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## 孕激素醋酸甲地孕酮和雌激素乙炔雌二醇 复合暴露对斑马鱼的生殖毒性\*

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**摘 要** 水生生物往往暴露于多种环境激素(如孕激素、雌激素)的混合物中,然而关于多种环境激素对鱼类的联合作用效应的研究较少.孕激素醋酸甲地孕酮(MTA)和雌激素乙炔雌二醇(EE2)是应用广泛的高活性药物,普遍存在于水环境中,二者均能引起鱼类的生殖毒性效应.本文研究了环境相关浓度的 MTA 和 EE2 复合暴露对斑马鱼的生殖毒性效应.将斑马鱼成鱼暴露于 MTA(33, 100 ng·L<sup>-1</sup>), EE2(5, 15 ng·L<sup>-1</sup>)以及二者的混合物(MTA + EE2; 33 + 5 ng·L<sup>-1</sup>, 100 + 15 ng·L<sup>-1</sup>) 21 d,结果显示,EE2(15 ng·L<sup>-1</sup>)单独及与 MTA(100 ng·L<sup>-1</sup>)复合暴露显著降低斑马鱼的产卵量;MTA、EE2 单独及复合暴露均显著降低雌鱼血浆中雌二醇(E2)、睾酮(T)及雄鱼血浆中 11-酮基睾酮(11-KT)的含量;EE2 单独及与 MTA 复合暴露导致斑马鱼卵巢的组织学变化(抑制卵子发生,诱导卵泡闭锁),但对精巢影响较小.此外,复合暴露组中斑马鱼的产卵量、血浆性激素含量、性腺组织学变化与 EE2 单独暴露组相比均无显著差异.本研究表明,MTA 和 EE2 复合暴露可引起斑马鱼的生殖毒性,其中 EE2 发挥主要毒性作用.本研究结果对于水环境中多种激素复合暴露的风险评估具有重要意义.

**关键词** 醋酸甲地孕酮, 乙炔雌二醇, 复合暴露, 生殖毒性, 斑马鱼.

## Reproductive toxicity caused by co-exposure of zebrafish to progestin megestrol acetate and estrogen 17 $\alpha$ -ethinylestradiol

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**Abstract:** Despite potential exposure of aquatic organisms to mixtures of environmental hormones (e.g., progestins, estrogens), very little is known about their binary activity in fish. Synthetic progestin megestrol acetate (MTA) and estrogen 17 $\alpha$ -ethinylestradiol (EE2) are widely used pharmaceutical agents with high bioactivity, and have been frequently detected in the aquatic

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environment. They both can cause reproductive toxicity in fish. In the present study, we investigated the combined reproductive effects of MTA and EE2 at environmentally relevant concentrations on zebrafish. Adult zebrafish were exposed to MTA (33 or 100 ng·L<sup>-1</sup>), EE2 (5 or 15 ng·L<sup>-1</sup>) or a mixture of both (MTA + EE2: 33 + 5 ng·L<sup>-1</sup>, 100 + 15 ng·L<sup>-1</sup>) for 21 d. Results demonstrated that the egg production was significantly reduced by exposure to 15 ng·L<sup>-1</sup> EE2 alone or combined exposure to 100 ng·L<sup>-1</sup> MTA and 15 ng·L<sup>-1</sup> EE2. Plasma concentrations of estradiol (E2) and testosterone (T) in females and 11-ketotestosterone (11-KT) in males were all significantly decreased after exposure to EE2 or MTA or the mixtures of both chemicals. Besides, exposure to EE2 or the mixtures led to histological alterations in the ovaries (inhibition of oogenesis, induction of atresia), but little effects were found on the testes. In addition, no significant difference was found in egg production, plasma concentrations of sex hormones and gonadal histological alterations in co-exposure groups when compared with those in EE2 single exposure groups. The present study suggests that co-exposure to environmentally relevant concentrations of MTA and EE2 could result in reproductive toxicity in zebrafish, and EE2 plays a predominant role in causing the dysfunctions. The present study has important implications for environmental risk assessment of combined exposure to environmental hormones present in aquatic environment.

**Keywords:** megestrol acetate, 17 $\alpha$ -ethynylestradiol, co-exposure, reproductive toxicity, zebrafish.

水生生物如鱼类往往暴露于多种环境激素中,其中活性最强的包括天然和人工合成的雌激素、雄激素和孕激素等<sup>[1-3]</sup>.已有大量研究报道雌激素和雄激素特别是人工合成的雌激素乙炔雌二醇(17 $\alpha$ -ethynylestradiol,EE2)对鱼类的毒理学效应<sup>[4-6]</sup>,然而对孕激素的相关研究较少<sup>[6-9]</sup>,对醋酸甲地孕酮(Megestrol acetate,MTA)的研究尤为有限<sup>[10-11]</sup>.尽管雌激素和孕激素往往联合用于避孕药<sup>[7]</sup>且在水环境中共存<sup>[12-13]</sup>,但目前仅有少数研究报道二者对鱼类的联合毒性效应<sup>[3, 14-15]</sup>.研究表明,EE2与孕激素左炔诺孕酮(Levonorgestrel,LNG)复合暴露对抑制黑头呆鱼(*Pimephales promelas*)产卵产生加和效应<sup>[15]</sup>.用EE2与孕激素甲基炔诺酮(Norgestrel,NGT)<sup>[16]</sup>或醋酸环丙孕酮(Cyproterone acetate,CPA)<sup>[9]</sup>复合暴露斑马鱼胚胎,复合暴露对基因转录水平的影响较单独暴露显著增强,一定程度上雌激素和孕激素表现出加和或协同作用.然而,用EE2与孕激素屈螺酮(Drospirenone,DRS)<sup>[8]</sup>或醋酸氯地孕酮(Chlormadinone acetate,CMA)<sup>[9]</sup>复合暴露斑马鱼胚胎,二者对HPG轴基因转录水平的作用相互独立或相互拮抗.可以看出,雌激素和孕激素复合暴露的毒性作用效应具有一定的复杂性,需开展更多研究.

MTA是一种人工合成的孕激素,广泛用于避孕药<sup>[7]</sup>、疾病治疗<sup>[17-18]</sup>、改善病人食欲<sup>[19-20]</sup>或作为促生长剂用于畜牧养殖业<sup>[7]</sup>等.目前,已在中国<sup>[12, 21-23]</sup>、日本<sup>[24]</sup>、瑞士<sup>[13, 25]</sup>和西班牙<sup>[26]</sup>等多个国家和地区的水环境中检测到MTA.MTA在污水处理厂进水和出水中的浓度分别为0.14—150 ng·L<sup>-1</sup>和0.1—20 ng·L<sup>-1</sup>,在地表水中的浓度为0.02—34 ng·L<sup>-1</sup>,其中在我国地表水中的浓度高达34 ng·L<sup>-1</sup><sup>[12]</sup>.EE2是一种人工合成的雌激素,广泛用于避孕药、生理替代治疗、前列腺癌与乳腺癌治疗等<sup>[4]</sup>.EE2广泛存在于世界各地的多种环境介质中<sup>[4]</sup>,在污水处理厂进水和出水中的浓度分别为ND(未检出)—3180 ng·L<sup>-1</sup>和ND(未检出)—176 ng·L<sup>-1</sup><sup>[4, 27-28]</sup>,在地表水中的浓度为ND(未检出)—127.9 ng·L<sup>-1</sup><sup>[4, 29]</sup>,其中在我国地表水中的浓度高达127.9 ng·L<sup>-1</sup><sup>[29]</sup>.

MTA<sup>[10-11]</sup>和EE2<sup>[30-33]</sup>在环境相关浓度下均能引起鱼类的生殖毒性.MTA在666 ng·L<sup>-1</sup>的浓度下能够显著降低斑马鱼的产卵量<sup>[10]</sup>,在低至33 ng·L<sup>-1</sup>的浓度下可显著降低斑马鱼血浆中性激素的含量<sup>[10-11]</sup>,在100、333 ng·L<sup>-1</sup>的浓度下阻滞斑马鱼卵巢中卵细胞的发育<sup>[11]</sup>.EE2在10—50 ng·L<sup>-1</sup>的浓度下可显著降低斑马鱼的产卵量<sup>[34-35]</sup>,在0.5—50 ng·L<sup>-1</sup>浓度下可显著降低斑马鱼血浆中性激素的含量<sup>[11, 15, 34, 36]</sup>,在10 ng·L<sup>-1</sup>浓度下可引起斑马鱼性腺组织学改变(抑制生殖细胞发育、诱导闭锁卵泡等)<sup>[11, 37]</sup>.此外,低浓度EE2暴露还可影响斑马鱼的性别分化<sup>[31]</sup>、生殖行为<sup>[30, 36]</sup>等.

研究表明,MTA和EE2在水环境中共存<sup>[12-13]</sup>,且均可对鱼类的生殖造成不良影响<sup>[4, 10]</sup>,然而目前关于二者复合毒性效应的研究较少<sup>[11]</sup>.为进一步阐明环境相关浓度的MTA和EE2的联合毒性效应,本文

以斑马鱼为模型,通过统计产卵量、测定血浆中性激素水平以及性腺组织学分析,研究了MTA和EE2复合暴露对斑马鱼的生殖毒性效应.研究结果将为水环境中多种激素(如孕激素、雌激素)复合暴露的风险评估提供新的科学依据.

## 1 材料与方法(Materials and methods)

### 1.1 试剂

醋酸甲地孕酮(MTA;CAS 595-33-5;纯度 $\geq 98\%$ )、乙炔雌二醇(EE2;CAS 57-63-6;纯度 $\geq 98\%$ )、二甲基亚砜(DMSO;纯度 $> 99.9\%$ )和MS-222均购自美国Sigma-Aldrich公司.氘代内标乙炔雌二醇-d4(Ethynylestradiol-2,4,16,16-d4,EE2-d4;Cat. No.350820-06-3;纯度 $> 98\%$ )和孕酮-d9(Progesterone-d9,P-d9;CAS 15775-74-3;纯度 $> 98\%$ )购自于Toronto Research Chemicals公司(North York,ON,Canada).雌二醇(E2)、睾酮(T)和11-酮基睾酮(11-KT)酶联免疫试剂盒购自美国Cayman Chemical公司(Ann Arbor,USA).用于分析化学测定的试剂均为HPLC级,其他试剂均为国产分析纯.

### 1.2 斑马鱼的饲养与暴露实验

性成熟的斑马鱼(6月龄,野生型,AB系)养殖于活性炭过滤后的自来水中,pH 7.0—7.4,水温控制在 $28 \pm 0.5\text{ }^\circ\text{C}$ ,光暗周期为14 h:10 h.每天喂食两次,间隔喂食片状饲料和刚孵化的丰年虫.暴露实验包括14 d的预暴露期(未给药暴露)和21 d的暴露期(给药暴露).在预暴露期开始前,驯养实验鱼2周.每个鱼缸(30 L规格,盛16 L水)放置成年雌、雄鱼各6条.预暴露期结束后,用MTA和EE2对斑马鱼进行半静态暴露21 d,包括以下暴露组:33、100  $\text{ng}\cdot\text{L}^{-1}$  MTA单独暴露组(记为MTA-33、MTA-100),5、15  $\text{ng}\cdot\text{L}^{-1}$  EE2单独暴露组(记为EE2-5、EE2-15),33 + 5  $\text{ng}\cdot\text{L}^{-1}$ 和100 + 15  $\text{ng}\cdot\text{L}^{-1}$  MTA + EE2复合暴露组(记为Comb-1、Comb-2).设置DMSO溶剂对照组(记为SC).每个处理组设置3个平行缸,各缸均含有0.001%(V/V)DMSO助溶剂.每隔24 h换水1次.

在暴露期结束后,所有实验鱼用0.03%(M/V)MS-222麻醉处理,用滤纸吸干鱼体表水分,称量并测量体长.断尾取血,并将血样置于肝素钠处理过的离心管中,离心后保存于 $-80\text{ }^\circ\text{C}$ .解剖出脑、肝脏和性腺,并分别称重,于液氮速冻之后保存于 $-80\text{ }^\circ\text{C}$ .体长和体重用于计算K值(Condition factor)和性腺指数(Gonad somatic indices,GSI).公式如下:

$$K(\%) = (\text{体重}/\text{体长}^3) \times 100\%$$

$$\text{GSI}(\%) = \text{性腺重} \times 100\%/\text{体重}$$

式中,体重、性腺重单位:g;体长单位:cm.

### 1.3 斑马鱼产卵量的统计

每天收集各缸所产鱼卵,并统计每天每条雌鱼的累积产卵量.在预暴露期,统计雌鱼的产卵能力并建立每缸雌鱼的产卵量基线数据<sup>[38]</sup>,确保每个处理组在进行正式暴露前具有相同的产卵基数.

### 1.4 血浆中性激素的抽提与测定

暴露结束后,麻醉实验鱼,断尾取血.每3尾鱼的血混合后作为一个平行样.混合血样于 $4\text{ }^\circ\text{C}$ 、 $7000\text{ r}\cdot\text{min}^{-1}$ 的条件下离心5 min后,取上清(10  $\mu\text{L}$ )用于抽提和测定.激素抽提方法参考已发表论文<sup>[11]</sup>.每个样品(10  $\mu\text{L}$ )加入390  $\mu\text{L}$  Milli-Q水,涡旋混匀,加入2 mL乙醚,混匀器混匀后于 $4\text{ }^\circ\text{C}$ 、 $5000\text{ r}\cdot\text{min}^{-1}$ 条件下离心10 min,取上清,再用2 mL乙醚按照上述方法抽提1次,将两次抽提上清收集于1个洁净的离心管内,氮气吹干后保存于 $-80\text{ }^\circ\text{C}$ .吹干后的样品加入EIA Buffer(Cayman Chemical公司的ELISA试剂盒内提供)复溶,按照试剂盒说明书测定样品中E2、T和11-KT的含量.3种性激素的检测限分别为:19  $\text{pg}\cdot\text{mL}^{-1}$ (E2),6  $\text{pg}\cdot\text{mL}^{-1}$ (T)和1.3  $\text{pg}\cdot\text{mL}^{-1}$ (11-KT).

### 1.5 性腺组织学观察及半定量统计

暴露期结束后,每组各取3条雌鱼和雄鱼,取出卵巢和精巢并在Bouin's固定液中固定24 h,保存在70%乙醇中用于下游实验.将样品脱水后包埋于石蜡中,沿性腺纵轴切成4  $\mu\text{m}$ 的切片,HE染色,制作成病理切片用于图像分析和观察.每个性腺样品在边缘和中间线之间至少等距离取6张横截面切片.使用装备Olympus Camedia C-505照相机的光学显微(Olympus MVX10)观察切片.

卵巢或精巢各发育期生殖细胞的划分根据之前文献报道的方法进行<sup>[39-40]</sup>.卵巢中生殖细胞划分为初级卵母细胞(Primary oocytes, PO),皮质肺泡卵母细胞(Cortical alveolar oocytes, CAO)、卵黄发生期的卵母细胞(Vitellogenic oocytes, VO)和闭锁滤泡(Atretic follicles, AF).精巢中的生殖细胞则划分为精原细胞(Spermatogonia, SPG)、精母细胞(Spermatocytes, SPC)和精细胞(Spermatides, SPD).卵巢和卵巢各发育期细胞的半定量统计根据已建立的方法进行<sup>[40]</sup>:对于卵巢,用某一发育期卵细胞的数量占所有卵细胞数量之和的百分比表示该发育期卵细胞所占的相对比例;对于精巢,应用 Image Pro Plus 6.0(Media Cybernetics Company, MD, USA)软件统计某一发育期精细胞的面积占所有精细胞总面积的百分比,用来表示该发育期精细胞所占的相对比例.

### 1.6 暴露水中 MTA 和 EE2 含量的测定

在暴露期的第一、二、三周,分别在换水前(T24)和换水后(T0)于各缸中取水样 500 mL,用于分析暴露水中 MTA 或 EE2 的实际浓度.MTA 和 EE2 的前处理和测定方法参考已发表的论文<sup>[11]</sup>.用 0.7  $\mu\text{m}$  的玻璃纤维滤膜(Whatman)过滤水样,按 1% 的体积比加入甲醛溶液以避免微生物的活动而降解目标检测物.向每个过滤好的水样中分别加入适量内标(100  $\mu\text{L}$  250  $\mu\text{g}\cdot\text{L}^{-1}$  P-d9 或 1  $\text{mg}\cdot\text{L}^{-1}$  EE2-d4).用 HPLC 级的甲醇和超纯水活化固相萃取柱(富集 MTA 用 Oasis HLB, 6 mL, 500 mg, Waters, USA;富集 EE2 用 C18 SPE cartridge, 500 mg, 6 mL, Supelco, USA).以 5—10  $\text{mL}\cdot\text{min}^{-1}$  的流速将上述水样加载到连有抽滤装置的 SPE 固相萃取柱中.水样加载完后真空抽干 SPE 柱.用 5 mL 乙酸乙酯(洗脱 MTA)或乙腈(洗脱 EE2)洗脱 SPE 柱,收集洗脱液于干净的玻璃管内.用缓慢的氮气流吹干玻璃管内洗脱液,然后再用 1 mL 40% 甲醇(溶解 MTA)或 0.5 mL 乙腈(溶解 EE2)重新溶解残留物,过 0.22  $\mu\text{m}$  滤膜,并将过滤液收集于 2 mL 棕色进样瓶,进行上机分析测试.利用高效液相色谱质谱联用仪(UPLC-Xevo TQ MS, Waters, USA)对样品进行测定,分别用  $\text{ESI}^+$  和  $\text{ESI}^-$  模式测定 MTA 和 EE2.测定 MTA 的流动相用甲醇(B相)和 0.1% 甲酸水(A相),测定 EE2 的流动相用含 0.1% 甲酸的甲醇(B相)和 0.1% 甲酸水(A相).MTA 的定性检测限(LOD)和定量限(LOQ)分别为 0.12  $\text{ng}\cdot\text{L}^{-1}$  和 0.40  $\text{ng}\cdot\text{L}^{-1}$ ,EE2 的 LOD 和 LOQ 分别为 0.30  $\text{ng}\cdot\text{L}^{-1}$  和 0.82  $\text{ng}\cdot\text{L}^{-1}$ .

### 1.7 数据处理与分析

所有数据均使用 SPSS 13.0 软件(SPSS, Chicago, IL, USA)进行处理和分析,用平均值  $\pm$  标准误(SEM)表示.分别 Kolmogorov-Smirnov 和 Levene's 检验方法对数据的正态分布和方差齐性进行检验.采用单因素方差分析(One-way ANOVA) Tukey's 多重比较法对各组均值之间的显著性水平进行分析.当  $P < 0.05$  时,即具有显著性差异;当  $P < 0.01$  时,则认定为差异极显著.

## 2 结果与讨论 (Results and discussion)

### 2.1 化学分析

在暴露期内,每周测定换水前(T24)和换水后(T0)各缸 MTA 和 EE2 的实际暴露浓度,结果见表 1.

表 1 各处理组中 MTA 和 EE2 的实测浓度

Table 1 The measured concentrations of MTA and EE2 in each exposure group

组别 Groups	MTA 实测浓度			EE2 实测浓度		
	Measured concentrations of MTA/( $\text{ng}\cdot\text{L}^{-1}$ )			Measured concentrations of EE2/( $\text{ng}\cdot\text{L}^{-1}$ )		
	T0	T24	RR/%	T0	T24	RR/%
SC	< LOD	< LOD	—	< LOD	< LOD	—
MTA-33	26.6 $\pm$ 0.7	13.7 $\pm$ 0.6	51.5	—	—	—
MTA-100	89.3 $\pm$ 2.1	65.2 $\pm$ 1.5	73.0	—	—	—
EE2-5	—	—	—	3.9 $\pm$ 1.2	3.1 $\pm$ 0.1	79.5
EE2-15	—	—	—	11.9 $\pm$ 0.2	8.7 $\pm$ 0.6	73.1
Comb-1	28.3 $\pm$ 1.2	13.5 $\pm$ 1.3	47.7	3.3 $\pm$ 0.2	2.5 $\pm$ 0.2	75.8
Comb-2	86.6 $\pm$ 2.6	64.4 $\pm$ 2.7	74.3	12.4 $\pm$ 0.5	9.0 $\pm$ 0.3	72.6

注: RR(%)为经过 24 h 暴露后 MTA 和 EE2 的残留率.LOD,定性检测限.“—”表示未检测.

Note: RR (%) indicates the residual ratio of MTA and EE2 after 24 h of exposure. LOD, limit of detection. “—” indicates that was not



determined.

对照组未检测出 MTA 或 EE2.刚换水后,MTA 和 EE2 的实测浓度分别为理论暴露浓度的 80.6%—89.3%和 66.0%—82.7%,基本接近理论暴露浓度.经过 24 h 后,暴露水中 MTA 和 EE2 的浓度均有所下降,残留率分别为 47.7%—74.3%和 72.6%—79.5%,这与水中微生物消耗、缸壁或鱼体吸附以及药物自然降解等因素有关<sup>[41]</sup>.

## 2.2 体质系数

经 21 d 暴露后,斑马鱼体质系数的变化情况见表 2.从表 2 可以看出,33 或 100 ng·L<sup>-1</sup> MTA 单独暴露并不影响雌、雄鱼的体长、体重、K 值和 GSI 等体质系数,这与先前已发表的结果相同<sup>[10]</sup>.5 ng·L<sup>-1</sup> EE2 单独暴露也不影响以上体质系数,但 15 ng·L<sup>-1</sup> EE2 单独暴露使得雌鱼的体重、K 值均显著上升且雌、雄鱼的 GSI 均显著下降(见表 2).有研究表明,用 50 ng·L<sup>-1</sup> EE2 暴露斑马鱼成鱼 14 d 可显著降低雌鱼的 K 值<sup>[42]</sup>.用 5—50 ng·L<sup>-1</sup> EE2 暴露斑马鱼成鱼 21—24 d 可导致雌、雄鱼的 GSI 显著下降<sup>[35, 37]</sup>.用 EE2 暴露黑头呆鱼、拟鲤(*Rutilus rutilus*)等其他鱼类,也发现雌、雄鱼的 GSI 显著被降低<sup>[43-44]</sup>.这些研究结果与本文的研究结果相同.从表 2 还可看出,33 ng·L<sup>-1</sup> MTA + 5 ng·L<sup>-1</sup> EE2 复合暴露并不影响雌、雄鱼的体长、体重、K 值和 GSI 等体质系数.100 ng·L<sup>-1</sup> MTA + 15 ng·L<sup>-1</sup> EE2 复合暴露显著升高雌鱼的体重、K 值,且显著降低雌、雄鱼的 GSI,但对雄鱼的体重、K 值无显著影响.此外,100 ng·L<sup>-1</sup> MTA + 15 ng·L<sup>-1</sup> EE2 复合暴露组雌鱼的 GSI 较 100 ng·L<sup>-1</sup> MTA 或 15 ng·L<sup>-1</sup> EE2 单独暴露组进一步显著降低,雄鱼的 GSI 较 100 ng·L<sup>-1</sup> MTA 单独暴露组也进一步显著降低.由于 MTA 对雌、雄鱼 GSI 并无显著影响,说明在复合暴露组中,对 GSI 的抑制作用主要由 EE2 暴露造成.

表 2 斑马鱼成鱼暴露于 MTA,EE2 及二者的复合物 21 d 后体质系数的变化

Table 2 Changes of somatic indices in adult zebrafish after exposure to MTA, EE2 and their combinations for 21 days

组别 Groups	雌鱼 Females				雄鱼 Males			
	体重 Body weight/g	体长 Body length/cm	K 值/%	性腺指数 GSI/%	体重 Body weight/g	体长 Body length/cm	K 值/%	性腺指数 GSI/%
SC	0.54±0.01	3.31±0.07	1.50±0.06	10.09±0.43	0.35±0.01	2.86±0.03	1.49±0.04	1.12±0.14
MTA-33	0.51±0.01	3.21±0.03	1.55±0.04	9.90±0.32	0.38±0.01	2.90±0.03	1.56±0.04	0.83±0.10
MTA-100	0.53±0.02	3.26±0.05	1.55±0.05	9.08±0.62	0.36±0.02	2.91±0.04	1.47±0.04	1.20±0.08
EE2-5	0.49±0.02	3.18±0.05	1.53±0.05	7.36±0.48	0.37±0.02	2.94±0.06	1.46±0.07	1.05±0.05
EE2-15	0.63±0.03*	3.21±0.03	1.90±0.06*	6.34±0.82*	0.37±0.01	2.81±0.03	1.65±0.04	0.59±0.14*
Comb-1	0.50±0.01	3.10±0.06	1.70±0.11	7.35±0.51	0.40±0.02	3.01±0.05	1.48±0.04	0.93±0.07
Comb-2	0.61±0.02*	3.21±0.03	1.86±0.07*	3.69±0.40**&&#	0.36±0.02	2.79±0.04	1.64±0.05	0.6±0.15* &

\*  $P < 0.05$  或 \*\*  $P < 0.01$  表示暴露组与对照组之间具有显著性差异; &  $P < 0.05$  或 &&  $P < 0.01$  表示复合暴露组与相应 MTA 单独暴露组相比具有显著性差异; #  $P < 0.05$  表示复合暴露组与相应 EE2 单独暴露组相比具有显著性差异.

\*  $P < 0.05$  or \*\*  $P < 0.01$  indicates significant difference between the exposure group and the control; &  $P < 0.05$  or &&  $P < 0.01$  indicates significant difference in the co-exposure group relative to the corresponding MTA single treatment group; #  $P < 0.05$  indicates significant difference in the co-exposure group relative to the corresponding EE2 single treatment group.

## 2.3 产卵量

在进行化学品暴露之前,统计了预暴露期各组雌鱼的产卵基数,发现各组雌鱼的平均累积产卵量之间并无显著性差异(图 1a),说明暴露之前各组雌鱼产卵能力相当.从图 1b 可以看出,33 或 100 ng·L<sup>-1</sup> MTA 单独暴露对斑马鱼的产卵无显著影响;Han 等<sup>[10]</sup>用 666 ng·L<sup>-1</sup> MTA 暴露斑马鱼 21 d 可显著降低斑马鱼的产卵量,说明 MTA 对斑马鱼产卵的影响具有剂量依赖效应,只有在较高浓度下才能引起斑马鱼的生殖损伤.大量研究表明,其他孕激素如孕酮(Progesterone, P4)、炔诺酮(Norethindrone, NET)、左炔诺孕酮(LNG)、去氢孕酮(Dydrogesterone, DDG)、去氧孕烯(Desogestrel, DSG)、孕二烯酮(Gestodene, GES)、屈螺酮(DRS)、醋酸氯地孕酮(CMA)等在一定浓度下均能抑制鱼类的产卵,造成生殖损伤<sup>[3, 6, 15, 41, 45-49]</sup>.

5 ng·L<sup>-1</sup> EE2 单独暴露也不影响斑马鱼的产卵;但单独暴露于 15 ng·L<sup>-1</sup> EE2 的斑马鱼从暴露期的第 10 d 起便不再产卵,从暴露期第 11 d 起直至暴露期结束,其产卵量较对照组均显著降低(图 1b).

Nash 等<sup>[34]</sup>发现用低剂量的 EE2 (0.5 或 5 ng·L<sup>-1</sup>) 暴露斑马鱼 40 d 并未对产卵量产生影响, 但用 50 ng·L<sup>-1</sup> EE2 暴露 10 d 便可使得斑马鱼完全停止产卵. Van den Belt 等<sup>[35]</sup>用 EE2 暴露斑马鱼 21 d, 发现 EE2 在 10 ng·L<sup>-1</sup> 浓度下可显著降低斑马鱼的产卵量, 在 25 ng·L<sup>-1</sup> 浓度下则完全使其产卵活动停止. 这些研究结果表明, EE2 对斑马鱼产卵的抑制具有明显的剂量依赖效应.

从图 1b 还可看出, 33 ng·L<sup>-1</sup> MTA + 5 ng·L<sup>-1</sup> EE2 复合暴露对斑马鱼的产卵无显著影响; 但经 100 ng·L<sup>-1</sup> MTA + 15 ng·L<sup>-1</sup> EE2 复合暴露的斑马鱼从暴露期的第 6 d 开始便不再产卵, 从暴露期第 7 d 起至暴露期结束, 其产卵量较对照组均显著降低. 此外, 与 100 ng·L<sup>-1</sup> MTA 单独暴露组相比, 100 ng·L<sup>-1</sup> MTA + 15 ng·L<sup>-1</sup> EE2 复合暴露组斑马鱼的产卵量在第 9 d 及第 11—21 d 均显著降低; 然而, 与 15 ng·L<sup>-1</sup> EE2 单独暴露组相比, 该复合暴露组斑马鱼的产卵量并无显著性差异. 此结果表明, 在该复合暴露组合中, 对斑马鱼产卵的抑制效应主要由 EE2 引起. 然而, Hua 等<sup>[11]</sup>发现当把 333 ng·L<sup>-1</sup> MTA 加入与 10 ng·L<sup>-1</sup> EE2 共同暴露斑马鱼时, 复合暴露对斑马鱼产卵的抑制作用较 EE2 单独暴露进一步增强. 虽然孕激素 (如 MTA) 和雌激素 (如 EE2) 的作用方式不同, 其中前者主要通过孕激素受体 (Progesterone receptor, PR) 发挥作用, 而后者是雌激素受体 (Estrogen receptor, ER) 的激动剂<sup>[15, 50]</sup>, 但二者均可导致相同的作用终点——抑制鱼类产卵 (或降低产卵量). 我们猜测, 当孕激素和雌激素复合暴露时, 可能独立发挥作用, 这样一定程度上能解释本研究与 Hua 等<sup>[11]</sup>研究结果的差异. Runnalls 等<sup>[15]</sup>发现, EE2 和孕激素左炔诺孕酮 (LNG) 复合暴露时并未发生相互作用 (拮抗或者协同), 二者对降低黑头呆鱼产卵量表现出加和效应. 这一结果一定程度上说明我们推测的合理性. 鉴于目前有限的研究结果, 雌激素和孕激素的联合作用效应有待进一步探索.

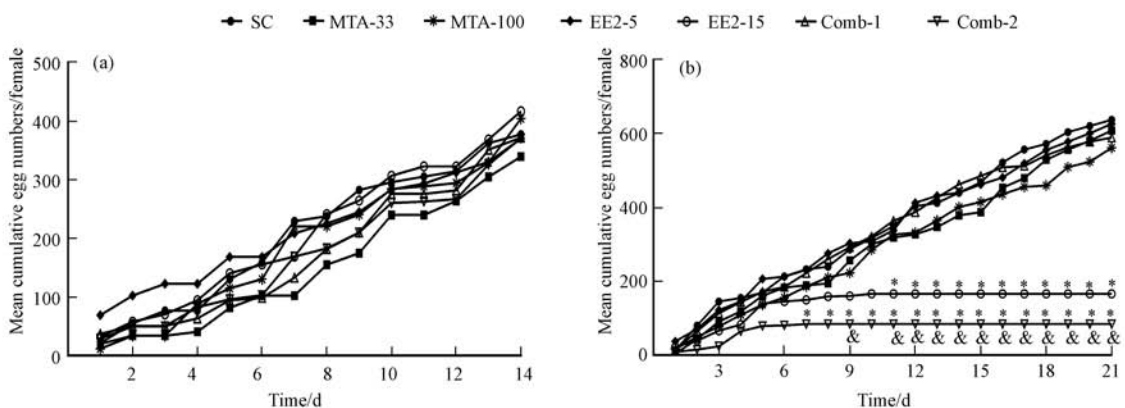


图 1 预暴露期 (a) 和暴露期 (b) 斑马鱼雌鱼的平均累积产卵量

(\*  $P < 0.05$  表示暴露组与对照组相比具有显著性差异;  $\& P < 0.05$  表示复合暴露组与相应 MTA 单独暴露组相比具有显著性差异)

Fig. 1 Mean cumulative egg production in female zebrafish during the pre-exposure (a) and exposure (a) period

(\*  $P < 0.05$  indicates significant difference between the exposure group and the solvent control;

$\& P < 0.05$  indicates significant difference in the co-exposure group relative to the corresponding MTA single exposure group)

#### 2.4 血浆性激素水平

在斑马鱼雌鱼中, 33、100 ng·L<sup>-1</sup> MTA 单独暴露使得血浆中 E2 的含量较对照分别显著下降了 35% 和 50% (图 2a), 同时使得 T 的含量较对照分别显著下降了 37% 和 41% (图 2b). 5 和 15 ng·L<sup>-1</sup> EE2 单独暴露使得雌鱼血浆中 E2 的含量较对照分别显著下降了 73% 和 83% (图 2a); 同时使得 T 的含量较对照分别显著下降了 44% 和 57% (图 2b). 33 ng·L<sup>-1</sup> MTA + 5 ng·L<sup>-1</sup> EE2 和 100 ng·L<sup>-1</sup> MTA + 15 ng·L<sup>-1</sup> EE2 复合暴露使得雌鱼血浆中 E2 的含量较对照分别显著下降了 77% 和 87% (图 2a); 同时使得 T 的含量较对照分别显著下降 49% 和 34% (图 2b). 此外, 33 ng·L<sup>-1</sup> MTA + 5 ng·L<sup>-1</sup> EE2 复合暴露使得雌鱼血浆中 E2 的含量较 33 ng·L<sup>-1</sup> MTA 单独暴露显著下降了 65%, 较 5 ng·L<sup>-1</sup> EE2 单独暴露下降了 16%, 但无显著性差异; 100 ng·L<sup>-1</sup> MTA + 15 ng·L<sup>-1</sup> EE2 复合暴露使得雌鱼血浆中 E2 的含量较 100 ng·L<sup>-1</sup> MTA 单独暴露显著下降了 75%, 较 15 ng·L<sup>-1</sup> EE2 单独暴露下降了 25%, 但并无显著性差异 (图 2a). 所有复合暴露中雌鱼血浆中 T 的含量较相应 MTA 单独暴露或 EE2 单独暴露均无显著性差异 (图 2b).

在斑马鱼雄鱼中, 33、100 ng·L<sup>-1</sup> MTA 单独暴露使得血浆中 11-KT 的含量较对照分别显著下降了

44%和47%;5、15 ng·L<sup>-1</sup> EE2 单独暴露使得血浆中 11-KT 的含量较对照分别显著下降了 77%和 94%; 33 ng·L<sup>-1</sup> MTA + 5 ng·L<sup>-1</sup> EE2 和 100 ng·L<sup>-1</sup> MTA + 15 ng·L<sup>-1</sup> EE2 复合暴露使得血浆中 11-KT 的含量较对照分别显著下降了 80%和 95% (图 2c).此外,33 ng·L<sup>-1</sup> MTA + 5 ng·L<sup>-1</sup> EE2 复合暴露使得雄鱼血浆中 11-KT 的含量较 33 ng·L<sup>-1</sup> MTA 单独暴露显著下降了 64%,较 5 ng·L<sup>-1</sup> EE2 单独暴露下降了 11%,但无统计学显著性差异;100 ng·L<sup>-1</sup> MTA + 15 ng·L<sup>-1</sup> EE2 复合暴露使得雄鱼血浆中 11-KT 含量较 100 ng·L<sup>-1</sup> MTA 单独暴露显著下降了 92%,较 15 ng·L<sup>-1</sup> EE2 单独暴露下降了 22%,但无显著性差异 (图 2c).

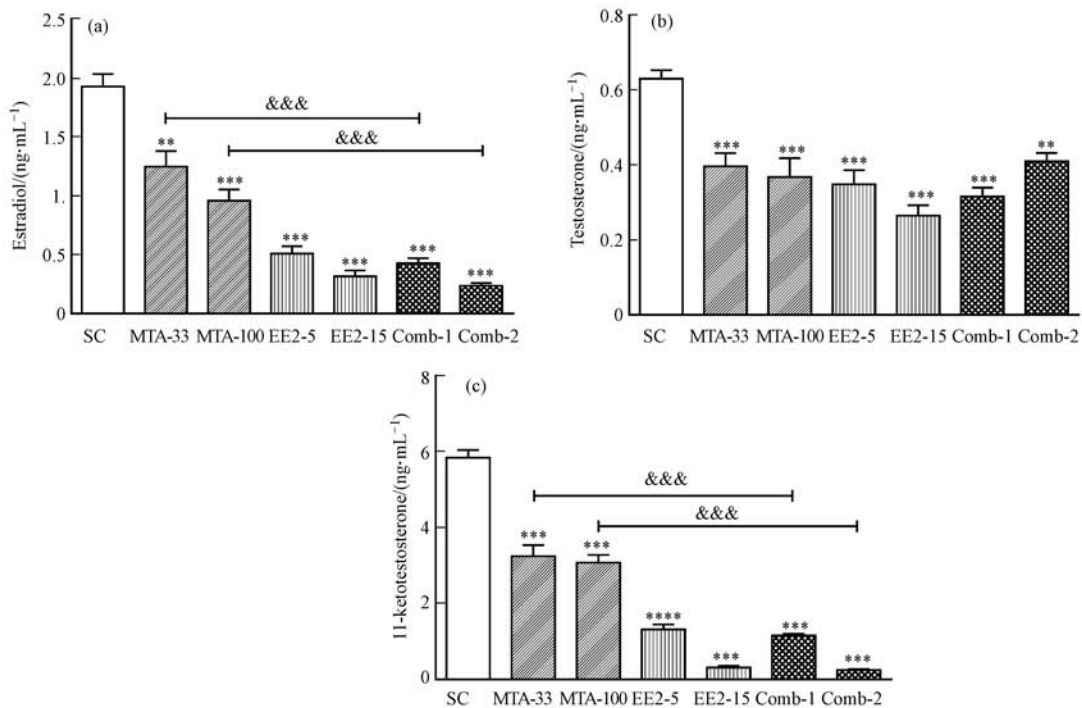


图 2 MTA,EE2 及二者复合暴露 21 d 对斑马鱼雌鱼血浆中雌二醇(E2) (a)、睾酮(T) (b) 及雄鱼血浆中 11-酮基睾酮 (11-KT) (c) 含量的影响

Fig.2 Effects on plasma concentrations of estradiol (E2) (a), testosterone (T) (b) in females and 11-ketotestosterone (11-KT) (c) in males in zebrafish after exposure to MTA, EE2 and their combinations for 21 days

性激素水平是鱼类对环境内分泌干扰物暴露反应较为敏感的指标.环境内分泌干扰物可通过干扰性激素水平来影响鱼类的生殖<sup>[51-53]</sup>.本研究中,MTA 单独暴露可使得雌鱼血浆中 E2、T 以及雄鱼血浆中 11-KT 的含量显著下降,这与先前的研究结果一致<sup>[10-11]</sup>.研究表明,孕激素如炔诺酮 (NET)<sup>[47]</sup>、左炔诺孕酮 (LNG)<sup>[6, 15, 54]</sup>、孕二烯酮 (GES)<sup>[6]</sup> 等也可显著降低鱼类血浆中性激素的含量.然而,用孕酮 (P4) 注射三倍体虹鳟鱼 (*Oncorhynchus mykiss*),或用其他孕激素如醋酸甲羟孕酮 (Medroxyprogesterone acetate, MPA)、去氧孕烯 (DSG)、屈螺酮 (DRS) 和去氢孕酮 (DDG) 对黑头呆鱼或斑马鱼进行水体暴露,均不干扰血浆中性激素的含量<sup>[3, 6, 55]</sup>.这些结果说明,使用不同孕激素和鱼类物种的研究结果存在一定差异.本研究中,EE2 单独暴露可显著降低斑马鱼雌鱼血浆中 E2、T 以及雄鱼血浆中 11-KT 的含量.其他研究也报道 EE2 在较低浓度下可显著降低鱼类血浆中性激素的含量<sup>[36, 43, 56-57]</sup>.复合暴露组斑马鱼雌鱼血浆中 E2 以及雄鱼血浆中 11-KT 的含量相对 MTA 单独暴露组显著下降,而相对 EE2 单独暴露组则没有差异,说明复合暴露组中性激素含量的降低主要由 EE2 引起,这一结果与 Hua 等<sup>[11]</sup>的研究结果一致. Runnalls 等<sup>[6]</sup> 也发现环境相关浓度的 EE2 和孕激素 LNG 复合暴露对黑头呆鱼血浆中 E2 和 11-KT 的抑制作用与 EE2 单独暴露类似. Atteke 等<sup>[55]</sup> 发现用 EE2 与孕酮 (P4) 混合注射和用 EE2 单独注射三倍体虹鳟鱼,鱼体血浆中性激素 (E2 和 E1) 含量上升的程度相当,但用孕酮 (P4) 单独注射并未引起这两种激素含量发生变化.这些结果说明,当用环境相关浓度的 EE2 与孕激素复合暴露鱼类时,对血浆中性激素含量的影响往往主要由 EE2 引起,这可能与 EE2 的活性很强有关.



## 2.5 性腺组织学

卵巢中各发育期卵细胞所占的相对比例见图 3a. 33 或 100  $\text{ng}\cdot\text{L}^{-1}$  MTA 单独暴露并不影响卵巢中各发育期卵细胞所占的比例(图 3a), 但 Hua 等<sup>[11]</sup>发现 MTA (100, 333  $\text{ng}\cdot\text{L}^{-1}$ ) 单独暴露可显著降低斑马鱼卵巢中晚期卵黄发生期的卵母细胞/成熟的卵母细胞所占的比例. 5  $\text{ng}\cdot\text{L}^{-1}$  EE2 单独暴露显著升高卵巢中初级卵母细胞(PO)所占的比例, 且显著降低皮质肺泡卵母细胞(CAO)所占的比例; 15  $\text{ng}\cdot\text{L}^{-1}$  EE2 单独暴露显著升高卵巢中初级卵母细胞(PO)和闭锁卵泡(AF)所占的比例, 且显著降低卵黄发生期卵母细胞(VO)所占的比例(图 3a). 这些结果说明 EE2 阻碍了斑马鱼卵巢中卵细胞的发育, 且加速了卵细胞的退化. 先前已有研究也表明 EE2 可抑制斑马鱼卵子发生的过程<sup>[34, 37]</sup>. 卵泡闭锁是卵细胞退化、重吸收的过程, 是清除损坏或多余卵细胞的一种方式, 某些环境压力或因素(如营养缺乏, 外源性化学品)可诱导闭锁卵泡的发生<sup>[58-59]</sup>. 先前在斑马鱼和青鳉鱼(*Oryzias latipes*)中的研究也发现 EE2 能够诱导闭锁卵泡<sup>[11, 37, 60]</sup>. 这些研究表明 EE2 是诱导鱼类卵巢产生闭锁卵泡的潜在环境因子. 此外, 33  $\text{ng}\cdot\text{L}^{-1}$  MTA + 5  $\text{ng}\cdot\text{L}^{-1}$  EE2 复合暴露使得卵巢中卵黄发生期卵母细胞(VO)所占的比例较对照显著降低; 100  $\text{ng}\cdot\text{L}^{-1}$  MTA + 15  $\text{ng}\cdot\text{L}^{-1}$  EE2 复合暴露使得卵巢中初级卵母细胞(PO)和闭锁卵泡(AF)所占的比例较对照显著升高, 同时使得卵黄发生期的卵母细胞(VO)所占的比例较对照显著降低(图 3a), 说明 MTA 和 EE2 复合暴露抑制了卵巢中卵母细胞的发育. 此外, 与 100  $\text{ng}\cdot\text{L}^{-1}$  MTA 单独暴露相比, 100  $\text{ng}\cdot\text{L}^{-1}$  MTA + 15  $\text{ng}\cdot\text{L}^{-1}$  EE2 复合暴露使得初级卵母细胞(PO)和闭锁卵泡(AF)所占的比例显著升高, 且皮质肺泡卵母细胞(CAO)和卵黄发生期卵母细胞(VO)所占比例显著降低; 与 15  $\text{ng}\cdot\text{L}^{-1}$  EE2 单独暴露相比, 100  $\text{ng}\cdot\text{L}^{-1}$  MTA + 15  $\text{ng}\cdot\text{L}^{-1}$  EE2 复合暴露并不影响卵巢中各发育期卵母细胞所占比例(图 3a). 这些结果表明, 复合暴露组中卵巢组织学的改变主要由 EE2 所导致.

在精巢中, MTA、EE2 单独暴露和 33  $\text{ng}\cdot\text{L}^{-1}$  MTA + 5  $\text{ng}\cdot\text{L}^{-1}$  EE2 复合暴露均不影响精巢中各发育期精子细胞所占的比例(图 3b). 在 100  $\text{ng}\cdot\text{L}^{-1}$  MTA + 15  $\text{ng}\cdot\text{L}^{-1}$  EE2 复合暴露组中, 精原细胞所占的比例较对照组或 100  $\text{ng}\cdot\text{L}^{-1}$  MTA 单独暴露组或 15  $\text{ng}\cdot\text{L}^{-1}$  EE2 单独暴露组均显著升高(图 3b). 相对卵巢, 本研究结果表明, 斑马鱼精巢组织学变化对 MTA、EE2 单独暴露及二者复合暴露的响应并不敏感. Hua 等<sup>[11]</sup>的研究表明 MTA、EE2 及二者的复合物对斑马鱼精巢发育没有影响, 也说明精巢组织学变化对 MTA 和 EE2 暴露并不敏感.

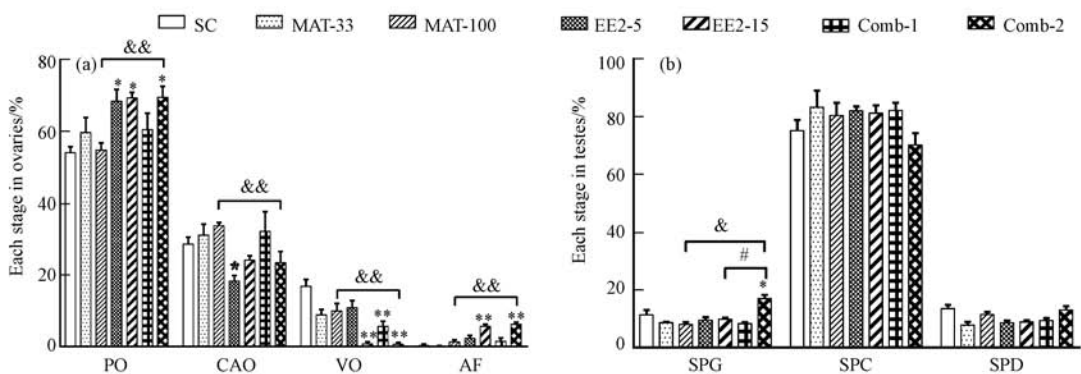


图 3 MTA、EE2 及二者复合暴露 21 d 对斑马鱼卵巢(a)和精巢(b)中各发育期生殖细胞所占比例的影响 (PO: 初级卵母细胞; CAO: 皮质肺泡卵母细胞; VO: 卵黄发生期的卵母细胞; AF: 闭锁卵泡; SPG: 精原细胞; SPC: 精母细胞; SPD: 精细胞)

Fig.3 Effects on percentages of germ cells at each stage in ovaries (a) and testes (b) of zebrafish after exposure to MTA, EE2 and their combinations for 21 days (PO: Primary oocytes; CAO: Cortical alveolar oocytes; VO: Vitellogenic oocytes; AF: Atretic follicles; SPG: Spermatogonia; SPC: Spermatocytes; SPD: Spermatids)

## 3 结论 (Conclusion)

(1) MTA (33, 100  $\text{ng}\cdot\text{L}^{-1}$ )、5  $\text{ng}\cdot\text{L}^{-1}$  EE2 单独暴露及较低浓度复合暴露均不影响斑马鱼的产卵量;



15 ng·L<sup>-1</sup> EE2 单独及与 100 ng·L<sup>-1</sup> MTA 复合暴露可显著抑制斑马鱼的产卵。

(2) MTA、EE2 单独及复合暴露均显著降低雌鱼血浆中 E2、T 及雄鱼血浆中 11-KT 的含量。

(3) MTA (33、100 ng·L<sup>-1</sup>) 单独暴露对斑马鱼性腺组织学无显著影响; EE2 单独及与 MTA 复合暴露可导致斑马鱼卵巢组织学变化(抑制卵子发生,诱导卵泡闭锁),但对精巢影响较小。

(4) 复合暴露组中斑马鱼的产卵量、血浆性激素含量、性腺组织学变化与 EE2 单独暴露组相比均无显著差异,说明复合暴露的毒性效应主要由 EE2 引起。

(5) 鉴于使用不同的浓度和复合方案,相同种类的孕激素和雌激素复合暴露导致的结果存在一定差异,雌激素和孕激素的联合作用模式尚值得深入研究。

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