Hg²⁺ 胁迫对浮萍体细胞 DNA 一级结构和抗氧化酶系的损伤

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摘要 主要从 DNA 一级结构及抗氧化酶系变化两方面, 研究了 Hg²⁺ 胁迫下浮萍体细胞的损伤。结果表明: 运用随机扩增多态性 DNA 法(Random amplified polymorphism DNA, RAPD)和 DNA 梯法(DNA Ladder) 5~10 mg·L⁻¹ Hg²⁺ 处理组可检测到基因组 DNA 的明显损伤; 20 mg·L⁻¹ Hg²⁺ 已导致细胞坏死; RAPD 法较 DNA Ladder 法更灵敏。本文还发现, 活性氧和抗氧化酶系很可能参与了浮萍体细胞凋亡过程。低浓度的 Hg²⁺ 胁迫可刺激抗氧化酶活性升高, 以清除体内活性氧, 而一旦活性氧水平超出一定阈值, 抗氧化酶活性急速下降, 导致细胞凋亡。

关键词 Hg²⁺ 浮萍 DNA 损伤 抗氧化酶 RAPD DNA Ladder

DAMAGE TO DNA PRIMARY STRUCTURE AND ANTIOXIDANT ENZYMES IN *LEMNA MINOR* INDUCED BY Hg²⁺

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Abstract The damage to *Lemna minor* cells induced by Hg²⁺ was studied in this article. Most damage occurred to DNA primary structure and changes in antioxidant enzyme activities were investigated by using RAPD and DNA ladder methods. The results showed that obvious damage to DNA was found in the process of apoptosis induced by 5-10 mg·L⁻¹ Hg²⁺, and 20 mg·L⁻¹ Hg²⁺ had already caused necrotic injury. The RAPD method was the more sensitive of the two methods, and so could be considered as an important method for monitoring apoptosis. The results also indicated that reactive oxygen species (ROS) and antioxidant enzymes are involved in the process of apoptosis. The activities of antioxidant enzymes could be stimulated to eliminate active oxygen by exposing the *Lemna minor* to low Hg²⁺ concentration; the cells declined rapidly when ROS were unable to be eliminated effectively.

Key words Hg²⁺, *Lemna minor*, DNA damage, Antioxidant enzymes, RAPD, DNA Ladder

Atmospheric deposition, geology and mineral resources seeping and untreated industrial effluent can cause heavy metal pollution in water. Hg²⁺, as one of the major heavy-metal pollutants, not only has poisoning effects on plant (Wu *et al.*, 1997), but also can enter the food chain, and serve as potential threat to the human health (Sugiyama, 1994). So far, the effects of heavy metal pollution on submerged plants focused mostly on the growth process and the physiological effects (Shi *et al.*, 2000; Korichera *et al.*, 1997; Zhang *et al.*, 1999). But few studies has been done on apoptosis of plants induced by heavy metal (Meyer & Heath, 1988; Schutzendubel *et al.*, 2001). Let alone the mechanism of DNA damage and changes of antioxidant enzyme activities in *Lemna minor* L. cell.

Lemna minor L. is a kind of fluitante belonging to Lemnaceae. It widely spreads over China and is very sensitive to the pollutants, so it is generally thought as a sensitive plant for environment monitoring (Tong, 1997). The aim of this work was to study the damage mechanism of DNA primary structure and antioxidant enzymes in the apoptosis process induced by Hg²⁺ in *Lemna minor* L., and to investigate the toxic mechanism of heavy metal to plants.

1 Materials and methods

1.1 Material and culture method

Lemna minor L. was collected in June 2001 (25 °C) in Xingdian town, Nanjing. The samples in good growth potential were selected and pre-cultured in the

deionized water for 2 d. Then they were treated with different Hg^{2+} concentration of 0, 5, 7, 10, 20 $\text{mg}\cdot\text{L}^{-1}$ at 9:00 in the morning. All the specimen were cultivated in Forma-3744 light culture box(made in USA). Temperature was(25.0 ± 0.5) $^{\circ}\text{C}$ /(15.0 ± 0.5) $^{\circ}\text{C}$ (day/night), light intensity was 70 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and photoperiod was 12 h/12 h. Since the sixth day, the leaves, 5 mm in diameter, were used to detect DNA damage and antioxidant enzyme activities. Each experiment was repeated for three times.

1.2 DNA extraction

0.2 g of leaf-tissue was firstly washed in sterile deionized water for 2 min. After being air dried, the sample was ground into powder in liquid-nitrogen with mortar and pestle. DNA was extracted from the sample using a modified CTAB (Cetyltrimethylammonium bromide) procedure(Rogers & Bendich, 1985), some of which was ameliorated in this paper. The powdered leaf-tissue was added to 0.6 ml CTAB extraction buffer(2% CTAB; 1.4 $\text{mol}\cdot\text{L}^{-1}$ NaCl; 1% PVP; 100 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl, pH 8.0; 20 $\text{mmol}\cdot\text{L}^{-1}$ EDTA; 0.2% 2-mercaptoethanol). The suspension was incubated in a water bath at 60 $^{\circ}\text{C}$ for 45 min with occasional shaking, then cooled to room temperature and extracted with one vol. chloroform/isoamyl alcohol(24:1) for 10 min. After centrifugation at 5 600 g for 10 min, the supernatant was extracted by chloroform/isoamyl alcohol extraction once more. The two phases were separated by centrifugation, and 2.5 vol. dehydrated alcohol was added to the supernatant, - 20 $^{\circ}\text{C}$ depositing for 2 h. Then the mixture was centrifugatized at 8 600 g for 15 min. The pellet was washed with 70% ethanol twice, then dissolved in sterilized TE (1 mol/L Tris-HCl 0.1 $\text{mol}\cdot\text{L}^{-1}$ EDTA, pH 8.0). The solution was electrophoresed in 1.2% agarose gel which had been stained with ethidium bromide (0.4 $\mu\text{g}\cdot\text{ml}^{-1}$) for about 1.5 h at 80V. After then, the gel was photographed under ultraviolet light using GDS-760 image-analysis system (UVP).

1.3 RAPD amplification

One hundred decamer oligonucleotide primers(kit I,

H, G, M and N) for RAPD analysis were purchased from Operon Technologies Inc.(Alameda, CA, USA). These primers were used to amplify the control group and the cultivated groups respectively, selecting primers of more effectivity and dependable repeatability. DNA amplification was based on the protocol reported by Williams *et al.* (1990). 5 μl of template DNA (20-25 ng) was amplified in 25 μl PCR mixture consisting of 2.5 μl 10 $\times$ PCR buffer, 2.5 $\text{mmol}\cdot\text{L}^{-1}$ MgCl_2 , 2 $\text{mmol}\cdot\text{L}^{-1}$ dNTP, 0.8 $\mu\text{mol}\cdot\text{L}^{-1}$ primer and 1U Taq polymerase. For DNA amplification, PTC-100 DNA thermocycler was programmed for 4 min at 94 $^{\circ}\text{C}$; followed by 40 cycles of 1 min at 94 $^{\circ}\text{C}$, 1 min at 36 $^{\circ}\text{C}$, 2 min at 72 $^{\circ}\text{C}$, then terminating with 7 min at 72 $^{\circ}\text{C}$. The reactions were ended with an indefinite hold at 12 $^{\circ}\text{C}$. The RAPD fragments were separated on 1.2% agarose gel by electrophoresis in 1X TAE buffer(40 $\text{mmol}\cdot\text{L}^{-1}$ Tris-acetate, 2 $\text{mmol}\cdot\text{L}^{-1}$ EDTA, pH 8) and stained with ethidium bromide. Finally, the gel was photographed under ultraviolet light using GDS-760 image-analysis system(UVP).

1.4 Antioxidant enzymes extraction and activity determination

The extraction and activity determination of SOD, POD, CAT were measured spectrophoto - metrically according to the method of Trevor and Fletcher(1994).

2 Results

2.1 The morphological damage in *Lemna minor* L. leaves under Hg^{2+} stress

Lemna minor L. is very sensitive to Hg^{2+} , because its leaf is thin and in direct contact with water. After cultivated for 6 d, the control group grew normally, while, the group treated with 5 $\text{mg}\cdot\text{L}^{-1}$ Hg^{2+} was already chlorotic from the edge of leaves, going with adventitious roots desquamation, and many black blotches and wilting occurred in the leaves treated with 7 $\text{mg}\cdot\text{L}^{-1}$ Hg^{2+} . As far as the group treated with 20 $\text{mg}\cdot\text{L}^{-1}$ Hg^{2+} was concerned, a large number of dark-yellow and dead-speckles distributed in both edge and center of leaves, the leaf area constricted and raised in the center(Table 1).

Table 1 External characters and comparision between the sensitivity of DNA Ladder and RAPD

Exposing concentration (Hg^{2+})	External characters	DNA Ladder detection	RAPD analysis (compared with the control group)
CK	Leaves were green, adventitious roots were developed	-	-
5 $\text{mg}\cdot\text{L}^{-1}$	Leaves were chlorotic, adventitious roots desquamated	Not obvious	Distinct difference
7 $\text{mg}\cdot\text{L}^{-1}$	Leaf area constricted, adventitious roots desquamated	Obvious	Distinct difference
10 $\text{mg}\cdot\text{L}^{-1}$	Leaves turned dark-yellow, adventitious roots desquamated	Clearer	Distinct difference
20 $\text{mg}\cdot\text{L}^{-1}$	Leaves were necrotic, adventitious roots desquamated	DNA was cleaved randomly	Distinct difference

Fig.1 Results of DNA damage presented by DNA Ladder method
M :DNA marker ;1 :The control group 2 :Treated with 5 mg·L⁻¹ Hg²⁺
3 :Treated with 7 mg·L⁻¹ Hg²⁺ 4 :Treated with 10 mg·L⁻¹ Hg²⁺ 5 :
Treated with 20 mg·L⁻¹ Hg²⁺

2.2 The DNA damage in *Lemna minor* L. leaves under Hg²⁺ stress

Through DNA Ladder detection , we found that the injury degree of DNA fragmentation was getting more serious with the rise of Hg²⁺ concentration. As shown in Fig. 1 , total DNA of the control group was a complete band , and no perceptible DNA Ladder could be seen in the group treated with 5 mg·L⁻¹ Hg²⁺ ,either. However , DNA Ladder appeared with the increasing concentration of Hg²⁺ . In the group treated with 7 mg·L⁻¹ Hg²⁺ , total DNA was degraded into approximately 180-200 bp and its multiples , and a typical DNA Ladder appeared on agarose gel electrophoresis. And DNA Ladder was more obvious when treatment concentration was 10 mg·L⁻¹ Hg²⁺ , which proved to be apoptosis. As far as the group treated with 20 mg·L⁻¹ Hg²⁺ was concerned , irregular break appeared on agarose gel electrophoresis. It seemed that total DNA had been cleaved randomly , which proved to be necrotic(Ryerson & Heath ,1996).

Through RAPD analysis , twelve of one hundred decamer primers , OPI-03、OPI-06、OPI-12、OPI-17、OPH-20、OPM-01、OPM-03、OPM-04、OPM-06、OPN-04、OPN-05 and OPN-06 showed distinct polymorphic fingerprints between the control group and the contaminated ones (Table 2). The same results were obtained in the repeated experiments for three times. We supposed that the diversity of polymorphic fingerprints was resulted from the damage of DNA , not the differences between individuals of *Lemna minor* L. Take the group treated with 5 mg·L⁻¹ Hg²⁺ for example , after amplified by OPN-06 , OPM-01 (Fig. 2 , 1、2、7、8) , DNA fragment reduced compared with the same site of the control group , which might because of DNA oxidative lesion , partial destroyed or strand breaks. Whereas ,when amplified by OPN-04 , OPM-06 (Fig.2 , 3、4、5、6) , new DNA fragments appeared compared with those in control group , which might because of DNA mismatched , and so on. In brief , different primer could detect the different sites of primary structure damage in DNA.

Table 2 Sequence of random arbitrary primers used in the experiment

Primer	Sequence(5' - 3')	Primer	Sequence(5' - 3')
OPI-03	CAG AAG CCC A	OPM-01	GGT GGT GGC T
OPI-06	AAG GCG GCA G	OPM-03	GGG GGA TGA G
OPI-12	AGA GGG CAC A	OPM-04	GGC GGT TGT C
OPI-17	GGT GGT GAT G	OPM-06	CTG GGC AAC T
OPN-04	GAC CGA CCC A	OPH-20	GGG AGA CAT C
OPN-05	ACT GAA CGC C	OPN-06	GAG ACG CAC A

Fig.2 Results of DNA damage presented by RAPD method
M :DNA Marker 1 2 :OPN-06 3 4 :OPN-04 5 6 :OPM-06 7 8 :
OPM-01 , in which 1 3 5 7 :Treated with 5 mg·L⁻¹ Hg²⁺ 2 4 6 8 :The control group

Fig.3 Effect of Hg²⁺ pollution on SOD activity

2.3 Changes of antioxidant enzyme activities in *Lemna minor* L. leaves under Hg²⁺ stress

As can be seen from Fig. 3 , in the group treated with 5 mg·L⁻¹ Hg²⁺ , the activity of SOD reached the peak value , increasing by 15.66% of control. The SOD activity of the group treated with 7 mg·L⁻¹ Hg²⁺ was lower than that of the control group , and then it decreased markedly with the increasing of Hg²⁺ concentration (*R* = -0.827 , *p* < 0.05 *). In the group treated with 20 mg·L⁻¹ Hg²⁺ , the activity of SOD was only 85.14% of the control group.

As shown in Fig. 4 ,the activity of POD increased slowly (*R* = 0.986 , *p* < 0.01 **). When treated with 10 mg·L⁻¹ Hg²⁺ , it reached the peak , with 89.58% higher than that of the control. However , the group treated with 20 mg·L⁻¹ Hg²⁺ declined to 80.71% of the control group.

As can be seen from Fig.5 , the activity of CAT was

more sensitive , it declined steadily with the increase of the Hg^{2+} concentrations. The CAT activity of the group treated with $5 \text{ mg} \cdot \text{L}^{-1} \text{Hg}^{2+}$ was 8.36% lower than that of the control group , and then it decreased rapidly($r = -0.989$, $p < 0.01^{**}$). In the case of the group treated with $20 \text{ mg} \cdot \text{L}^{-1} \text{Hg}^{2+}$, the CAT activity was only 39.13% of the control group.

Fig. 4 Effect of Hg^{2+} pollution on POD activity

Fig. 5 Effect of Hg^{2+} pollution on CAT activity

3 Discussion

The previous researches demonstrated that the method of DNA Ladder used in the detection of cells apoptosis *in vitro* was effective , because cells *in vitro* were similarly injured and cells apoptosis was induced simultaneously(Mengoni *et al.* 2000). However , apoptosis of cells *in vivo* was induced in the different time , so apoptosis was detected difficultly by DNA Ladder detection . In our studies , DNA ladder could be detected easily during cell apoptosis of *Lemna minor* L. treated with 7-10 $\text{mg} \cdot \text{L}^{-1} \text{Hg}^{2+}$. However , it almost couldn 't be detected in apoptosis induced by the concentration of $5 \text{ mg} \cdot \text{L}^{-1} \text{Hg}^{2+}$. So in the case of low concentrations , we selected one hundred primers for RAPD analysis. The results showed that twelve primers could detect the damage of DNA. The reason was that the polymorphic DNA had been amplified by PCR , using appropriate random primers. Therefore , the DNA damage and diversity of polymorphic fingerprints could be detected rapidly by RAPD technology. It also was a sensitive method in detecting the damage of DNA primary structure during apoptosis of plant cells.

Many cells apoptosis could be induced by the accumulation of reactive oxygen species (ROS), which had been suggested in many studies(Xia *et al.* ,1999 ;Lu *et*

al. ,1996). There was an effective defense mechanism of antioxidation in cells , including SOD ,CAT and POD , which could scavenge ROS out effectively. Cells could be in the normal condition when the balance was maintained between oxidation and antioxidation. Once the balance was disturbed , the function would be affected ,and which might lead to apoptosis. It was showed in our studies that Hg^{2+} at low concentration could cause a rapid increase of the anti-oxidant enzymes activity to scavenge the reactive oxygen , while the activity decreased distinctly when reactive oxygen exceeded a certain level , which would cause the plant cell apoptosis. The mechanisms of ROS ' damage to organism mainly related to the damage of DNA , especially the oxidative damage of mtDNA(Zeng & Zhang , 1995). The oxidative damages of mtDNA might cause some changes of the primary structure of DNA ,such as base deletion , base modification and insertional mutagenesis , in which base deletion was more universa(Halliwell & Aruoma ,1993). ROS can also induced nuclear DNA base deletion , base modification , single and double-stranded DNA breakage , interstand DNA cross-links and so or(Lu *et al.* , 1996). So we could propose that the appearance of DNA Ladder during cell apoptosis was likely concerned with ROS.

The latest studies abroad showed that heavy-metal pollution had a pronounced effect on the intrinsic DNA polymorphic fingerprints in RAPD analysis , namely the damage of DNA primary structure(Atienzar *et al.* 2001 ; Mengoni *et al.* ,2000). Our studies showed that Hg^{2+} could result in the damage of mtDNA and nuclear DNA primary structure , such as nucleotide fragment lost , mismatched , and so on (Fig. 1 , 2) , and lead to cell metabolism disorder and cell apoptosis in the end.

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