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二噁英引起斑马鱼下颌短小及其与 *Sonic hedgehog* 基因的关联*

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摘　要:环境污染物二噁英中毒性最强的2,3,7,8 – 四氯二苯并对二噁英(2,3,7,8-tetrachlorodibenzo-*p*-dioxin,、以下简称TCDD)经由芳烃基受体(aryl hydrocarbon receptor, Ahr)引起啮齿类口唇开裂,斑马鱼下颌短小等典型特征. .本试验研究了TCDD引起的下颌短小与形态发育基因 *Sonic hedgehog*(*shh*)的关系. 用0 – 1.0 μg/L 的TCDD给受精后24 h(24 hpf)的斑马鱼胚染毒直至观察并进行形态学观察及原位杂交. 结果观察到TCDD引起斑马鱼的下颌短小与其浓度相依存,同时观察到TCDD染毒群的 *Shh* 基因表达以及类似基因 *tiggy-winkle hedgehog* (*twhh*)的低下. 观察 *Shh* 缺失的斑马鱼变异体 *Syu*,或给正常斑马鱼染毒,添加 *Shh* 阻断药 Cycopaman Cyclopamine,可以同样观察到斑马鱼的下颌短小. 本试验表明TCDD引起的下颌短小与 *shh* 和 *twhh* 表达是相关联的. 同时也说明斑马鱼有可能作为二噁英类污染物的生物学毒性评价生物.
关键词:*Shh* 基因;二噁英;畸形;下颌;斑马鱼

Correlation Between Short Lower Jaw in Zebrafish Embryos Induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and *Shh* Gene

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Abstract: Dioxins including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are persistent, bioaccumulative toxicants that are widely distributed in the environment. TCDD induced cleft palate in the mice and short lower jaw in the zebrafish (*Danio rerio*) by aryl hydrocarbon receptor (AhR) which is most toxic in the Dioxins. In this experiment, we investigated the correlation between TCDD induced short lower jaw and *Sonic hedgehog* (*Shh*) a development gene in the zebrafish embryos during early development, it has many advantages as a vertebrate toxicological model. Embryos is exposed to TCDD (0 – 1 μg/L) at 24 h post fertilization (24 hpf) until the time of observation for studying by morphology and *in situ* hybridization. Embryos treated with TCDD showed marked short lower jaw and reduction of *shh* and *twhh* expression in lower developing jaw. This inhibitory effect was dependence on exposed concentration. TCDD concentrations 0.3, 0.5 and 1.0 g/L exerted significant effects that were first detected at 84, 72, and 60 hpf, respectively. But 0.1 μg/L TCDD did not have any effect until 120 hpf. *Shh* function defective mutant embryos (*Syu*) with relatively normal circulation around developing jaw showed marked retardation of jaw growth. When cyclopamine, an inhibitor for *shh*, was applied, lower jaw growth was significantly inhibited. It was found that TCDD affected chondrogenesis in the head in later stages of zebrafish development by alcian blue stain. TCDD shortened the length of Mechel's cartilage. Similarly, the short Mechel's cartilage was found in the *syu* embryos and cyclopamine treated embryos. These results indicate the close correlation between hedgehog expression and jaw growth retardation by TCDD in developing zebrafish embryos. It is also suggested zebrafish may become a biomarker of dioxin toxicity.
Keywords: *Shh*; TCDD; dioxin; malformation; jaw; zebrafish

多氯联苯(Polychlorinated biphenyls,简称PCB)、多氯代二苯并二噁英(polychlorinated dibenzo- *p*-dioxin,

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简称 PCDD)和多氯代二苯并呋喃(polychlorinated dibenzofuran,简称 PCDF)等二噁英类化合物具有急性毒性、致癌性、致畸性、免疫毒性和生殖毒性等多种毒性. 这些化合物即使在常规仪器难以检出的极微量水平,也可引起毒性,且在自然环境中难以降解,所以由此产生的环境污染已成为重大的世界性环境问题[1]. 在二噁英类中以 2,3,7,8 - 四氯代二苯并对二噁英(2,3,7,8-tetrachlorodibenzo-*p*-dioxin、TCDD)的毒性最强,广泛引起各种动物的强烈毒性[2]. 其中小鼠口唇裂、包括下颌在内的头部骨骼畸形以及血液循环障碍被广泛认同[3,4]. 二噁英类首先与细胞质内的芳烃基受体(Aryl hydrocarbon receptor、以下简称为 AhR)相结合,再与核内的转录因子 AhR nuclear translocator(Arnt)形成异型配偶体,进入细胞核内特异的与 DNA 相应领域(dioxin response element、DRE)结合,引起一系列基因转录调节的变化[5]。,其中以细胞色素 P4501A(CYP1A)转录的增强研究较多. 但此后的毒性机制仍然不很明了[6].

有关鱼类下颌发育短小,虹鳟(*Oncorhynchus mykiss*)[7]、青鳉(*Oryzias latipes*)[8]以及斑马鱼(*Danio rerio*)[9,10]等被相继作了定性报告. 而相关毒性的成因通常认为是,经由 AhR 以及 CYP1A 的诱导,引起血液循环障碍等各种毒性,进而由于血液循环障碍引起下颌短小[7]. 但 Teraoka 等研究表明 TCDD 引起的下颌已经短小的斑马鱼下颌部的血液循环量并没有发生变化[10],因此考虑 TCDD 可能直接影响相关基因的调控,引起下颌短小.

斑马鱼的 *Sonic hedgehog*(*Shh*)基因,以及类似基因 *Tiggy-winkle hedgehog*(*Twhh*)已被确认与头部颜面、肢体发育有密切的关系[11-13]. *shh* -/- 基因敲出小鼠的头部构成物缺损[14]. *Shh* 在鱼类牙鲆(*Paralichthys olivaceus*)的上颚、下颌表达[15]. 因此考虑 *Shh* 与上颚和下颌的增殖、分化相关[16]. 本试验初次详细定量了 TCDD 引起的下颌短小,确认了 *Shh* 在斑马鱼下颌部表达,研究了以及以 TCDD 浓度为依存的下颌短小,以及下颌短小与的 *Shh* 基因的关系.

1 材料和方法

1.1 药物

2,3,7,8-tetrachlorodibenzo-*p*-dioxin(TCDD)来自 Cambridge isotope Laboratories, Inc. IL,其他的药物分别来自 SIGAM 或日本和光纯药.

1.2 采卵及染毒

受精卵根据 Westerfield 的方法[17],将斑马鱼(AB 系)雌雄分别培养,并以 L: D = 14: 10 光周期促熟,实验前一天混养. 自然交配后,采集受精卵并放入盛有斑马鱼林格尔液(ZR 液: 38.7 mmol/L NaCl,1.0 mmol/L KCl,1.7 mmol/L HEPES,2.4 mmol/L $CaCl_2$, pH 7.2)的培养皿中,置于 28.5℃孵化箱(LTI - 600ED)中孵化. 受精后 24 h(24 hpf,24 hours post- fertilization)开始染毒,染毒时使用 3 ml 培养皿,TCDD 溶于二甲基亚砜(DMSO),按 0.1、0.3、0.5 和 1.0 μg/L TCDD 的浓度梯度及 DMSO 的最终浓度为 0.1% 加入药物,定容到 3 ml,每个培养皿并放入 10 个受精卵,置于 28.5℃孵化箱内续孵化. 用含有 0.1% DMSO 的 ZR 液饲育的卵作为溶媒对照.

图 1 斑马鱼下颌测量方法(左侧为正值, 右侧为负值)

Fig. 1 A ventral view of 2 zebrafish embryos above panel illustrate the measure jaw growth(positive value, left and negative value, right)

1.3 下颌长度的测定

从 48 hpf 至 144 hpf,将染毒鱼苗和对照鱼苗用 MS222 麻醉,放在 3% 的 Carboxymethylcellulose 羧甲基纤维素钠盐/ZR 中固定,再用带有标尺的实体显微镜进行测定. 测定时,把幼鱼水平仰卧放置,以眼球前端为基准值(0 μm),下颌的前端与眼球的距离为下颌的长(图 1).

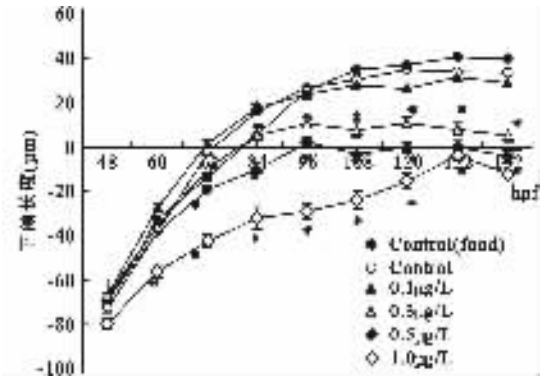

图 2 不同浓度的 TCDD 对斑马鱼幼鱼下颌长度的影响. Control(food)为摄食对照组. 图中表示的下颌长度为 10 个胚胎的平均值 ± 标准差(* $p < 0.05$)

Fig. 2 The effect of different concentration on low jaw lengths in zebrafish fry. The Congtrol(food) was feeding group. Height of each bar and associated vertical line is mean ± S. D (n = 10 embryos/treatment). * $p < 0.05$.

1.4 Alcian-blue 软骨染色

胚胎用 10% 的福尔马林进行固定 24 h,于染色液 (0.1% Alcian blue 8GX/80% 甲醇/20% 醋酸) 中染色 6 h,然后用 75%,50% 的酒精以及蒸馏水各清洗 3 h,再用消化液(0.05% 的胰酶/饱和 tetraborate 四硼酸盐)以及 1% KOH/30% H_2O_2 各处理 1 h,最后在 70% 的甘油中观察及保存.

1.5 原位杂交

按 Barth 的方法进行[18]. 用蛋白酶 K(10 μg/ml)室温消化 15 min,然后加入杂交液 (50% 甲酰胺,5 × SSCx,2mg/ml Torula RNA, 200 μg/ml 肝素) 放于 65℃的恒温箱中 1 h(预杂交),再用含有 *Shh* 或 *Thwhh* 探针的杂交缓冲液更换,放于恒温箱中过夜. 用 2 × SSCx、0.2 × xSSC 在 65℃恒温箱中各清洗 30 min,再用顺丁烯二酸 buffer (100 mmol/L 顺丁烯二酸,150 mmol/L NaCI,0.1% 吐温 20,pH 7.5)清洗 15 min. 用 blocking buffer(2% blocking reagent, 100 mmol/L 顺丁烯二酸,150 mmol/L NaCI,0.1% 吐温 20,0.1% Trito x - 100,pH7.5)在室温清洗 1h,放入含有 4000 倍稀释的抗 - DIG 抗体(Roche)并置于 4℃冰箱中反应过夜. 在清洗夜 NTMT(100 mmol/L Tris-HCI,pH 9.5, 100 mmol/L NaCI, 50 mmol/L $MgCI_2$, 0.1% 吐温 20) 中静置 15min,使用 BM purple AP substrate precipitating (Roche)发色.

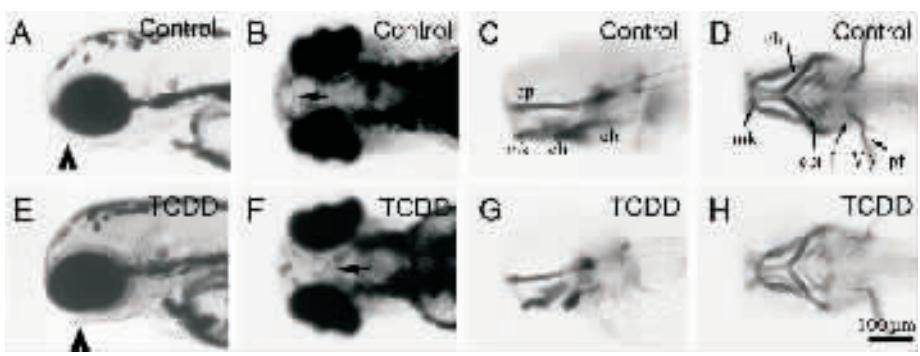

图 3 TCDD 染毒引起的下颌短小. 鱼胚从 24 hpf 至 72 hpf 用 0.1% DMSO (Control) 或 TCDD (1μg/L.) 染毒 (A, B, E, F). 为 72 hpf 所观察到的下颌生长状态. 于 72 hpf 之后 用福尔马林固定后进行观察,同时, 进实施 alcian blue 软骨染色(C, D, G, H). A, B, E, F 中的箭头表示下颌前端开口部. A, C, E, G 为侧面像,B, D, F, H 为腹测像. 简写:cb (1 - V),第一到第五角鳃骨;ch,角舌骨;ep,筛骨;mk,麦克氏软骨;pf,胸鳍. 比例尺 = 100 μm.

Fig. 3 Retardation of lower jaw growth by TCDD. Embryos were exposed to 0.1% DMSO (Control) or TCDD (1 g/L.) from 24 hpf until observation at 72 hpf (A, B, E, F). After fixation in formalin, these embryos were stained with alcian blue (C, D, G, H). Arrows in A, B, E, F indicated the position of mouth opening. A, C, E, G: lateral views. B, D, F, H: ventral views. Abbreviations: cb (1 - V), first to fifth ceratobranchial; ch, ceratohyal; ep, ethmoid plate; mk, Meckel's cartilage; pf, pectoral fin. Bar = 100 μm.

1.6 统计方法

试验所得数据表示的是为平均值 ± 标准差 (SD), 平均值间进行 t - 检验 ($p < 0.05$).

2 结果

2.1 TCDD 引起的下颌短小

在以往的报道中,描述了 TCDD 可引起头部的畸形,下颌的短小,但没有定量的结果. 本试验用不同浓度 TCDD 染毒,详细测定了下颌的长度. 在 0.1 μg/L 染毒组与对照组相比没有观察到显著的影响,0.3 μg/L 在 84 hpf 以后、0.5 μg/L 染毒群在 72 hpf 以后、1 μg/L 染毒群在 60 hpf 以后,与对照组相比较,有显著差异($p < 0.05$)(图 2). 通过软骨染色,可以观察到 TCDD 不但对下颌前端的 Meckel 软骨麦克氏软骨有抑制作用,对其他的头部软骨也有同样的抑制作用(图 3).

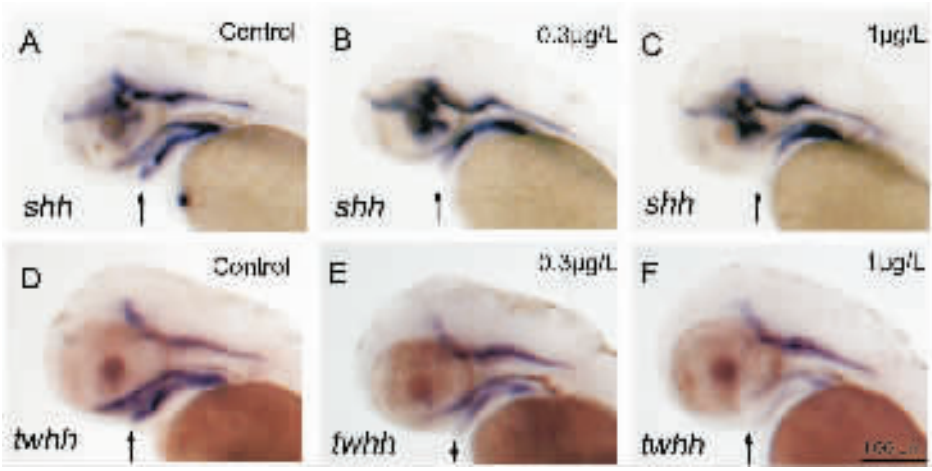

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图 4　*Shh* 及其类似体在发育期斑马鱼下颌部位的表达. 54 hpf 胚胎固定于 0.4% PFA,然后实施 *shh*(A－C), *twhh*(D－F)的整体原位杂交,箭头表示下颌. 比例尺 = 100 μm

Fig. 4 Expressions of hedgehogs and their signaling molecules in jaw region of developing zebrafish. Embryos were fixed with 0.4% PFA at 54 hpf and used for whole mount *in situ* hybridization of *shh* (A-C), *twhh* (D-F)

2.2 TCDD 染毒对 *Shh* 以及 *Twhh* 基因的影响

在对照组,*shh* 从 48hpf 的在下颌原基正常表达. 但 55 hpf 时在下颌处的表达最为明显. *Shh* 的表达部位为神经底板、上颚以及下颌. 除了斑马鱼的 *Shh* 以外还有 *Twhh* 在类似的区域表达,具有与 *Shh* 类似的功能[19]. 0.3 μg/L TCDD 对 *Shh* 和 *Twhh* 表达没有显著的影响,但从 0.5 μg/L 开始,在下颌部的表达显著减弱或部分消失 (图 4).

2.3 *Shh* 对下颌长度的影响

上面提到 *shh* 和 *twhh* 表达被 TCDD 特异的抑制,下面探讨了 *Shh* 与下颌发育的关系.

2.3.1 *syu* 的观察　命名为 *sonic you*(*syu*)的变异体是斑马鱼 *shh* 的点状变异,失去了 *Shh* 活性[20]. 因此用变异体来研究 *Shh* 机能欠缺与下颌短小之间的关系. 实验结果表明,*syu* 下颌显著的短小(图 5). 通过软骨染色可以观察到极为严重的骨发育障碍(图 6).

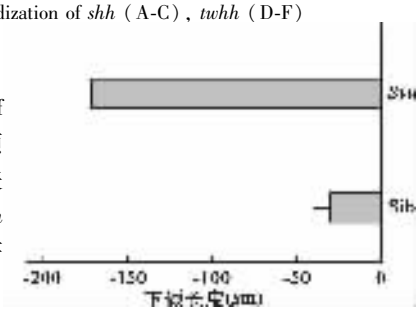

图 5 72hpf syu 变异体(Syu)下颌长度与同腹幼鱼(Sib)比较. 图中表示的下颌长度为 10 个胚胎的平均值 ± 标准差, * $p < 0.05$

Fig. 5 Comparing lower jaw length of syu embryos with their siblings (Sib) at 72 hpf. Height of each bar and associated vertical line is mean ± S. D (n = 10 embryos/treatment). * $p < 0.05$.

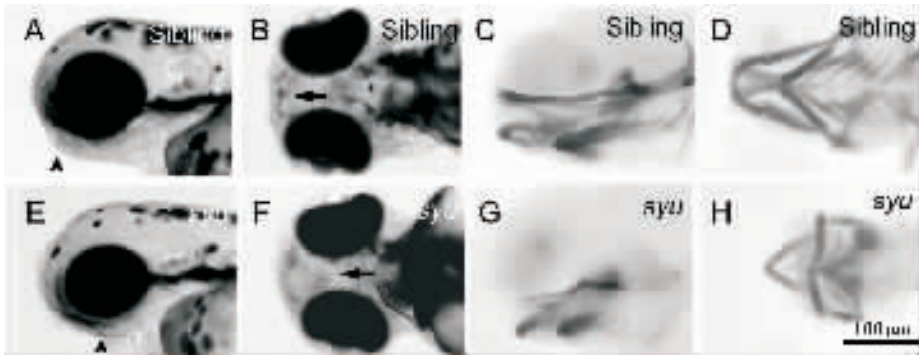

图 6 下颌生长不良的 *Shh* 功能欠缺的 *sonic you*(*syu*)变异体 以及同腹胚胎(Sib)下颌生长的抑制. A, B, E, F 为 72 hpf *sonic you*(*syu*) 和同腹幼鱼(Sibling). C, D, G, H 为 72 hpf *syu* 胚及其同腹胚的 alcian blue 染色. A, C, E, G 为侧面像, B, D, F, H 为腹测像. 箭头表示下颌的前端. 比例尺 100μm

Fig. 6 Impairment of lower jaw growth in *Shh* function defective mutant. Larvae of *sonic you* (*syu*) and their siblings were observed at 72 hpf (A, B, E, F). Images of alcian blue staining with 72 hpf *syu* embryos and their siblings (C, D, G, H) were also presented. A, C, E, G: lateral views. B, D, F, H: ventral views. Arrows and arrow heads indicate anterior edge of lower jaw. Bar = 100μm.

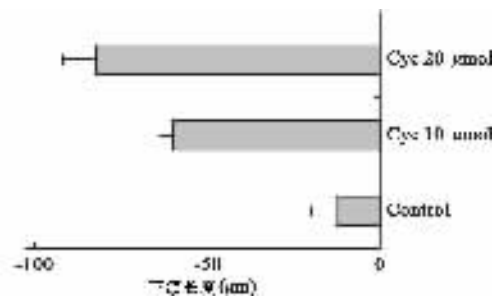

图 7 Cyclopamine 对 72 hpf 斑马鱼下颌生长长度的影响. 图中表示的下颌长度为 10 个胚胎的平均值 ± 标准差(* $p < 0.05$).

Fig. 7 The effect of Cyclopamine on developmental length of zebrafish lower jaw at 72 hpf. Height of each bar and associated vertical line is mean ± S·D ($n = 10$ embryos/treatment)(* $p < 0.05$).

2.3.2 Cyclopamine(环杷明)的影响 *Syu* 对下颌原基的影响有可能是在发育早期阶段便已经开始,致使其严重影响骨的发育. 用对 *Shh* 有阻碍作用的 Cyclopamine[21] 进行了相关试验. 结果表明,从下颌伸长的时期的 48hpf 到 72 hpf ,以 10 μmol 及 20μmol 的 cyclopamine 染毒 24h,便可以观察到差异显著的下颌短小(图 7). 软骨染色的结果也显示 Meckel、(麦克氏软骨) ethmoid plate、(筛骨)、ceratohyal(角舌骨)等软骨的短小,及其 Ceratobranchial(角鳃骨)的缺失(图 8).

3 讨论

本试验详细定量测定了 TCDD 引起下颌短小的长度,及其与 *Shh* 的关联. 以往,TCDD 引起的硬骨鱼下颌短小只是主观的判定,而下颌短小以及其他头部的畸形的起因被认为是 TCDD 引起血液循环障碍的二次症状. 例如,Henry 等首次探讨了斑马鱼的毒性,认为下颌短小是因为浮肿的二次症状[9],Hornung 等认为 TCDD 引起的下颌短小与心脏的重量减轻相关联[7]. 而 Teraoka 等测定了斑马鱼初期胚胎下颌的血流量,至 96hpf 没有观察到 TCDD 对下颌血液循环的阻作用[10],推测引起下颌短小有可能是因为 TCDD 对下颌原基的直接作用.

Shh 基因在下颌原基表达[22],在下颌表达的程度与 TCDD 的染毒浓度相依存,染毒浓度越高,*Shh* 表达的程度越小. *Shh* 机能缺失的变异体 *syu* 表现出极其短小的下颌以及整体头部的发育不全,说明 *Shh* 基因的完全缺失,除下颌以外,对头部的其他组织亦可引起不良的影响. *Shh* 阻断剂 Cyclopamin 的染毒引起的下颌短小,存在明显的浓度依存性,考虑 Cyclopamin 对 *Shh* 抑制的程度也同样与浓度相关联. 本试验强烈暗示 TCDD 抑制了 *Shh* 基因以及类似体 *twhh* 基因的表达. 同时说明 *Shh* 和 *Twhh* 对下颌的发育起重要的作用. TCDD 抑制 *Shh* 表达的机理尚不明确,Prasch 等报道去除 AhR2 基因(AhR-MO), 可以抑制 TCDD 引起的包括软骨发育在内的各种发育障碍,表明 *Shh* 基因是 AhR2 的下流控制基因[23]. 有必要研究其他在下颌表达的基因如 *Goosecoid*、*AhR2* 等与 *Shh* 的关系,*Shh* 受体的 *Ptc*1、*Ptc*2、转录基因 *Gli*1、*Gli*2 以及衡量 *Shh* 蛋

白质表达的免疫染色等等. 短下颌与 Shh 的抑制相关,而 *Shh* 的抑制是否造成局部的细胞凋亡,进而引起下颌短小尚有待进一步研究. 总结以上结果表明,*Shh* 对下颌的生长发育起重要的作用,TCDD 引起下颌短小与 *Shh* 表达量的减少相关联. 进而也表明 TCDD 引起的下颌短小有可能作为低浓度二噁英类化合物生物毒性评价的一个参考指标.

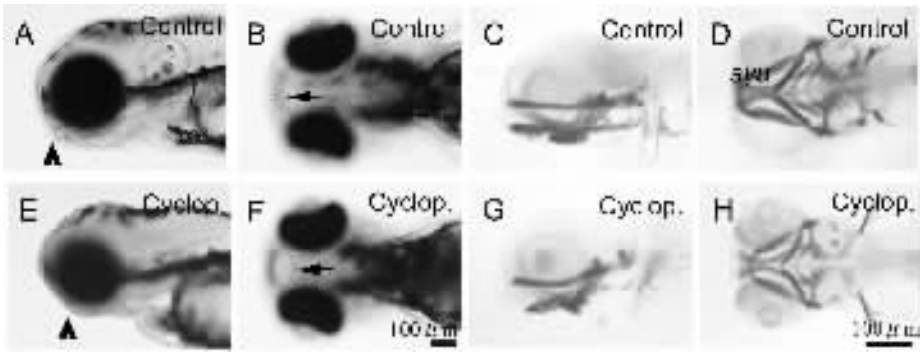

图 8 *Shh* 阻断剂对下颌发育短小的影响阻断剂对下颌发育的抑制. 鱼胚从 48 hpf 至 72 hpf 用 0.1% DMSO (Control) 或 Cyclopamin (10 – 20 μm: Cyclop.) 染毒,A, B, E, F 为 72 hpf 所观察到的下颌生长状态. 于 72 hpf (A, B, E, F),之后用福尔马林固定,进行 alcian blue 软骨染色(C, D, G, H). A, B, E, F 中的箭头表示下颌前端开口部. A, C, E, G 为侧面像,B, D, F, H 为腹测像.

Fig. 8 4 Retardation of lower jaw growth by chemical inhibitor for hedgehog signaling. Embryos were exposed to 0.1% DMSO (Control) or cyclopamine (10 – 20 μm: Cyclop.) from 48hpf until observation at 72 hpf (A, B, E, F). After fixation in formalin, these embryos were stained with alcian blue (C, D, G, H). Arrows in A, B, E, F indicate the position of mouth opening. A, C, E, G: lateral views. B, D, F, H: ventral views.

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