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草甘膦暴露对斑马鱼胚胎发育的毒性效应及机制

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摘要: 为了探究环境中草甘膦的毒性效应及致毒机理。将斑马鱼(*Danio rerio*)胚胎暴露于草甘膦(1、10、100 $\mu\text{g}\cdot\text{L}^{-1}$) 72 h, 观察并记录胚胎孵化率、存活率、畸形率、心率, 测定 ATPase 活性及胚胎发育相关基因(*Tbx5*、*Nkx2.5*、*Tnnt2*、*Bmp2b*、*Ihh*、*Shh*)表达水平。结果显示, 高浓度组(10 $\mu\text{g}\cdot\text{L}^{-1}$ 和 100 $\mu\text{g}\cdot\text{L}^{-1}$) 中斑马鱼胚胎存活率和孵化率显著降低, 心包水肿率、脊柱弯曲率和卵黄囊水肿率显著升高; 胚胎发育相关基因的转录水平发生了改变, 在 1 $\mu\text{g}\cdot\text{L}^{-1}$ 组中, *Nkx2.5* mRNA 水平无明显变化, 但 *Tbx5*、*Tnnt2*、*Bmp2b*、*Shh* 水平明显升高, 而 *Ihh* 水平明显降低; 在 10 $\mu\text{g}\cdot\text{L}^{-1}$ 和 100 $\mu\text{g}\cdot\text{L}^{-1}$ 组中, *Tbx5*、*Nkx2.5*、*Bmp2b*、*Ihh*、*Shh* 的 mRNA 表达显著降低; 在 10 $\mu\text{g}\cdot\text{L}^{-1}$ 和 100 $\mu\text{g}\cdot\text{L}^{-1}$ 暴露组 Na^+/K^+ -ATP 酶和 Ca^{2+} -ATP 酶活性均受到显著抑制。较高浓度的草甘膦对于鱼类具有显著的发育毒性, 毒性效应机制与胚胎发育相关基因转录水平变化及酶活性抑制有关。依据试验结果, 草甘膦 LOEC 为 10 $\mu\text{g}\cdot\text{L}^{-1}$, NOEC 为 1 $\mu\text{g}\cdot\text{L}^{-1}$, 环境中的草甘膦残留量达到一定浓度($\geq 10 \mu\text{g}\cdot\text{L}^{-1}$) 时对鱼类构成潜在威胁, 在实际应用中应全面考察环境草甘膦可能残留的浓度。

关键词: 草甘膦; 斑马鱼; 发育毒性; 毒性机制

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Developmental Impact of Glyphosate Exposure on Zebrafish Embryos and Mechanism Involved

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Abstract: In order to explore the toxic effects of glyphosate and underlying mechanism, zebrafish (*Danio rerio*) embryos were exposed to glyphosate at 1, 10 and 100 $\mu\text{g}\cdot\text{L}^{-1}$ for 72 h, and embryonic hatching rate (48 h post-fertilization), survival rate, malformation rate, heart rate, ATPase activity and the transcription of development-related genes (*Tbx5*, *Nkx2.5*, *Tnnt2*, *Bmp2b*, *Ihh*, *Shh*) were examined. Both survival rate and hatching rate were significantly reduced in the high concentration groups (10 $\mu\text{g}\cdot\text{L}^{-1}$ and 100 $\mu\text{g}\cdot\text{L}^{-1}$), the rates of pericardial edema, spine curvature and yolk sac edema were all significantly increased. The transcriptional levels of development-related genes were altered, in the 1 $\mu\text{g}\cdot\text{L}^{-1}$ group, *Nkx2.5* mRNA levels showed no significant change, the mRNA levels of *Tbx5*, *Tnnt2*, *Bmp2b*, *Shh* were significantly increased, while the transcription of *Ihh* were significantly de-

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creased. Significantly decreased mRNA levels of *Tbx5*, *Nkx2.5*, *Bmp2b*, *Ihh*, *Shh* were observed in the 10 $\mu\text{g}\cdot\text{L}^{-1}$ and 100 $\mu\text{g}\cdot\text{L}^{-1}$ groups. The activities of Na^+/K^+ -ATPase and Ca^{2+} -ATPase were significantly inhibited in the 10 $\mu\text{g}\cdot\text{L}^{-1}$ and 100 $\mu\text{g}\cdot\text{L}^{-1}$ groups. Higher concentrations of glyphosate showed significant developmental toxicity for zebrafish, and the mechanisms underlying toxic effect might be related to the changes in the transcription level of genes related to embryonic development and the inhibition of the enzyme activities. Based on the results of this study, the LOEC of glyphosate is 10 $\mu\text{g}\cdot\text{L}^{-1}$ and NOEC is 1 $\mu\text{g}\cdot\text{L}^{-1}$. Thus, glyphosate residue in the environment at a certain concentration ($\geq 10 \mu\text{g}\cdot\text{L}^{-1}$) would pose a potential threat to fish. In practical application, the residual concentration of glyphosate in the environment should be fully investigated.

Keywords: glyphosate; zebrafish; developmental toxicity; mechanism

草甘膦(glyphosate)是1971年由Monsanto公司开发的一种内吸传导的非选择性芽后除草剂,其原理是通过抑制植物莽草酸(shikimic acid)途径中关键酶的合成,从而影响植物芳香族氨基酸(aromatic amino acid)的生物合成,使蛋白质合成受到抑制,导致植物死亡。由于这条生化途径在高等生物中并不存在,通常认为草甘膦对包括人类在内的哺乳动物是安全的,美国环境保护局(United States Environmental Protection Agency, US EPA)将草甘膦列为第三类有毒物质(低毒性)^[1]。草甘膦因具有高效、低毒、广谱的特点,成为世界上应用最广、使用量最大的农药品种,年销售值一直居农药之首^[2]。

除草剂多为有一定残效期(1~5年)的化合物,可在生物机体内富集,对水体中的鱼类构成严重威胁^[3]。在中国,每年有145万t的各种类型农药被使用^[4],仅有1%农药有效作用于靶生物^[5],其余大部分通过沉降、雨水冲刷等途径进入土壤和农田水环境。水环境中草甘膦分布较广,在河流、饮用水源水、海水、地下水和雨水中都曾检测到草甘膦的存在。2011年范瑾煜等^[6]测定太湖中草甘膦含量为0.113 $\mu\text{g}\cdot\text{L}^{-1}$ 。在美国的河流中检测到草甘膦质量浓度达到了2.2 $\mu\text{g}\cdot\text{L}^{-1}$ ^[7];在加拿大南部的安大略湖的最高浓度达40.8 $\mu\text{g}\cdot\text{L}^{-1}$ ^[8],在德国的江河中检出最高浓度3.2 $\mu\text{g}\cdot\text{L}^{-1}$ ^[9];在法国雨水管道中检出最高浓度75~90 $\mu\text{g}\cdot\text{L}^{-1}$ ^[10]。草甘膦及制剂对生物及人体的危害不可忽视^[11],越来越多的研究表明,草甘膦及其制剂对多种水生生物和哺乳动物都具有毒性作用^[12-14]。草甘膦及其制剂低浓度暴露显示出对鲫鱼(*Carassius auratus*)的生态毒性^[15];不同浓度的草甘膦对鲫鱼染毒后,血红蛋白和血红细胞形成受抑制^[16];泥鳅(*Misgurnus anguillicaudatus*)暴露于草甘膦后,外周血淋巴细胞和粒细胞均升高^[17];草甘膦制剂对银鲶鱼(*Rhamdia quelen*)的新陈代谢和酶活性产

生显著影响^[18];草甘膦可导致银鲶鱼机体自由基增加、天冬氨酸氨基转移酶(aspartate aminotransferase, AST)活力升高和线粒体损伤^[19];将金鱼暴露于低剂量(2.5~20 $\text{mg}\cdot\text{L}^{-1}$)草甘膦96 h后,金鱼组织中超氧化物歧化酶(superoxidase dismutase, SOD)、谷胱甘肽过氧化物酶(glutathione peroxidase, GST)、谷胱甘肽还原酶(glutathione reductase, GR)和葡萄糖-6-磷酸脱氢酶(glucose-6-phosphate dehydrogenase, G-6-PD)活力受到抑制^[20-21]。草甘膦对青鳉(*Oryzias latipes*)暴露显出肝脏毒性^[22];罗非鱼(*Oreochromis mossambicus*)暴露于浓度为15 $\text{mg}\cdot\text{L}^{-1}$ 的草甘膦后可导致其肾脏病变^[23];草甘膦暴露可导致虹鳟(*Oncorhynchus mykiss*)幼鱼出现损害,且表现时间-效应关系^[24];成年雌性斑马鱼(*Danio rerio*)暴露于65 $\mu\text{g}\cdot\text{L}^{-1}$ 草甘膦15 d后可使类固醇生成因子-1(steroidogenic factor, SF-1)表达升高^[25]。

随着草甘膦及其代谢物在水环境中的残留量逐年增加,生物富集作用使其影响更加长期,对水环境造成严重威胁,其潜在致毒作用有必要长期关注。研究显示,草甘膦对草鱼(*Ctenopharyngodon idella*)、鲢鱼(*Hypophthalmichthys molitrix*)、鲫鱼(*Carassius auratus auratus*)和斑马鱼(*Danio rerio*)的96 h-LC₅₀分别为0.2518、0.2588、0.2599和0.879 $\text{mg}\cdot\text{L}^{-1}$ ^[26-27],依照毒性分类标准属于高毒农药。

斑马鱼与人类基因同源性高达85%^[28],且具有发育周期短、体外受精、胚胎透明易观察、单次产卵数高等优势,已成为研究环境污染物对水生生物影响的良好模型^[29-31]。研究表明,斑马鱼的胚胎发育受多种基因调控,心脏及骨骼发育在胚胎至仔鱼阶段可初步完成,而胚胎时期心脏及骨骼发育与相关基因表达量直接相关,*Tbx5*是心肌细胞分化与心脏系统传导功能重要因子^[32]; *Nkx2.5*是触发初始心肌细胞分化的关键因素, *Tnnt2*编码心肌肌钙蛋白^[33];

Bmp2b 是骨骼发育中背板早期形成的重要因子;*Shh*、*Ihh* 与早期软骨组织形成关系密切^[34]。本课题根据环境中检测出的草甘膦浓度范围^[6-10]设置浓度梯度,研究其对斑马鱼胚胎的毒性效应,并以 *Tbx5*、*Nkx2.5*、*Tnnt2*、*Bmp2b*、*Shh*、*Ihh* 等为目标基因,采用聚合酶链式反应(polymerase chain reaction, PCR)初步分析其分子机制,对草甘膦的生态风险评估以及水体污染的生物监测提供指导,期望为进一步制定草甘膦的毒性判断标准提供参考数据。

1 材料与方法 (Materials and methods)

1.1 主要试剂

草甘膦(纯度 $\geq 99\%$,山东西亚化学股份有限公司),先以纯水配制浓度为 $1\text{ mg}\cdot\text{L}^{-1}$ 储备液, $4\text{ }^{\circ}\text{C}$ 避光保存。蛋白含量测定、ATP 酶活性测定、RNA 提取、逆转录、荧光定量 PCR 等试剂盒均购于南京建成生物工程研究所,引物由福州擎科生物技术有限公司合成;其他试剂均为分析纯试剂。

1.2 主要仪器

体视显微镜(尼康, SMZ745T),光照培养箱(上海一恒, THZ-100),酶标仪(上海闪谱生物科技有限公司, ReadMax-1200),实时荧光定量 PCR 仪(Archimed, TMX4),全自动数码凝胶成像分析仪(培清科技, JS-2012),琼脂糖凝胶电泳仪(北京六一生物技术有限公司, DYY-8C),低温高速离心机(SCILO-GEX, CF1524R)。

1.3 实验用鱼

斑马鱼(AB 品系,购于福州百维斯生物科技有限公司),根据 Westerfield^[35]的方法饲养于武夷学院圣农实验中心的斑马鱼养殖循环系统(上海海圣)。循环养殖水(曝气除氯并经活性炭过滤的自来水),水温(26 ± 1) $^{\circ}\text{C}$,光照周期为 14 h 光照/10 h 黑暗,喂食新鲜孵化的丰年虫(*Artemia nauplii*),日常每天早晚喂 2 次,计划配鱼前一周,每天早中晚喂 3 次。

1.4 胚胎收集

实验前 1 天晚上,将雌雄鱼按照 1:1 比例放置在孵化槽中,并用隔板将雌雄分开,第 2 天早上约 9 点打开隔板,开灯给予光照刺激使其交配产卵。0.5~1 h 内收集鱼卵,用斑马鱼养殖液清洗并在显微镜下观察,选取受精胚胎用于暴露实验。

1.5 暴露试验

设置终浓度为 1、10、100 $\mu\text{g}\cdot\text{L}^{-1}$ 3 个试验组,1 个空白对照组,每组设 3 个平行($n=3$)。实验前将草

甘膦用培养液($3.5\text{ g}\cdot\text{L}^{-1}\text{ NaCl}$, $0.05\text{ g}\cdot\text{L}^{-1}\text{ NaHCO}_3$, $0.05\text{ g}\cdot\text{L}^{-1}\text{ KCl}$, $0.05\text{ g}\cdot\text{L}^{-1}\text{ CaCl}_2$)配制染毒液,分别置于直径 90 mm 培养皿(暴露培养液 30 mL, 50 枚斑马鱼胚胎)。暴露期间(28 ± 0.5) $^{\circ}\text{C}$ 恒温培养,每隔 12 h 更换等量等浓度的培养液并清理死亡胚胎, 72 hpf (hours post-fertilization)收样。

1.6 相关形态学指标统计及计算

于斑马鱼胚胎 12、24、36、48、72 hpf 时,观察胚胎的存活情况(肉眼观察胚胎变白或者显微镜下观察胚胎絮状凝结、心脏不跳动即可判定死亡),统计存活率。48 hpf 时,统计孵化率(胚胎出膜并能游动视为已孵化), 72 hpf 时统计畸形率(主要指心包水肿、卵黄囊水肿和脊柱弯曲),并测量心率(heart rate, HR)。

存活率 = 存活胚胎数/总胚胎数 $\times 100\%$; 孵化率 = 已孵化胚胎数/总胚胎数 $\times 100\%$; 畸形率 = 畸形胚胎数/存活胚胎数 $\times 100\%$ 。

心率的统计方法:每个处理组设 3 个平行,每个平行随机测 5 条仔鱼。在 40 倍焦距光学显微镜下,计算不同实验组的心跳速率,显微镜下对心房和心室的搏动次数进行记录并摄影,记录胚胎 20 s 内心跳次数,计算 1 min 心率^[36]。

1.7 ATP 酶活性测定

采用暴露 72 hpf 的仔鱼以超纯水荡洗后用液氮速冻,保存于 $-80\text{ }^{\circ}\text{C}$ 冰箱。实验前将样品于冰盒上解冻,按 1:9 的比例加入生理盐水,在匀浆器内仔细研磨。准备好的匀浆液在冷冻离心机上,在 $4\text{ }^{\circ}\text{C}$ 下,以 $4\ 000\text{ r}\cdot\text{min}^{-1}$ 离心 10 min,取上清液测定蛋白含量和酶活性。

蛋白含量测定、 Na^+/K^+ -ATP 酶活性、 Ca^{2+} -ATP 酶活性的测定均按试剂盒说明进行操作。计算酶活力时,定义每小时每毫克组织蛋白的组织中 ATP 酶分解 ATP 产生 1 μmol 无机磷的量为一个 ATP 酶活力单位($\mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{h}^{-1}$)。

1.8 荧光定量 PCR 测定

RNA 提取、逆转录、荧光定量 PCR 测定均使用试剂盒。内参基因选择 *efl α* ,各基因引物采用 primer5 软件自行设计(表 1),经 Basic Local Alignment Search Tool (BLAST)比对后,送由福州擎科生物技术有限公司合成。荧光定量 PCR 的结果采用相对定量法计算,基因的表达式 $F=2^{-\Delta\Delta C_T}$ 。

1.9 数据分析

结果采用平均值 $\pm$ 标准误差(Mean $\pm$ SE)表示,应用 SPSS 软件对数据进行单因素方差分析(one-way

ANOVA),组间数据用 Duncan 法进行显著性水平分析,设定 $P<0.05$ 表示差异显著。

2 结果 (Results)

2.1 草甘膦暴露对斑马鱼胚胎存活率及孵化率的影响

斑马鱼胚胎经草甘膦暴露 48 h,胚胎存活率与空白对照组(94%)比较,100 $\mu\text{g}\cdot\text{L}^{-1}$ 暴露组显著降低,降低至 82% (图 1)。胚胎孵化率空白对照组为 82%,经草甘膦暴露后 1 $\mu\text{g}\cdot\text{L}^{-1}$ 组无显著差异,10 $\mu\text{g}\cdot\text{L}^{-1}$ 、100 $\mu\text{g}\cdot\text{L}^{-1}$ 组孵化率显著降低,分别降至 42% 和 37% ($P<0.05$) (图 2)。

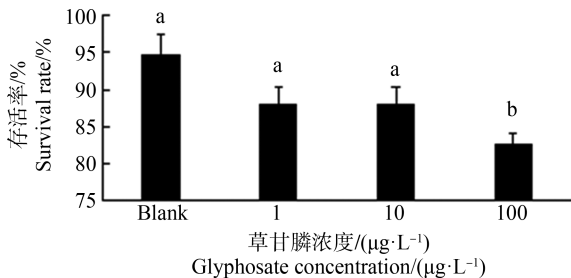


图 1 暴露 48 h 对斑马鱼胚胎存活率的影响

注:数据表示为平均值 $\pm$ 标准误差, $n=3$; 上标字母不同的组间具有显著性差异($P<0.05$)。

Fig. 1 Mortality rate in zebrafish embryos exposed for 48 h
Note: Data are presented as mean $\pm$ S.E., $n=3$; means of the treatment not sharing a common letter are significantly different ($P<0.05$).

表 1 荧光定量 PCR 序列
Table 1 Sequences of forward and reverse primers used for RT-PCR

目标基因 Target gene	引物序列(5' ~ 3') Primer sequences (5' ~ 3')	扩增片段长度/bp Primer size/bp
<i>eflα</i>	F:CTTCTCAGGCTGACTGTGC	358
	R:CCGCTAGCATTACCCTCC	
<i>Nkx2.5</i>	F:AGCATCCAACC TTCACAGTCC	173
	R:AAAAACATCCCAGCCAAACC	
<i>Tbx5</i>	F:ATTCGCCGATAACAAATGG	138
	R:CGCCTTGACGATGTGGAT	
<i>Tnnt2</i>	F:GGAAATCATCAGCCTCAAAGAC	141
	R:CATGTAACCGCCGAAGTGCA	
<i>Ihh</i>	F:GCTCACGCCGAACACTCAA	190
	R:GCCGTCTTCATCCCAAC	
<i>Shh</i>	F:GGCTGTTTCCCAGGTTC	149
	R:GGTCTTGCGTTTCTATGAC	
<i>Bmp2b</i>	F:AAAAGCCGAGGAGAAAGCAC	222
	R:TGGGAATGTTGGAGTTGACC	

2.2 草甘膦暴露对斑马鱼仔鱼脊柱弯曲率及卵黄囊水肿率的影响

斑马鱼胚胎在空白组及 1 $\mu\text{g}\cdot\text{L}^{-1}$ 暴露组未出现脊柱弯曲。10 $\mu\text{g}\cdot\text{L}^{-1}$ 、100 $\mu\text{g}\cdot\text{L}^{-1}$ 组出现脊柱弯曲,且随着草甘膦暴露浓度增大,脊柱弯曲率显著上升,10 $\mu\text{g}\cdot\text{L}^{-1}$ 、100 $\mu\text{g}\cdot\text{L}^{-1}$ 暴露组分别为 6.6%、7.3%。卵黄囊水肿发生率随着草甘膦暴露浓度增大而显著增大,10 $\mu\text{g}\cdot\text{L}^{-1}$ 、100 $\mu\text{g}\cdot\text{L}^{-1}$ 暴露组分别增大 26.5%、33.3% ($P<0.05$)(图 3 ~ 图 6)。

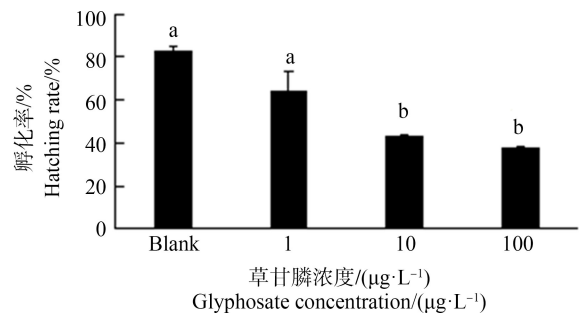


图 2 暴露 48 h 对斑马鱼胚胎孵化率的影响

注:数据表示为平均值 $\pm$ 标准误差, $n=3$; 上标字母不同的组间具有显著性差异($P<0.05$)。

Fig. 2 Hatching rate in zebrafish embryos exposed for 48 h
Note: Data are presented as mean $\pm$ S.E., $n=3$; means of the treatment not sharing a common letter are significantly different ($P<0.05$).

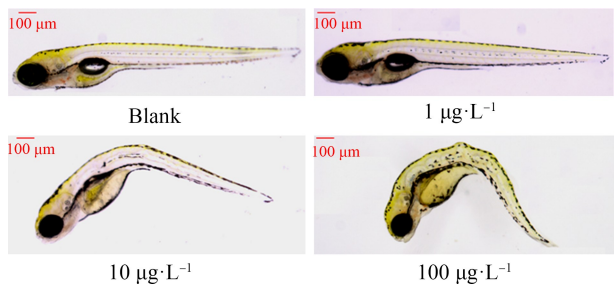


图 3 暴露 72 h 斑马鱼仔鱼脊柱弯曲

Fig. 3 Spinal curvature in zebrafish larvae exposed for 72 h

2.3 草甘膦暴露对斑马鱼仔鱼心率及心包水肿率的影响

由图 7 ~ 9 可知斑马鱼仔鱼心率及心包水肿情况。草甘膦暴露 72 h 斑马鱼仔鱼心率与空白对照组相比,仅 100 $\mu\text{g}\cdot\text{L}^{-1}$ 组显著上升 12.2% ($P<0.05$) (图 8)。由图 9 可知,空白对照组及 1 $\mu\text{g}\cdot\text{L}^{-1}$ 暴露组中未出现心包水肿,在 10 $\mu\text{g}\cdot\text{L}^{-1}$ 及 100 $\mu\text{g}\cdot\text{L}^{-1}$ 暴露组出现心包水肿,但发生率均不高,分别仅为 0.6% 和 2.6%,且由图 7 可知,10 $\mu\text{g}\cdot\text{L}^{-1}$ 组心包水肿程度较 100 $\mu\text{g}\cdot\text{L}^{-1}$ 组轻微。

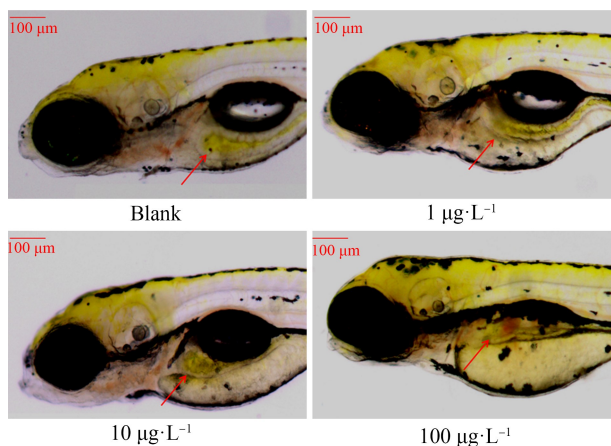


图4 暴露72 h 斑马鱼仔鱼卵黄囊水肿

Fig. 4 Yolk cyst in zebrafish larvae exposed for 72 h

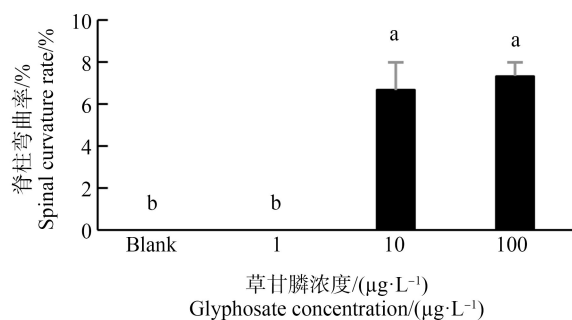


图5 暴露72 h 对斑马鱼仔鱼脊柱弯曲率的影响

注:数据表示为平均值 $\pm$ 标准误差, $n=3$; 上标字母不同的组间具有显著性差异($P<0.05$)。

Fig. 5 Spinal curvature rate in zebrafish larvae exposed for 72 h

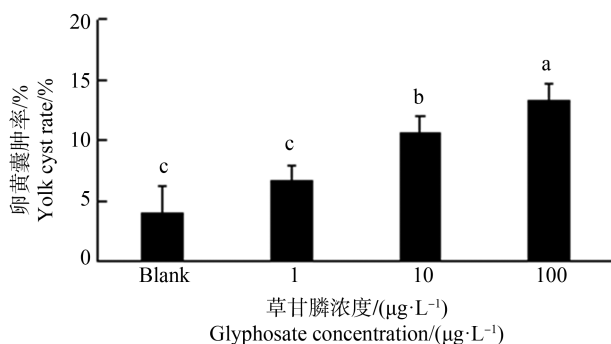
Note: Data are presented as mean $\pm$ S.E., $n=3$; means of the treatment not sharing a common letter are significantly different ($P<0.05$).

图6 暴露72 h 对斑马鱼仔鱼卵黄囊水肿率的影响

注:数据表示为平均值 $\pm$ 标准误差, $n=3$; 上标字母不同的组间具有显著性差异($P<0.05$)。

Fig. 6 Yolk cyst rate in zebrafish larvae exposed for 72 h

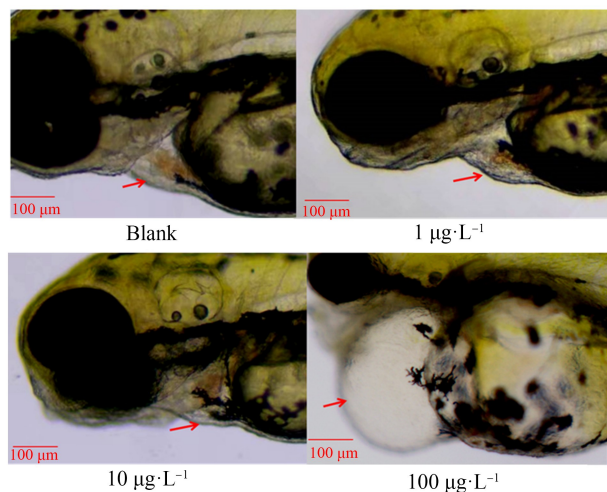
Note: Data are presented as mean $\pm$ S.E., $n=3$; means of the treatment not sharing a common letter are significantly different ($P<0.05$).

图7 暴露72 h 斑马鱼仔鱼心包囊水肿

Fig. 7 Pericardial cyst in zebrafish larvae exposed for 72 h

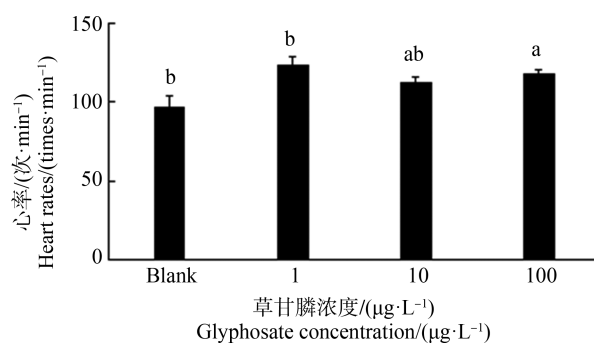


图8 暴露72 h 对斑马鱼仔鱼心率的影响

注:数据表示为平均值 $\pm$ 标准误差, $n=3$; 上标字母不同的组间具有显著性差异($P<0.05$)。

Fig. 8 Heart rates in zebrafish larvae exposed for 72 h

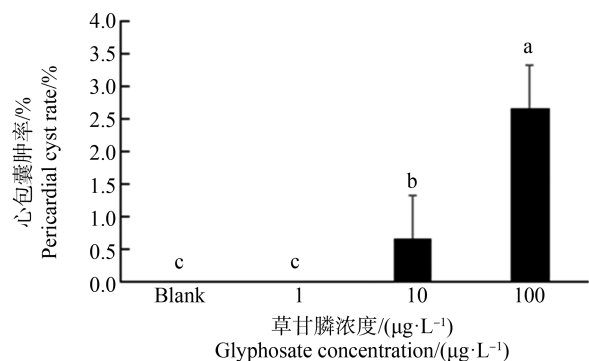
Note: Data are presented as mean $\pm$ S.E., $n=3$; means of the treatment not sharing a common letter are significantly different ($P<0.05$).

图9 暴露72 h 对斑马鱼仔鱼心包囊水肿率的影响

注:数据表示为平均值 $\pm$ 标准误差, $n=3$; 上标字母不同的组间具有显著性差异($P<0.05$)。

Fig. 9 Pericardial cyst rate in zebrafish larvae exposed for 72 h

Note: Data are presented as mean $\pm$ S.E., $n=3$; means of the treatment not sharing a common letter are significantly different ($P<0.05$).

2.4 草甘膦暴露对斑马鱼仔鱼 ATP 酶活性的影响

斑马鱼胚胎经草甘膦暴露 72 h, 在 $1 \mu\text{g} \cdot \text{L}^{-1}$ 暴露组仔鱼组织 Na^+/K^+ -ATP 酶活性及 Ca^{2+} -ATP 酶活性都与空白对照组无差异; 而在 $10 \mu\text{g} \cdot \text{L}^{-1}$ 及 $100 \mu\text{g} \cdot \text{L}^{-1}$ 暴露组均显著降低 ($P < 0.05$) (图 10)。

2.5 草甘膦对斑马鱼仔鱼相关基因表达的影响

斑马鱼胚胎暴露 72 hpf 后相关基因表达量如图 11 所示, 草甘膦暴露对仔鱼的多个基因产生了不同程度的影响。其中, 在低浓度 ($1 \mu\text{g} \cdot \text{L}^{-1}$) 暴露组中, *Bmp2b*、*Tbx5*、*Tnnt2* 和 *Shh* 等 4 个基因表达量上升 (上升量分别为 28%、45%、87% 和 86%, $P < 0.05$), 而 *Ihh* 表达水平降低 (下降量为 25%, $P < 0.05$), *Nkx2.5* 表达水平与空白组无差异。但当草甘膦浓度增大时, 在 $10 \mu\text{g} \cdot \text{L}^{-1}$ 及 $100 \mu\text{g} \cdot \text{L}^{-1}$ 暴露组中各相关基因表达水平均出现下调, 且存在剂量-效应关系, *Nkx2.5* 表达量下调比例最大, 达 82% ($P < 0.05$)。

3 讨论 (Discussion)

鱼类毒性试验是评价污染物环境风险的主要方法之一, 斑马鱼胚胎毒性检测实验中常出现的毒性反应是心包水肿、脊柱弯曲、卵黄囊水肿等发育畸形^[37]。有研究表明, 水肿会引起生物体代谢障碍, 损害器官功能, 是生物体抵御外界有毒物质入侵的一种反应^[38]。实际上, 脊柱弯曲、心包水肿、心脏形态和功能缺陷在暴露于杀菌剂和杀虫剂等化学物质的鱼类胚胎中相当常见^[39-42]。本试验中, 斑马鱼胚胎经草甘膦暴露后仔鱼有出现心包水肿、卵黄囊水肿及脊柱弯曲等反应, 显示草甘膦对斑马鱼胚胎具有显著的发育毒性。

心脏作为胚胎发育过程中最早发挥功能的器官, 也是鱼类胚胎试验的重要毒理学终点^[43-44], 完善的循环功能对于胚胎的正常发育起着关键作用^[45]。对于斑马鱼胚胎发育而言, 正常生长条件下, 48 hpf 时主要的器官系统完成形态发生并开始破膜孵化, 这是发育进程的一个重要转折点。当生物体内能量供给不足时, 心脏的正常工作会受到影响^[46]。有研究表明污染物的暴露会使斑马鱼胚胎心率上升或下降以适应心脏对能量的供给^[47]。本试验中, 斑马鱼胚胎经草甘膦暴露后仔鱼心率升高, 可推测此表现是为了满足心脏发育进程中能量需要的适应性反应。

ATP 酶在细胞内维持离子平衡和渗透压稳态方面具有重要作用^[48]。 Na^+/K^+ -ATP 酶是组成 Na^+/K^+ 泵的主要部分, Ca^{2+} -ATP 酶是质膜上钙离子转运系统中最重要部分。有研究分析, 受污染物低浓度

暴露时, 鱼体为了使机体免受氧化损伤, 抗氧化酶会被激活, 清除自由基; 但当污染物浓度超过机体耐受阈值, 产生的自由基过多会使细胞衰老加速, 导致酶活性降低^[49], 这也能反映能量代谢的变化^[50]。本试验结果显示低浓度 ($1 \mu\text{g} \cdot \text{L}^{-1}$) 草甘膦暴露中仔鱼的 Na^+/K^+ -ATP 酶和 Ca^{2+} -ATP 酶的活性无显著变化, 但在高浓度 ($10 \mu\text{g} \cdot \text{L}^{-1}$ 、 $100 \mu\text{g} \cdot \text{L}^{-1}$) 组中 2 种 ATP 酶活性均下降。有研究认为 ATP 酶的作用是水解高能化合物 ATP 而释放供生命活动所需的能量^[51], 造成胚胎发育异常的原因可能与 ATP 酶的活性抑制有关。本试验结果提示草甘膦暴露引起 Na^+/K^+ -ATP 酶和 Ca^{2+} -ATP 酶活性的降低也是影响斑马鱼胚胎心脏发育畸形的原因。

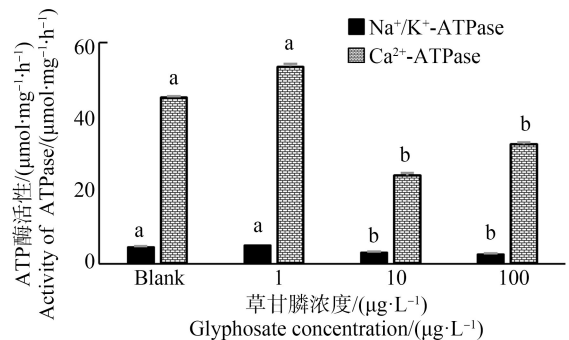


图 10 暴露 72 h 对斑马鱼仔鱼 Na^+/K^+ -ATP 酶和 Ca^{2+} -ATP 酶活性的影响

注: 数据表示为平均值±标准误差, $n=3$; 上标字母不同的组间具有显著性差异 ($P < 0.05$)。

Fig. 10 Activity of Na^+/K^+ -ATPase and Ca^{2+} -ATPase in zebrafish larvae exposed for 72 h

Note: Data are presented as mean±S.E., $n=3$; means of the treatment not sharing a common letter are significantly different ($P < 0.05$).

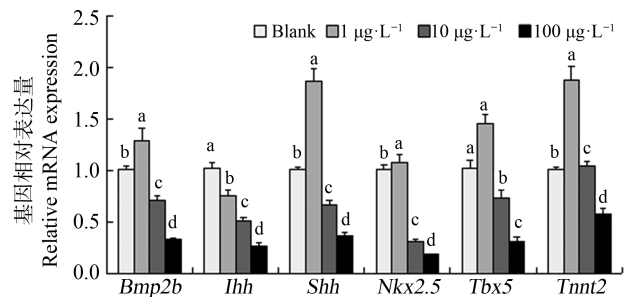


图 11 暴露 72 h 对斑马鱼仔鱼基因表达的影响

注: 数据表示为平均值±标准误差, $n=3$; 上标字母不同的组间具有显著性差异 ($P < 0.05$)。

Fig. 11 The transcription of genes in zebrafish larvae exposed for 72 h

Note: Data are presented as mean±S.E., $n=3$; means of the treatment not sharing a common letter are significantly different ($P < 0.05$).

斑马鱼胚胎发育由多种基因控制, *Tbx5* 是心脏发育的重要调节剂^[32,52], 在心肌细胞的分化^[53]以及心脏传导系统功能^[54]中起着至关重要的作用。有研究表明, *Tbx5* 表达异常导致小鼠严重畸形^[55], 人类 *Tbx5* 基因的突变导致心脏发育缺陷^[56]。本试验结果显示较高浓度草甘膦暴露后仔鱼出现 *Tbx5* 基因表达量下降, 这可能是心包发育异常的主要机制。

骨形态发生蛋白(bone morphogenetic protein, BMP)在脊椎动物中是骨骼形成的关键调节因子, *Bmp2* 是转化生长因子- β (TGF- β)超家族中的一员。在斑马鱼生长发育过程 *Bmp2* 直接参与了背板的早期形成^[34]。*Nkx2.5* 是触发初始心肌细胞分化的关键因素^[57], 可定性和定量确定心室的特征^[58-59]。心肌肌钙蛋白(由 *Tnnt2* 编码)参与心脏收缩功能, 是斑马鱼心肌分化所必需的^[33]。本课题组前期研究表明, 环境污染物通过干扰斑马鱼胚胎的转录会导致斑马鱼胚胎心脏发育和功能缺陷^[41-42]。本试验显示, 高浓度组($100\ \mu\text{g}\cdot\text{L}^{-1}$)中 *Nkx2.5* 表达量下调且比例最大, 而 *Bmp2b*、*Tnnt2* 表达量上调。*Bmp2b* 是 *Nkx2.5* 的上游基因^[51], *Nkx2.5* 表达量降低表明草甘膦可能会阻止心肌细胞的发育, 从而引起心脏发育畸形^[34], 由图 7 可知, 高浓度组出现极大程度的心包水肿, 推测此畸形出现与 *Nkx2.5* 表达量受抑制关系最大。

Hh(Hedgehog)基因家族在胚胎发育中对软骨形成起着重要作用, *Shh*(Sonic hedgehog)、*Ihh*(Indian hedgehog)是 Hh 基因家族 2 个重要成员, 早期胚胎发育中这 2 个基因在内胚层的正确表达与软骨组织相关^[33]。课题组前期的研究表明污染物暴露干扰斑马鱼 *Shh*、*Ihh* 和 *Bmp2* 的转录水平从而引起骨发育缺陷^[60]。本试验结果显示草甘膦暴露出现脊柱弯曲, 尤其是高浓度组发生率最高(7.3%)且弯曲程度最大(图 3)。高浓度组仔鱼 *Bmp2b*、*Nkx2.5* 的表达量受抑制, 表明草甘膦暴露导致心脏发育紊乱, 进而影响骨骼的发育。

环境浓度的草甘膦暴露导致斑马鱼胚胎明显的胚胎毒性, 包括存活率下降、脊柱弯曲、心包水肿、卵黄囊肿; 酶活性测定显示, 心包水肿和骨骼畸形与 Na^+/K^+ -ATPase 和 Ca^{2+} -ATPase 活性的降低有关; 荧光定量 PCR 分析显示, 草甘膦暴露改变了与胚胎心脏发育(*Tbx5*、*Nkx2.5*、*Tnnt2*)、骨骼发育(*Bmp2b*、*Shh*、*Ihh*)相关基因的转录。值得注意的是, 不同浓度的草甘膦对斑马鱼胚胎发育毒性强弱有差异, 较

低浓度($1\ \mu\text{g}\cdot\text{L}^{-1}$)对斑马鱼胚胎相对安全, 但浓度达 $10\ \mu\text{g}\cdot\text{L}^{-1}$, 甚至 $100\ \mu\text{g}\cdot\text{L}^{-1}$ 时出现显著毒性效应。可以认为, 环境中的草甘膦残留量达到一定浓度($\geq 10\ \mu\text{g}\cdot\text{L}^{-1}$)时对环境生物构成潜在威胁。依据本试验结果, 以斑马鱼胚胎为对象, 草甘膦的最低观察效应浓度(lowest observed effect concentration, LOEC)可表示为 $10\ \mu\text{g}\cdot\text{L}^{-1}$, 无观察效应浓度(no observed effect concentration, NOEC)可表示为 $1\ \mu\text{g}\cdot\text{L}^{-1}$ 。该指标可为草甘膦风险评估提供参考值, 在草甘膦的实际应用中应全面考察环境可能残留浓度。

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