DNA-Protein Crosslinks Induced by Microcystin-LR in Hepatic Cells of Mice

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Abstract: This study investigated the changes of DNA-protein crosslinks (DPC) in hepatic cells of mice that exposed to microcystin-LR (MCLR). Twenty male Kun Ming mice were divided into four groups: one control and three exposure groups. The control group were treated with saline and the exposure groups were treated with 3.0, 6.0 and 12.0μg·kg⁻¹ MCLR respectively by injected intraperitoneally (i.p.) for 7d. Results showed that the DNA-protein crosslinks induced by MCLR exposures at 3.0, 6.0 and 12.0μg·kg⁻¹ were significant (p<0.01, p<0.01, p<0.05) compared with the control, and the 6.0μg·kg⁻¹ exposure group was found to induce DNA-protein crosslinks most. In conclusion, MCLR can induce DNA-protein crosslinks in hepatic cells of mice at the concentrations of 3.0, 6.0 and 12.0μg·kg⁻¹.

Keywords: microcystin-LR; mouse; hepatic cells; DNA-protein crosslinks

微囊藻毒素-LR 致小鼠肝细胞 DNA-蛋白质交联作用的研究

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摘要: 为探讨微囊藻毒素-LR 致小鼠肝细胞的 DNA-蛋白质交联作用, 将 20 只昆明雄性小鼠随机分为 4 组: 1 个对照组和 3 个染毒组, 采用腹腔注射进行染毒 7d, 染毒剂量分别为 3.0, 6.0 和 12.0μg·kg⁻¹, 检测小鼠肝细胞 DNA-蛋白质交联程度。结果显示, 3.0, 6.0 和 12.0μg·kg⁻¹ 微囊藻毒素-LR 均可导致小鼠肝细胞显著的 DNA-蛋白质交联作用(与对照组相比, p<0.01, p<0.01, p<0.05), 当微囊藻毒素为 6.0μg·kg⁻¹ 时, 这种作用最明显。

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1 Introduction

Contamination of natural waters by cyanobacterial blooms is a worldwide problem, causing serious water pollution and public health hazard to humans and livestock (Oudra et al., 2001). Blooms of cyanobacteria in ponds and water reservoirs have been associated with acute, usually lethal toxicity in various species of domestic animals and wildlife in addition to cases of illness in humans (Falconer et al., 2005) due to the entrance of the toxins in the aquatic environment after cell lysis. Microcystins (MCs) are found to be the most common group of cyanotoxins and have more than 70 variants (Fasbner et al., 2002; 2007). These variants have a general structure comprised of five D-amino acids that exhibit minor structural variations and a pair of variable L-amino acids. Microcystin-LR (L and R represent the variable amino acids Leucine and Arginine, respectively) is one of the most commonly studied and toxic analogues, which has an LD₅₀ value of 0.05mg·kg⁻¹ in mice by intraperitoneal injection (Dawson, 1998).

In vertebrate animals, the main targets of microcystins are the hepatic cells, where they irreversibly inhibit protein phosphatases (PP) of the serine/threonine family, especially PP1 and PP2A. Acutely, this effect causes cytoskeleton disarrangement of hepatocytes, which leads to hepatic failure. Chronically, sublethal doses of microcystins can promote tumors in tissues such as skin and liver (Humpage et al., 2000). DNA-protein crosslinks are induced by a variety of endogenous and exogenous agents (including, paradoxically, agents that are known to cause cancer as well as agents that are used to treat cancer), and they have not yet paid as much attention as other types of DNA damage. Excessive DNA-protein crosslinks is considered to be a hige risk factor. Due to the poor repair capacity of cells, DPC complexes may be present during DNA replication and cause a loss and inactivation of important genes such as tumor suppressor genes.

In view of these considerations, the aim of the present study was to evaluate if exposure to microcystin-LR (MCLR) can induce DNA-protein crosslinks in hepatic cells using KCl-SDS assay. Furthermore, the results of the study will be very useful to illustrate carcinogenesis mechanisms of MCLR.

2 Materials and methods

2.1 Animals

Twenty male Kun Ming mice (25~30g) were purchased from the Experimental Animal Center of Henan Province and kept in a well-ventilated room (maintained at (23±1)°C, 12h light/12h dark cycles, with free access to water and standard pellet diet) for a week before use.

2.2 Reagents and apparatus

The cyanobacterial hepatotoxin MCLR was obtained from Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) and test solutions were prepared in saline solution. Calf thymus DNA and fluorescence dye Hoechst 33258 were purchased from Sigma. SDS and proteinase K were purchased from Merk. Acridine Orange. Low temperature centrifuge (Eppendorf -5415R) and fluorescence spectrophotometer (RF-5301PC, Shimadzu).

2.3 Animal treatment with MCLR

Taking into account the values of LD₅₀ obtained in mouse by other authors (Fawell et al., 1999), the dosages of the toxin MCLR were set at 3.0, 6.0 and 12.0μg·kg⁻¹. Twenty healthy mice were divided at random into 4 groups of 5 animals each group. In 3 testing groups, the animals were administered i. p. injection of MCLR (1mL) at doses of 3.0, 6.0 or 12.0μg·kg⁻¹ (body weight) every day for 7 days. The control group was injected the same volume of vehicle, 0.9%, w/v saline solution in same condition. During the exposure animals were allowed to drink and eat.

2.4 Cell separate

After exposure, mice were killed and the livers were quickly removed, immediately washing out the
blood with ice-cold 0.9% saline solution. The tissues were cut into homogenate using an ophthalmic scissors. After homogenate was filtrated via filter paper, the percolate was centrifuged for 5min at 1500r · min⁻¹, and then cells were resuspended in 0.5mL PBS. The resulting cell suspension was used to determine DNA-protein crosslinks levels.

2.5 Measurement of DPC using KCl-SDS assay

The KCl-SDS assay was initially developed by Zhitkovich et al. in 1992 (Zhitkovich et al., 1992) and modified by Costa et al. in 1996 (Costa et al., 1996) for detecting DPC in whole cells. The method utilizes harsh treatments to dissociate non-covalent DNA-protein binding (2% SDS, heat at 65℃) and selectively precipitates stable DNA-protein complexes by adding KCl. In this study the KCl-SDS assay was applied to detect MCLR-induced DPC according to Zhitkovich et al. (1992) and Chakrabarti et al. (1999) with some modification. The separated cells were lysed with 0.5mL of 2% SDS solution with gentle vortexing. The mixture was heated at 65℃ for 10min and 100mL of 2.5mol·L⁻¹ KCl in 10mmol·L⁻¹ Tris-HCl, pH 7.4, was added, followed by passing the mixture six times through a 1-mL polypropylene pipette tip to favor shearing of DNA to a uniform length. Since SDS binds tightly to protein but not to DNA, the free protein and protein-DNA complexes precipitated while free DNA remained in the supernatant. The SDS-KCl precipitate (containing the protein and DNA-protein crosslinks complexes) was formed by cooling the samples on ice for 5min and was collected by centrifugation at 10000rpm for 5min in an Eppendorf microfuge. The supernatants containing the unbound fraction of DNA were collected in tubes with sign. The pellets (containing DPC) were washed three times by resuspending each time in 1mL washing buffer (0.1mol·L⁻¹ KCl, 0.1mmol·L⁻¹ EDTA, and 20mmol·L⁻¹ Tris-HCl, pH 7.4) followed by heating the sample at 65℃ for 10min, chilling on ice for 5min, and centrifuging as before. The supernatants from each wash step were pooled with previous unbound fractions. The final pellet was resuspended in 1mL proteinase K solution (0.2mg·mL⁻¹ solubled in wash buffer) and digested for 3h at 50℃. The supernatants were collected by centrifugation at 12000rpm for 10min (the supernatant contained DNA previously involved in DNA-protein crosslinks). Supernatant (1mL) containing the unbound fraction of DNA or 1mL supernatant containing DNA previously involved in DNA-protein crosslinks was then mixed with 1mL freshly prepared fluorescent dye Hoechst 33258 (400ng·mL⁻¹ solubled in 20mmol·L⁻¹ Tris-HCl), respectively, and then the tubes were allowed to stand for 30min in the dark. The fluorescence of the sample was then measured in a RF-5301PC fluorescence spectrofluorimeter with excitation wavelength at 350nm and emission wavelength at 450nm. The DNA content of the sample was then determined quantitatively from a corresponding DNA standard curve generated using calf thymus DNA. The DNA-protein crosslinking coefficient (DPC coefficient) was estimated as a ratio of the percentage of DNA involved in DNA-protein crosslinks to the percentage of DNA involved in DNA-protein crosslinks and unbound fraction of DNA.

2.6 Statistical analysis

The results were expressed as means±SEM, and one-way analysis of variance (ANOVA) and t-test were applied in statistical analysis. p value <0.05 was considered as the significant difference.

3 Results

The effect of MCLR-induced DPC in hepatic cells of mice was analyzed. Table 1 shows the results. It can be seen that the DPC coefficients in MCLR exposure groups is higher than that of control group. There is significant difference in the DPC coefficient between the groups exposure with 3.0, 6.0 and 12.0μg·kg⁻¹ of MCLR and control group (p<0.05; p<0.01). Furthermore, the DPC coefficient is highest at the dose of 6.0μg·kg⁻¹.
Table 1 DPC coefficients of hepatic cells in control and MCLR-exposed mice

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>DPC coefficient (±2σ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>0.010±0.004</td>
</tr>
<tr>
<td>3.0μg·kg⁻¹</td>
<td>5</td>
<td>0.060±0.018</td>
</tr>
<tr>
<td>6.0μg·kg⁻¹</td>
<td>5</td>
<td>0.103±0.015</td>
</tr>
<tr>
<td>12.0μg·kg⁻¹</td>
<td>5</td>
<td>0.036±0.005</td>
</tr>
</tbody>
</table>

Note: *, **: MCLR exposure groups compared with control group, p<0.05, p<0.01

4 Discussion

In the last decade, public health concern about cyanobacteria and cyanotoxins has increased in many countries, owing to their frequent occurrence. Microcysts are a group of heptaptide hepatotoxins produced by cyanobacteria in eutrophic freshwater, many of which are potent hepatotoxins, with microcystin-LR being one of the most abundant and toxic variants in blooms. Ding et al. (1999) reported that MC-LR was able to induce DNA damage in primary cultured rat hepatocytes. Similar results were obtained by Mankiewicz et al. (2002) in human lymphocytes. More recently it was reported that MCLR induced DNA single strand breaks (SSBs) in HepG2 cells (Zegura et al., 2006). MC-LR was considered tumor promoter.

As a key event of tumors induced by chemical poisons, DPC is considered to be the primary and directly genotoxic effect of poisons. In normal cell there is a basic level of DPC, which is concerned with DNA replication and transcription and is necessary for the cell growth. However, excessive DPC, which is produced by environmental pollutants and carcinogens, is a high risk factor. Unlike DNA strands breaks, DPC is more difficult to be repaired in the cells. Due to the poor repair capacity, DNA-protein complexes may be present during DNA replication and cause a loss and inactivation of the important genes such as tumor suppressor genes (Barker et al., 2005).

The results showed that 3.0μg · kg⁻¹ MCLR could make DPC coefficients of hepatic cells significantly increase compared with the control (p<0.01). When MCLR concentration was 6.0μg · kg⁻¹, DPC coefficients of hepatic cells significantly increase, compared with the control and 3.0μg · kg⁻¹ group (p<0.01). This is because liver is the major target organ. When MCLR concentration was 3.0μg · kg⁻¹, most MCLR congregated in hepatic cells which could initiate reactive oxygen species formation. Proteins might be crosslinked to DNA through oxidative free radical. When MCLR concentration was increased to 6.0μg · kg⁻¹, added toxin was in hepatic cells, which made this kind of damage was more serious. The results also showed that DPC coefficients of hepatic cells in 12.0μg · kg⁻¹ group were lower than those of 3.0μg · kg⁻¹ and 6.0μg · kg⁻¹ groups, but they were still higher than those of control (p<0.05). These phenomena can be explained that the major type of DNA lesion is DPC, when MCLR concentration is low. When MCLR concentration increased to 12.0μg · kg⁻¹, the DPC coefficient descended and the type of DNA lesion may be DNA-DNA crosslinks or DNA break. Exposure of mice to 3.0, 6.0 and 12.0μg · kg⁻¹ MCLR could induce excessive DPCs in hepatic cells. The covalent crosslinking of proteins to DNA is expected to interrupt DNA metabolic processes such as replication, repair recombination, transcription chromatin remodeling, etc. DPC accumulation may be associated with liver cancer induced by MCLR.

The results allow us to conclude that i.p. injected MCLR can induce DPC in hepatic cells. Although the i.p. administration is not the most direct way to simulate the common contact of animals or humans, it can be considered simple and useful toxicological exposure way. Exposure of animals and humans to low concentrations of microcysts is much more frequent than lethal intoxication events. Therefore, its effects on the diverse organs are certainly necessary for future study.

References
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cortical cells and its antagonism by specific amino acids and magnesium ion [J]. Toxicology and Applied Pharmacology, 154: 245–255


