应用微核试验和单细胞凝胶电泳技术来检测农药对青蛙蝌蚪及成体的遗传毒性

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摘要: 应用青蛙红细胞微核试验和单细胞凝胶电泳试验研究了两种新型杀虫剂– 吡虫啉和抑食肼对青蛙蝌蚪和成体的遗传毒性, 结果表明: 当吡虫啉为 2mg/L 时, 蝌蚪红细胞微核率与对照组相比, 无显著性差异($p > 0.05$); 浓度升高到 8mg/L 时, 微核率与对照组相比, 有显著性差异($p < 0.05$); 当浓度为 32mg/L 时, 微核率与对照组相比, 有极显著性差异($p < 0.01$); 并有明显的剂量– 效应关系($r = 0.9843$)。而抑食肼在浓度为 2.5mg/L 和 10mg/L 时, 微核率与对照组相比, 无显著性差异($p > 0.05$); 当浓度增至 40mg/L 时, 微核与对照组相比, 有极显著性差异($p < 0.01$); 吡虫啉与抑食肼各浓度组对青蛙红细胞的 DNA 损伤与阴性对照组相比, 都有极显著性差异($p < 0.01$), 且具有明显的剂量– 效应关系($r = 0.960$, $r = 0.990$)。

关键词: 吡虫啉, 抑食肼, 黑斑蛙, 蝌蚪, 微核, 单细胞凝胶电泳试验(又名彗星试验)
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农田中施用的农药可以通过各种途径进入自然水体, 污染水环境, 并对非靶生物造成潜在危害。青蛙是敏感的生物群体和水生栖息环境质量指示生物, 利用蝌蚪或成体作为水环境污染的监测生物具有重要科学价值[1—5]。另一方面, 青蛙是害虫的天敌之一, 对于农田的生态防治起重要作用[6,7]。农田中施用的农药会直接或间接对蝌蚪或成体青蛙造成影响, 特别是农药的致突变性, 它不但影响青蛙的生活力和捕食能力, 而且还会对下代产生影响。因此, 研究农药对蝌蚪或成体的遗传毒性可以作为农药安全性评价的重要内容, 具有重要的科学意义。微核试验和单细胞凝胶电泳试验技术– 彗星试验(Comet assay)在检测化学物质的遗传毒性方面具有诸多优点, 广泛地应用于遗传毒理学研究[8—18]。陈军建等建立了规范化的青蛙蝌蚪红细胞微核试验, 而有关青蛙体细胞彗星试验目前国内尚未见报道。

吡虫啉和抑食肼是我国近期研制合成的新型杀虫剂, 具有良好的杀虫效果[19—22]。但它们对非靶生物青蛙的分子生态毒理学的研究尚未见报道。本文在研究了它们对蝌蚪红细胞微核率影响的基础上, 并尝试建立青蛙红细胞的彗星试验, 来研究它们对青蛙成体的 DNA 损伤情况, 为评价这两种新型杀虫剂对农田生态系统的影响及其合理安全使用提供科学的依据。

1 材料与方法

1.1 受试生物 黑斑蛙(*Rana nigronaculata* Hallowell)蝌蚪, 采自南京市风景区紫金山无污染的水塘中。蛙龄 1.5 个月左右。大小一致, 体长: 3.75±0.11cm, 体重 0.461±0.06g。成体青蛙, 购于市场, 体重: 60±15g。

1.2 试验方法 黑斑蛙蝌蚪红细胞微核试验[1—4]: 将采来的黑斑蛙蝌蚪放入曝气池中暂养 2—3d 后, 去掉不健康的蝌蚪, 然后逐渐将暂养水换成稀释水进行驯化(7d, 水温 25±1℃)。自然死亡率< 1%。试验前 24h 停止喂食。用曝气 24h 以上的自来水, 分别配成 3 个不同的浓度, 吡虫啉为 2、8 和 32mg/L; 抑食肼为 2.5、10.0 和 25mg/L; 并用曝气的自来水作对照。试验容器为 5L 的玻璃器皿, 每缸实验液为 4L, 每个浓度放 10 只蝌蚪, 染毒 7d, 期间不喂食。

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染毒后, 取蝌蚪, 用纱布将体表水擦干, 剖腹取蝌蚪心脏制片, 将血加到预先滴有小牛血清的载玻片上, 甲醇固定, 10% Giemas (pH= 6.89) 染色 20min, 风干。镜检, 每个浓度组观察 7 个动物, 每个蝌蚪制备 1 张血涂片, 每个片子观察计数 2000 个细胞以上。计算红细胞的微核千分率(‰), 并记录结果。

黑斑蛙红细胞彗星试验[8—17]: 成蛙在实验室驯养 7d, 选体色鲜艳活泼健康的青蛙, 用乙醚麻醉青蛙并固定在解剖台上, 剪开腹腔露出心脏, 用肝素钠处理过的注射器迅速插进心脏取血。然后用 Hank's 液稀释到大约为 10^5—10^6 个细胞/mL 浓度。分装小的离心管, 每管 0.9mL。并以苔酚蓝染色观察细胞存活率。加入受试物, 使吡虫啉的最终浓度为: 0.05、0.1、0.2、0.5mg/L, 抑食肼的最终浓度为: 5、25、50、100mg/L(土温- 80 为助溶剂)。另设一空白(蒸馏水) 和阴性对照组(土温- 80, 浓度 1%), 35℃恒温培养箱染毒 2h, 染毒完毕。离心去掉上清液, 置于冰块上冷却待用, 以苔酚蓝染色观察染毒后的细胞存活率, 以反映受试物对细胞的毒性。彗星测试按王民生[11] 报道的方法进行, 其程序为: 单细胞悬液滴于显微镜载玻片(毛面) 上铺成"三明治"结构的凝胶片, 凝胶片浸没于碱性溶液中消化 1h, 经电泳、中和、荧光染色后, 用显微镜观察、计数; 对 DNA 损伤程度进行分级, 分级标准为: 无损伤(0 级), 轻度损伤(1 级), 中度损伤(2 级), 重度损伤(3 级), 极其严重损伤(4 级)。计算细胞损伤率和专用单位(Arbitrary units)[13,17]。DNA 专用单位 $U=\sum i \times n_i$, n_i 为第 i 级损伤细胞数, 是一种衡量 DNA 链损伤程度的特有单位, 是把不同的分级加以换算统计, 得到 DNA 损伤的总体水平。统计方法应用 X^2 检验。

2　结果与讨论

吡虫啉和抑食肼对青蛙蝌蚪微核试验和成体彗星试验的结果分别见表 1 和表 2。

表 **1**　吡虫啉与抑食肼对黑斑蛙蝌蚪红细胞微核率的影响

Tab. 1　Effects of imidacloprid and RH-5849 on micronuclei frequency in tadpole erythrocytes of the frog Hallowell

受试物 Chemicals	剂量 Dosage(mg/L)	动物数 Number of animals(个)	观察细胞数 Number of cells observed	微核率 Micronucleus frequencies(‰)
对照 control		7	14380	1.46±0.64
吡虫啉	2	7	14149	1.70±0.48
Imidacloprid	8	7	14163	2.40±0.34*
	32	7	14112	3.75±0.84**
抑食肼	2.5	7	14079	1.56±0.57
RH-5849	10	7	14128	1.70±0.52
	40	7	14136	3.55±1.11**

* $p < 0.05$, ** $p < 0.01$, (与对照组相比)。

表 **2**　吡虫啉与抑食肼对青蛙红细胞 **DNA** 的损伤

Tab. 2　Number of frog erythrocyte in each damage degree and the DNA damage scoring in control and treat groups

受试物 Chemicals	暴露浓度 Exposure Dosage(mg/L)	受损细胞分级 Number of cells in each damage grade(Mean±SD) 0	1	2	3	4	损伤率 Damage Percentage(%)	AU*
吡虫啉	0.05	76.3±12.3	23.7±8.9	0±0	0±0	0±0	23.7**	23.7
Imidacloprid	0.1	23.5±6.4	66.7±13.4	9.8±3.2	0±0	0±0	76.47**	86.27
	0.2	13.0±6.1	60.9±12.4	21.7±4.5	4.3±1.2	0±0	86.96**	117.39
	0.5	7.8±1.3	17.6±4.5	41.2±12.5	31.4±8.9	2.0±0.5	92.16**	201.96
抑食肼	5	75.5±14.5	24.5±7.4	0±0	0±0	0±0	24.5**	24.5
RH-5948	25	32.7±4.5	55.8±14.5	11.5±3.2	0±0	0±0	67.31**	78.85
	50	14.8±3.4	37.7±4.5	36.1±7.8	11.5±3.6	0±0	82.5**	144.26
	100	5.2±0.9	10.3±1.2	41.4±9.6	41.4±9.4	1.7±0.8	94.83**	224.14
蒸馏水 Distilled water	(空白)	97.3±7.8	2.7±0.9	0±0	0±0	0±0	2.7	2.7
Tween 80	1%	96.2±8.6	3.8±1.2	0±0	0±0	0±0	3.8	3.8
丝裂霉素 Mitomycin C	100ug/L	2.4±0.6	7.3±1.6	39.0±4.3	41.5±7.3	9.8±3.7	97.56**	248.78

* DNA 专用单位: $U=\sum i \times n_i$, n_i 为第 i 级损伤细胞数; ** $p < 0.01$(用 X^2 检验)。

从表1中可以看出, 当吡虫啉和抑食肼浓度低时, 对蝌蚪红细胞微核率影响不大, 与对照组相比无显著性差异($p > 0.05$); 浓度升高到一定程度时, 则对微核率有明显的影响, 与对照组相比有显著性差异($p < 0.05, p < 0.01$); 并有明显的剂量- 效应关系($r = 0.9843$)。而且这两种农药在高浓度时, 不但蝌蚪红细胞微核率增加, 还表现在双微核和多微核细胞增多, 有的细胞发生核碎裂现象。

苔酚蓝染色观察表明, 染毒前后, 细胞的存活率度在90%, 说明系遗传毒性而非细胞毒性。从表2中可以看出, 吡虫啉与抑食肼各浓度组对青蛙红细胞的DNA损伤与阴性对照组相比, 都有极显著性差异($p < 0.01$), 且具有明显的剂量- 效应关系($r = 0.960, r = 0.990$)(图1, 2)。

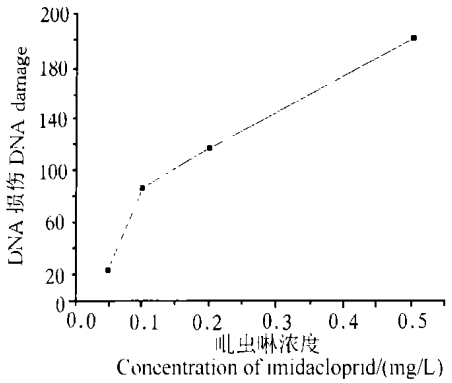

图1 吡虫啉对青蛙红细胞DNA损伤剂量- 效应关系

Fig. 1 The dose-responsing relation ship of imidacloprid on frog erythrocyte cells

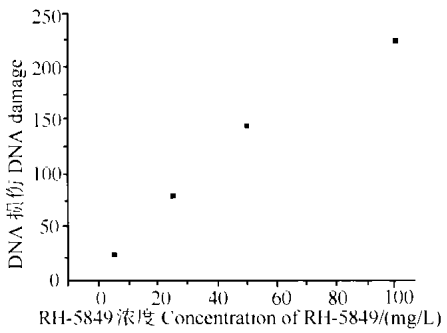

图2 抑食肼对青蛙红细胞DNA损伤剂量- 效应关系

Fig. 2 The dose-responsing relation ship of RH-5849 on frog erythrocyte cells

由上述可知, 吡虫啉与抑食肼在浓度低(吡虫啉: 2mg/L, 抑食肼: 10mg/L)时对蝌蚪红细胞的微核率基本上没有影响, 而青蛙红细胞的彗星试验结果则显示, 在吡虫啉为0.05mg/L、抑食肼为5mg/L情况下就已经对DNA具有损伤作用, 而且具有明显的剂量- 效应关系。这是由于这两种检测方法测试的终点(End-points)不同, 微核试验检测的是染色体断裂、染色体丢失, 染色体不分离、细胞分裂延迟和凋亡等多个终点, 与细胞的分裂密切相关。在微核试验中细胞要经过两个细胞周期, 有损伤DNA等的修复过程, 而且只有在DNA双链完全断裂的情况下, 不能完全修复的染色体断裂片才形成微核[1—3, 18]。虽然在浓度很低时, 这两种农药已经对DNA造成了一定程度的损伤, 但是生物活细胞都具有修复DNA损伤的能力, 结果不一定形成微核; 而彗星试验则与细胞的分裂与否无关, 只要化学物质对DNA有损伤作用, 就能在显微镜下形成明显的彗星, 且彗星拖尾的长度与DNA损伤的程度成线性相关[14]; 在青蛙红细胞彗星试验中染毒2h后, 细胞还来不及对损伤DNA进行修复, 就进入消化液中进行消化, 终止了细胞的修复作用。所以, 彗星试验的敏感性(就引起可见效应的化学物质浓度而言)比微核试验要高得多, 同时它还可以检测出化学物质对细胞DNA单链损伤作用, 以及细胞对DNA损伤的修复能力等[13, 14, 16], 可知青蛙红细胞单细胞凝胶电泳试验是一种敏感的检测DNA损伤的技术和一种很好的分子生态毒理学方法[5, 14]。该方法的建立, 对揭示农药对水生生物体的潜在危害及作用本质具有重要意义, 同时也为水环境中致突变物的检出提供了一种新的敏感而准确可靠的生物检测方法[5, 15], 在环境研究中将有着广泛的应用前景, 特别是它与蝌蚪红细胞微核试验的综合研究对于化学物质致突变性评价和水环境质量的评价具有重要意义[12]。

吡虫啉和抑食肼对蝌蚪红细胞微核率的最低影响浓度(LOEC)分别为8mg/L和40mg/L; 对青蛙成体DNA有损伤作用的浓度分别为0.05mg/L和5mg/L。将微核试验和单细胞凝胶电泳试验结果进行比较, 发现蝌蚪红细胞在一定程度范围内对DNA损伤具有修复作用; 青蛙红细胞单细胞凝胶电泳技术是一种敏感的农药致突变性或水环境致突变物生物检测方法。

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GENOTOXICOLOGICAL STUDIES OF TWO PESTICIDES TO TADPOLES AND FROGS OF *RANA NIGRINACULATA* HALLOWEII BY MICRONUCLEI TEST AND SINGLE CELL GEL ELECTROPHORESIS ASSAY

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Abstract: Imidacloprid [1-(6-chloro-3-pyridylmethyl)-N-nitro-imidazolidin-2-ylideneamine] and RH-5849[2′-benzoyl1′-tert-butylbenzoylhydrazine] are two novel pesticides being used in China. Imidacloprid, which act as an agonist at the nicotinic acetylcholine receptor, is highly effective against many sucking insects including ricehoppers, aphids, thrips and white flies. RH-5849, a nonsteroidal ecdysone agonist, which act similar to 20-hydroxyecdysone by binding to the ecdosone receptor, have been found to be very effective against lepidopteran pests in vegetables, cotton, and cereals. To our knowledge, their effects on the aquatic and agricultural ecosystems have not been fully investigated. Amphibians are important organisms in the aquatic and agricultural ecosystems; they are among the most important natural enemies of many agricultural pests. Because of their sensitivity to changes of their habiat and that their larvae live in the aquatic environment, the amphibians were regarded as bio-indicators of aquatic and agricultural ecosystems, and broadly used as typical test organisms in evaluating the effects of chemicals on the aquatic and agricultural ecosystems. This study was initiated to combine micronucleus test (MN) and comet assay to assess the genotoxicity of the two pesticides on the amphibians from different endpoints. The objective was to achieve a more comprehensive understanding of the effects and potential risks of the pesticides on the aquatic and agricultural ecosystems.

Amphibians, *Rana nigronaculata Hallowell* was selected as the test organisms. They were acclimated to the conditions of the laboratory for 7 days before the tests. The tadpoles were one and half months old, with body lengths of 37.5±1.1mm and body weights of 461±60mg and the frogs with body weights of about 100 g were chosen for the tests.

After being tamed for 7 days in the laboratory, the tadpoles of *R. nigronaculata* were exposed to different levels of the two pesticides for 7 days. The concentration of DO in the solutions was maintained at no less than 8.5 mg/L with bubble aerator, and the temperature of solutions was controlled at 20±1℃, and the solutions were replaced with freshly prepared solution of the same concentration every 24hr. After the seven-day exposure, blood was taken from each of the tadpoles by cardiac puncture and one smear was prepared per animal. Fixed in methanol and stained with 5% of Giemsa in Sorensen buffer (pH 6.98), the smears were screened under a microscope. The erythrocytes with one or more micronuclei were conuted for a total of at least 2000 erythrocytes per tadpole and seven random animals were screened in each group.

The procedure of comet assay was basically the same as that described by Singh et al. Modifications due to the uniqueness of the biological material studied and due to the equipment available were relatively minor. Blood samples were collected from *R. nigronaculata* frogs by decapitation followed by immediately placing the animals in a 10% solution of Hank's balanced salt solution. The survival rate of erythrocytes after the isolation was higher than 95% as examined by trypan blue exclusion test. After 1hr of exposure at 20℃ in a 5% CO_2 atmosphere, the cells were collected by centrifugation (10 min. 3000rpm, at 4℃) and washed twice with Hank's balanced salt solution (at 4℃) to minimize possible damage repair. Samples were immediately placed on ice for comet assay. After lysing, electrophoresis and neutralization, the slides were stained with ethidium bromide(EB). For evaluation of DNA damage, 100 cells per slide were analyzed at 400× magnification under a "TMD-EF" fluorescent microscope (Nikon, Japan). The cells were scored visually and given scores 0 (undamaged), 1, 2, 3 or 4(maximally damaged) according to tail intensity (size and shape). Thus, the total

score for 100 comets ranges from 0 (all undamaged) to 400 (all maximally damage). The percentage of damaged cells was calculated and the results analyzed with the χ^2 test. The "AUs" was used to express the extent of DNA damage and were calculated as follows:

$$Arbitrary\ units = \sum_{i=0}^{4} n_i \times i \quad (1)$$

Where n_i is number of cells in damage degree i(0, 1, 2, 3, 4).

The results showed that there were not differences in the frequencies of micronuclei when the concentrations of Imidacloprid was 2mg/L and the concentrations of RH-5849 were 2.5mg/L and 10mg/L($r > 0.05$), but Imidacloprid at or above 8mg/L and RH-5849 at 32mg/L, there were evident differences ($r < 0.05$ or $r < 0.01$), compared with the control groups. And the dose-effect relationship was observed evidently ($r = 0.9843$). The results of the comet assay showed that the distributions in the damage grades in all the pesticide-treated groups were significantly different from the control ($p < 0.01$). DNA damage scores expressed as arbitrary units (AUs) increased with the exposure levels of the two pesticides and in the tests dose-effect relationships were observed for both imidacloprid($r^2 = 0.92$) and RH-5849 ($r^2 = 0.98$). The MN test and comet assay revealed potential adverse effects of the two pesticides on DNA in the erythrocytes of amphibians, *R. nigronaculata*. As the amphibian *R. nigronaculata* is a sensitive organism suitable for acting as the bio-indicator of aquatic and agricultural ecosystems, the combination of acute toxicity test, MN test and comet assay in this study provided valuable information to evaluate the pesticides' risks to aquatic and agricultural ecosystems. And the results showed that the comet assay has a broad prospect in determining the mutagen in the environment.

Key words: Imidacloprid; RH-5849; *Rana nigronaculata* Hallowell; Tadpole; Micronuclei; Single cell gel electrophoresis assay (comet assay)