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厦门大学

博 士 学 位 论 文

甲基对硫磷和镉联合胁迫斑马鱼脑的毒性效应及其蛋白质组学研究

Joint Toxicological Effects and Proteomics in Zebrafish Brain under the Combination Stress of Methyl Parathion with Cadmium

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摘 要

对多种污染物产生联合毒性效应的研究已成为环境科学发展的重要方向之一。目前，有关污染物的生物效应研究多数还是集中在单个化合物，因而有关预测和治理多种毒物对生物产生联合毒性的研究，越来越受到环境科学家的关注和重视，特别是有关有机磷农药和重金属的联合毒性效应研究。本文选取了在水体污染中比较有代表性的甲基对硫磷(MP)和重金属镉(Cd)为共污染物，以经典的模式生物斑马鱼(*Danio rerio*)为实验材料，从生理生化指标的变化、蛋白的差异表达等几方面对MP和Cd对斑马鱼脑组织的联合胁迫效应进行了较为系统的研究。

首先，对 MP 和 Cd 分别胁迫斑马鱼后产生的急性毒性效应进行分析。结果显示 MP 对斑马鱼的半致死浓度(96 h-LC_{50})为 6.3 mg/L，Cd 对斑马鱼的 96 h-LC_{50} 为 8.3 mg/L；利用气相色谱 (GC)和电感耦合等离子体质谱法 (ICP-MS)对 MP 和 Cd 共同存在时对彼此在斑马鱼脑中的富集影响进行了分析，结果表明两者在前 24 h 相互促进富集，24 h 到 96 h 抑制彼此的富集，这说明 MP 和 Cd 共存时，联合毒物对斑马鱼的生存和毒性产生新的综合效应。

其次，本文研究了 MP 和 Cd 联合作用下对斑马鱼脑组织中超氧化物歧化酶（SOD）、过氧化氢酶（CAT）、谷胱甘肽转移酶（GST）和乙酰胆碱酯酶（AChE）活性的影响，以探讨 MP 和 Cd 对斑马鱼的毒害效应以及应激机制，同时筛选出联合胁迫的酶蛋白标志物。结果表明，MP 和 Cd 的联合胁迫能引起 SOD、CAT、GST 和 AChE 的的活性产生显著的变化趋势，酶活的变化与毒物的富集存在一定的相关性：MP 和 Cd 的联合胁迫在前 24 h 促进了彼此的富集，使得毒性增加，进而抑制了酶的活性；从 24 h 到 96 h 联合胁迫作用抑制了彼此的富集，诱导更多的解毒酶发挥作用，使得酶活增加，从而降低了联合毒性，联合效应表现为拮抗作用。这四种酶活的变化趋势说明，斑马鱼脑产生的毒性效应是 MP 和 Cd 共同作用的结果，提示该系列指标可以作为 MP 和 Cd 胁迫的生物标志物。

再者，选用差异蛋白质组学及相关分析技术筛选与鉴定由 MP 和 Cd 联合胁迫下斑马鱼脑组织表达的差异蛋白，并进一步筛选出关键蛋白，以探讨 MP 和 Cd 的联合效应和胁迫机制。质谱鉴定的 16 个差异蛋白质按功能分为五大类：细胞

代谢、信号传导、受体系统、细胞骨架和应激蛋白，其中代谢相关蛋白占到 50%，表明在 MP 和 Cd 的胁迫下，斑马鱼脑组织内的代谢过程受到了很大的影响；MPI、GSTM3、TCRa-V54、Keratin8 和 Hsp 8 在所有胁迫组中的表达量变化提示它们可能是作为有害物质胁迫下诱导产生的共同蛋白，可以作为监测环境污染情况的生物共性标记物；ALDOCB、DPYSL2 和 NDUFS1 在 MP 单一胁迫组中的表达量呈现特异性上调，说明三者在机体抵抗 MP 的胁迫毒性方面起着重要的作用，潜在着作为监控有机磷农药污染程度的蛋白指示物；UQCRFS1 和 RPSA 在 Cd 单一胁迫组中的表达量呈现特异性上调，认为可能参与 Cd 的解毒效应，适合作为监测 Cd 污染程度的特异性标志物；ALDH9A1A、 SSADH、NDRG3、GSTM3 和 ATP5A1 的表达有利于细胞的解毒，它们在联合胁迫组中的表达量呈现显著上调，提示联合作用下细胞的解毒能力增强，使得联合毒性减弱，说明这五种蛋白与联合效应作用有关，潜在着作为联合毒物效应的标志物；另外，TCRa-V54 在单一胁迫组完全被抑制表达，而在联合胁迫组中只是表达下调，说明联合毒性对免疫系统的伤害小于单一毒性引发的伤害，从一定程度上也说明联合毒性减弱，二者联合胁迫时的毒性存在相互作用。上述描述的关键蛋白质对后续开展联合毒理机理的研究提供了科学依据和实验证据。实时定量 PCR 分析表明基因在 mRNA 的表达变化与相应蛋白表达变化水平呈正相关，western blotting 表明 TCRa-V54 的表达与双向电泳的分析结果一致。这些实验结果进一步提高了已筛选与鉴定的差异蛋白的可信度。

最后，选择在 MP 和 Cd 的联合毒性中起着重要解毒作用的 GSTM3 作为研究对象，对其进行了重组表达的研究。根据毕赤酵母的密码子偏好性，对其密码子进行优化，根据蛋白酶 GSTM3 的氨基酸顺序合成了相应的 cDNA，并成功构建了毕赤酵母表达载体 pPIC9-GSTM3；将其通过电击转化整合入毕赤酵母 GS115 基因组中，实现了 GSTM3 蛋白在重组毕赤酵母宿主菌 G115 中的分泌表达；重组表达的 GSTM3 活性测定为 192 U/mgprot，为正常斑马鱼脑组织中 GSTM3 活性（20.62 U/mgprot）的 9.3 倍，这说明毕赤酵母 GS115 可以作为表达斑马鱼脑组织中 GSTM3 的合适宿主，并可用于研究 GST 的解毒机制和分析其作为揭示有机磷农药和重金属联合毒性效应的蛋白标志物的可行性。

关键词：联合毒性效应；甲基对硫磷与镉；差异蛋白质组学

Abstract

Combined pollution has become one of the important directions of research in environment science at present. Before now most of the research has concentrated on the action of single compound against organisms. Due to the presence of multiple pollutants in natural environments, environmental scientists are paying more attentions on the joint toxicological effects, especially about the combination of organophosphorus pesticides with heavy metals. In the present study, methyl parathion (MP) and Cd were chosen as two pollutants which are widely found in the aquatic environment, and zebrafish (*Danio rerio*) was chosen as experimental object. The joint toxicological effects of MP with Cd were analyzed, including changes of physio-biochemical markers, differential expression of proteins and other aspects.

Firstly, the acute toxicological effects of MP or Cd on zebrafish were examined. The single 96-h LC_{50} value of MP on zebrafish was 6.3 mg/L, and that of Cd was 8.3 mg/L. In order to provide insights into the interaction between them, the bioaccumulation of MP and Cd in the tissues after 96 h of joint exposure was analyzed by GC and ICP-MS. The results of bioaccumulation showed that MP and Cd interacted mutually. They promoted mutual bioaccumulation in the 24 h, and inhibited mutual bioaccumulation from 24 h to 96 h. It indicates that Cd and MP can affect mutual toxicity on zebrafish when exposed to mixtures of them.

Secondly, the activities of superoxide dismutase (SOD), catalase (CAT), GST (gultathione S transferase) and AChE (acetylcholineserase) in zebrafish brain tissues were assessed, so as to discuss the joint toxicities of MP and Cd and the associated response of zebrafish, along with sieving out suitable biomarkers of the pollution. The results showed that activities of all four enzymes under joint stress changed significantly when exposed to all treatments， and the changes is related with the bioaccumulation of MP and Cd in combination. The promoted bioaccumulation of MP and Cd in 24 h makes the joint toxicity increased, thereby inhibiting the enzymes activities; the inhibited bioaccumulation of MP and Cd from 24 h to 96 h induces more enzymes to play a role in detoxification, so the enzymes activities are increased and the joint toxicity is reduced. The joint toxicological effect in 96 h is antagonism. Changes of enzyme activities under the joint stress for 96 h suggest the toxicological

effects on zebrafish are affected by the interaction between MP with Cd, indicating that they could be biomarkers of the joint pollution of MP with Cd.

Thirdly, differential proteomics and related analytical technologies were applied to sieve out and identify the differential proteins in zebrafish brain under the combination of MP with Cd. And it's hoped to find the key proteins associated with the combination of the two contaminants and evaluate their joint effects. As a result, 16 proteins were identified using MS. These proteins are involved in cell metabolism, signal transport, receptor activity, cytoskeleton assembly and stress proteins. Among them, proteins related with metabolism are 50%, indicating the process of metabolism in zebrafish tissues is disturbed greatly under the stress of MP and Cd. According to the expression level change of MPI, GSTM3, TCRa-V54, Keratin8 and Hsp 8 in all treatments, it reveals that they could be common biomarkers of MP or Cd or joint pollutions; On the basis of the specific up-regulation of ALDOCB, DPYSL2 and NDUFS1 in the single MP treatment, it suggests they might be the main specific biomarkers of MP toxicity; Due to the specific up-regulation of UQCRFS1 and RPSA in the single Cd treatment, it's considered to be related with Cd detoxification, and they could be specific and suitable biomarkers of Cd exposure. Expressions of ALDH9A1A, SSADH, NDRG3, GSTM3 and ATP5A1 are in favor of cell detoxification. The remarkable up-regulation of them in combination indicates cells improve the detoxification to make the joint toxicity of MP with Cd less than the single toxicity. It proves they are related with the joint effects of MP with Cd, and could be chosen as biomarkers of the combined pollution. In addition, the expression of TCRa-V54 in the single treatment (completely inhibited to express) and the combination (down-regulated), indicates the joint toxicological effects is existed.The analysis of these proteins contributes to provide useful insights and experimental evidences for the joint toxicological mechanism between different chemicals. Meanwhile, the results of real-time quantitative PCR showed the regulation of most genes at the mRNA level correlated well with that of the corresponding proteins, and the western blotting results showed the expression level in joint action was down-regulated, which was in good accordance with that of 2-DE. These results further confirm our confidence in the proteins identified using proteomic approaches.

Lastly, GSTM3 was chosen to study the recombinant expression, because of its significance of detoxification in joint toxicity between MP and Cd. According to the preferential codon of *Pichia pastoris*, the cDNA of GSTM3 was designed and

synthesized based on the amino acid sequence of GSTM3. And the expression vector of pPIC9-GSTM3 was constructed successfully. It was then transformed into the *Pichia pastoris* GS115 by electroporation. The result showed GSTM3 was successfully secreted and expressed in recombinant *Pichia pastoris* G115. The activity of GSTM3 from recombinant expression was 192 U/mgprot, which was 9.3 times of that from normal zebrafish brain tissues (20.62 U/mgprot). It indicates *Pichia pastoris* G115 can be the suitable host to express the GSTM3. The expression system could be used to make clear the detoxification mechanism of GSTM3, and analyze its feasibility as a molecular biomarker of the joint toxicological effects of organophosphorus pesticide with heavy metals.

Keywords: joint toxicological effects; Methyl parathion and Cadmium; Differential proteomics;