td>324.1</td>
</tr>
</tbody>
</table>

* ... Rats administered by 33, 66 or 100 % of mouldy wheat.
** ... Rats administered by 33, 66 or 100 % of mouldy wheat and supplemented by vitamins.

Leu – Leucocytes; Ery – Erythrocytes; Trombo – Thrombocytes; Hb – Hemoglobin; Ht – Haematocrit; MVC – Maximum voluntary contraction; MCH – Mean corpuscular hemoglobin; MCHC – Mean cell hemoglobin concentration;
Further we studied the effect of supplementation of naturally mouldy wheat on MTs level in rats. In mouldy wheat we detected presence of following fungi species: Mucor spp., Absidia spp., Penicillium spp., Aspergillus spp. and Fusarium spp. Moreover we identified and determined content of mycotoxins in mouldy wheat as follows: deoxynivalenol (80 ± 5 µg per kg of mouldy wheat), zearalenone (56 ± 3 µg/kg), T2-toxin (20 ± 2 µg/kg) and aflatoxins as a sum of B1, B2, G1 and G2 (3.9 ± 0.2 µg/kg). To our knowledge the influence of mycotoxins on MTs level in brain tissues has not been investigated yet. Level of MTs determined in rats treated with 66% of mouldy wheat was significant lower compared to control ones (Fig. 2). On the other hand rats treated with 100% of mouldy wheat had less MTs but not significantly. The decrease of MTs content in brains of rats administered by mouldy wheat can be associated with toxic effect of fungi on rats, because we also observed slight decrease in average weight gain (Vasatkova et al. 2009). Dexamethasone had similar effect on MTs level in brain tissues of rats (Mendez-Armenta et al. 2003). Supplementation of vitamins to rats fed by mouldy wheat had adverse effect on MTs level compared to rats with no extra supplementation by vitamins. Rats fed by 33, 66 and 100% of mouldy wheat and supplemented by vitamins had higher MTs level compared to control ones, however, only experimental group fed by 100% of mouldy wheat differed significantly (Fig. 2). Higher supplementation by vitamins probably resulted in the enhancing of protective mechanisms against toxic effects of fungi. This stimulation also influenced MTs synthesis. MTs synthesis in brain tissue of rats can be also enhanced by kainic acid (Kim et al. 2003). We attempted to evaluate this presumption by treating of rats with one fungi specie – Ganoderma lucidum L. Based on the results obtained it can be concluded that vitamins supplementation has no effect on MTs level in brain tissues of rats treated or non-treated with Ganoderma lucidum L.

Based on the results obtained in this study it can be concluded that both mycotoxins and vitamins have considerable effect on level of MT in brain tissues. It can be assumed that the administered substances markedly influence redox metabolism, which could negatively influence numerous biochemical pathways including metallothionein synthesis. The effect of mycotoxins on other essential substances of redox metabolism such as reduced and oxidized glutathione can be also considered.

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REFERENCES
