· 实验技术及其应用 ·

亚慢性苯并［a］芘染毒对转人载脂蛋白 E4 基因小鼠 tau 蛋白磷酸化及轻度认知功能障碍的影响

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摘要：目的 探讨亚慢性苯并[a]芘(benzo[a]pyrene, BaP)染毒对 HAPOE4 基因型小鼠 tau 蛋白磷酸化及轻度认知功能障碍的影响。**方法** 野生型和转人 APOE4 基因型(HAPOE4)的 C57BL/6 雄性小鼠各 24 只，每种类型小鼠随机分为溶剂组(等体积比橄榄油)、BaP 染毒低剂量组(2.5 mg/kg)、BaP 染毒高剂量组(6.25 mg/kg)，每组 8 只。染毒方式为腹腔注射，隔天称重后染毒，染毒周期为 90 d。采用 Morris 水迷宫试验检测小鼠学习记忆能力；采用悬尾实验检测小鼠抑郁状态；采用甘氨酸银浸染色法观察小鼠脑组织切片中神经原纤维缠结情况；采用免疫组织化学法检测小鼠海马组织中 APOE、低密度脂蛋白受体相关蛋白 1(LDL receptor - related protein 1, LRP1)、tau、p - tau(ser199)、p - tau(ser396)的表达情况。以上计数资料染毒处理比较时均使用方差分析方法，基因型之间比较时均使用两样本 t 检验方法，验证染毒处理与基因型之间交互作用时均使用析因分析方法。**结果** 水迷宫行为学实验结果：定位航行实验结果：各组逃避潜伏期时长随着训练天数增加而下降，且同一染毒剂量 HAPOE4 型小鼠逃避潜伏期高于 WT 型。第 4、5 天组间逃避潜伏期有差异；APOE4 基因与染毒均会导致小鼠穿越平台次数与目标象限停留时间下降，但 APOE4 基因与染毒之间交互作用无统计学意义。悬尾实验结果：APOE4 基因与染毒对小鼠静止不动时间有交互作用且均会导致小鼠静止不动时间增加。甘氨酸银浸镀实验结果：APOE4 基因与染毒均会导致小鼠染色加深变多。免疫组织化学结果显示：APOE4 基因与染毒对 APOE、LRP1、tau、p - tau(ser199)、p - tau(ser396)的 MOD 值有交互作用且均会导致平均光密度值(Mean optical density, MOD)值升高。**结论** BaP 和 APOE4 基因影响小鼠空间学习记忆能力与抑郁状态，BaP 和 APOE4 基因对小鼠悬尾实验静止不动时间存在交互作用，表明 BaP 和 APOE4 基因联合作用可能对小鼠抑郁状态有影响；BaP 和 APOE4 基因对小鼠 APOE、LRP1 及 tau 蛋白异常磷酸化存在交互作用，表明 BaP 亚慢性染毒会导致 HAPOE4 基因型小鼠 tau 蛋白磷酸化及轻度认知功能障碍，其原因可能是 BaP 与 APOE4 基因联合作用导致小鼠 APOE 和 LRP1 升高所致。

关键词：BaP；APOE4 基因；LRP1；tau 蛋白磷酸化；轻度认知功能障碍
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Effects of subchronic benzo[a]pyrene toxicity on tau protein phosphorylation and mild cognitive dysfunction in HAPOE4 genotype mice

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Abstract: Objective To investigate the effect of subchronic benzo[a]pyrene (BaP) toxicity on tau protein phosphorylation and mild cognitive dysfunction in HAPOE4 mice. **Methods** 24 male C57BL/6 wild - type and HAPOE4 mice were used in this study, 12 of each type. The mice were randomly divided into three groups: vehicle group (olive oil), low - dose BaP - treated group (2.5 mg/kg), and high - dose BaP - treated group (6.25 mg/kg), with 8 mice in each group. The mice were injected with BaP via the peritoneal cavity and weighed before and after injection. The exposure period was 90 days. The Morris water maze test was used to detect the mice's learning and memory abilities, the tail suspension test was used to detect the mice's depressive state, the silver glycinate dip staining was used to observe the neurofibrillary tangles in mouse brain tissue sections, and immunohistochemistry was used to detect the protein expression of APOE, LRP1, tau, p - tau (Ser199), and p - tau (Ser396) in the hippocampus of the mice. ANOVA was used for the comparison of the above count data, the two -

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sample t – test was used for the comparison between genotypes, and the factorial analysis method was used to verify the interaction between the treatment and genotype. **Results** The results of the water maze behavior experiment: the results of the positioning navigation experiment: the escape latency length of each group decreased with the increase of training days, and the escape latency of HAPOE4 mice with the same dose of poison was higher than that of WT type. There were differences in escape latency between groups on the 4th and 5th days. Both genotype and toxicity could lead to a decrease in the number of crossings of the platform and the residence time in the target quadrant, but there was no significant interaction between genotype and toxicity. The results of tail suspension experiment: genotype and poisoning had an interactive effect on the immobility time of mice, and both of them would lead to an increase in the immobility time of mice. The results of silver glycinate dipping experiment: both genotype and poisoning would cause the dyeing to deepen and increase in mice. The results of immunohistochemistry showed that genotype and poisoning had an interactive effect on the MOD values of APOE, LRP1, tau, p – tau (ser199) and p – tau (ser396), and all of them led to an increase in MOD values. **Conclusion** BaP and APOE4 genes affected spatial learning and memory ability and depression in mice, and BaP and APOE4 genes interacted with the immobility time of tail suspension experiments, indicating that the combined effect of BaP and APOE4 genes may have an effect on the depressed state of mice; and BaP and APOE4 genes interacted with abnormal phosphorylation of tau protein in mice, indicating that subchronicBaP infection could lead to tau protein phosphorylation and mild cognitive dysfunction in mice with HAPOE4 genotype, which may be caused by the combined effect of BaP and APOE4 genes resulting in the increase of APOE and LRP1 in mice.

Keywords: BaP; APOE4; LRP1; Tau phosphorylation; MCI

多环芳烃(polycyclic aromatic hydrocarbons, PAHs)是有机物不完全燃烧产生的一类污染物,被怀疑是神经毒性物质[1]。几项临床研究表明 PAHs 与儿童神经发育障碍有关,并报告了 PAHs 暴露与神经心理症状(如焦虑、抑郁和注意力缺陷)风险之间的正相关性[2]。苯并[a]芘(benzo[a]pyrene, BaP)是多环芳烃家族的代表物质,可通过食物、饮水和空气进入到人体内[3]。近年来,越来越多的人类和动物研究报告 BaP 暴露可引起神经毒性[4],本课题组前期研究发现 BaP 暴露会导致小鼠轻度认知功能障碍、tau 蛋白异常磷酸化[5]。然而,BaP 诱导轻度认知功能障碍的确切机制仍不清楚。

载脂蛋白 E(apolipoprotein E,APOE)基因多态性是晚发型阿尔茨海默病(alzheimer disease,AD)的一个主要遗传危险因素,与常见的 APOE3 等位基因相比,APOE4 等位基因增加了 AD 的发病风险[6]。tau 蛋白是一种微管相关蛋白质,在正常神经元的成熟、脑组织的发育及学习和神经元可塑性方面发挥着重要作用[7]。在正常的成年脑组织中,tau 蛋白极少被磷酸化;但在神经退行性疾病中,tau 蛋白处于非正常的磷酸化状态[8]。最新研究发现 APOE4 增强 tau 介导的神经变性并引起小胶质细胞的突触吞噬作用[9]。

低密度脂蛋白受体相关蛋白 1(low – density lipoprotein receptor – related protein 1, LRP1)在脑的不同区域高度表达,并作为 APOE 的主要受体发挥作用[10]。研究发现应激诱导海马 LRP1 可通过激活 Akt 信号通路,促进突触可塑性,增加微管动力学,导致 tau 蛋白磷酸化增加[11]。越来越多的证据表明,APOE 影响 tau 病理学、tau 介导的神经变性和小胶质细胞对 AD 相关病理学的反应[12-13]。

目前尚无关于 BaP 是否参与 APOE 致 tau 蛋白异常磷酸化的报道。本研究通过对 WT 型小鼠和 HAPOE4 基因型小鼠进行 BaP 亚慢性染毒,探究亚慢性 BaP 染毒对于 HAPOE4 基因型小鼠 tau 蛋白异常磷酸化及轻度认知功能障碍的影响。

1 材料和方法

1.1 *动物* 选用成年 C57BL/6 雄性小鼠,一类为通过同源重组,将小鼠 APOE 基因进行人源化修饰的小鼠(Cat. NO. NM – HU – 190002, HAPOE4),从上海南方模式生物有限公司购得;二类为 WT 型 C57BL/6 小鼠,购自(斯贝福,北京)。每类型小鼠随机分为溶剂组、低剂量染毒组和高剂量染毒组,每组 8 只。小鼠体重为(24 ± 2) g,饲养于光暗循环的标准清洁动物房,温度为(22 ± 2)℃,饮水饮食自由,用标准化饲料喂养(购自斯贝福,北京)。实验过程遵循国际兽医学编辑协会《关于动物伦理与福利的作者指南共识》(动物伦理审查编号:2020GLL037)。

1.2 *染毒* 小鼠适应性饲养一周后,隔天腹腔注射染毒,染毒剂量为低剂量组 2.5 mg/kg、高剂量组 6.25 mg/kg,隔天称量体重后染毒,溶剂组注射同体积比的橄榄油,染毒周期为 90 天。

1.3 *实验方法*

1.3.1 Morris 水迷宫 实验在高 45 cm、直径 100 cm 的圆形水池中进行。将水池依据内壁的标识分为 4 个象限,于目标象限的中间位置水面下 0.5 ~ 1 cm 处放置平台。前 5 天进行定位航行训练。第 6 天撤离水池中平台后记录小鼠穿越平台次数以及目标象限

停留时间。

1.3.2 悬尾实验 实验在一个尺寸为 50 cm ×50 cm ×60 cm 的透明实验箱中进行,确保箱内无其他干扰因素。将小鼠尾巴固定于悬尾支架上,头部朝下,与悬尾箱底面保持约 30 cm 的距离。实验开始时,连续录制 6 min 的视频,观察并记录小鼠后 4 min 内的不动时间,以评估其行为状态。

1.3.3 甘氨酸银浸染色 为了检测小鼠脑组织中神经元纤维缠结,在将小鼠处死后立即将脑组织在 4% 多聚甲醛中固定,然后将样品石蜡包埋并切成 5 μm 厚的切片。根据甘氨酸银染色试剂盒(Servicebio,中国)的说明书对脱蜡和再水化的切片进行染色。最后进行小鼠脑组织海马区域显微镜镜检以及图像采集。

1.3.4 免疫组织化学 一抗抗体动物种属均为兔,二抗抗体为辣根过氧化物酶标记山羊抗兔 IgG(购自华安生物),将小鼠处死后立即将脑组织在 4% 多聚甲醛中固定,并进行贯状面切片,组织石蜡切片;烤片;过缸;水化;抗原修复;3% H_2O_2 室温孵育 30 min;用组化笔画圈阻水;封闭;孵一抗;孵二抗;DAB 显色;苏木素复染;1% 盐酸酒精分化 1 ~3 s,自来水冲洗后用 PBS 返蓝,再用自来水冲洗;过缸;脱水;中性树胶加封盖玻片,晾干后镜下观察并拍照。用 imagej 软件分析图像得到累计光密度值(Integrated optiondensity, IOD)与阳性面积值(Area),将 IOD 值与 Area 值相比得出平均光密度(mean density,MOD)作为结果。

1.3.5 统计学分析 所有的计量资料以 $\bar{x} \pm s$ 表示,采用 SPSS 22.0 软件进行统计分析。组间比较采用析因设计的方差分析,检验水准 $\alpha = 0.05$。

2 结 果

2.1 小鼠染毒前后体重变化情况 染毒期间各组小鼠正常饮水、进食,精神状态、活动度无明显差异,体重均正常增长。染毒前后各组小鼠体重变化差异均无统计学意义($F = 2.219$, $P = 0.070$; $F = 1.765$, $P = 0.141$)。(见表 1)

表 1 小鼠染毒前后体重变化($\bar{x} \pm s$, $n = 8$)

Table 1 Changes in body weight of mice before and after exposure of mice($\bar{x} \pm s$, $n = 8$)

基因型及染毒剂量(mg/kg)	染毒前(g)	染毒后(g)
WT +0	24.38 ±0.63	32.07 ±0.89
WT +2.5	24.67 ±0.65	31.85 ±0.78
WT +6.25	24.12 ±0.51	32.08 ±0.80
HAPOE4 +0	24.02 ±0.51	31.21 ±0.89
HAPOE4 +2.5	24.61 ±0.46	31.17 ±0.80
HAPOE4 +6.25	24.70 ±0.57	31.63 ±1.0
F 值	2.219	1.765
P 值	0.070	0.141

2.2 Morris 水迷宫 逃避潜伏期结果(秒,s):随着训练天数增加,各组逃避潜伏期时长下降。训练第 3、4、5 天,方差分析结果显示各基因型小鼠组内差异有统计学意义。(见图 1)

目标象限停留时间结果(秒,s):同一基因型组内小鼠目标象限停留时间均随染毒剂量增加而增加且差异有统计学意义。(见图 2)

穿越平台次数结果:同一基因型组内小鼠穿越平台次数均随染毒剂量增加而增加且差异有统计学意义,2.5 mg/kg 染毒处理 HAPOE4 型小鼠穿越平台次数低于同处理 WT 型小鼠且差异有统计学意义。(见图 2)

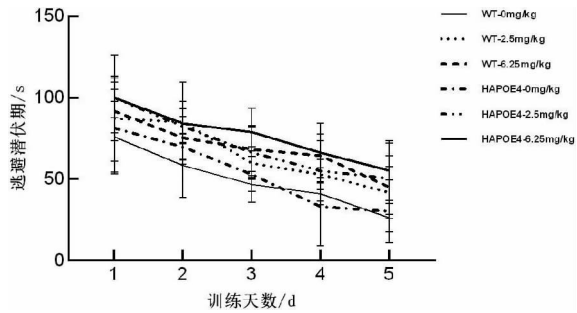
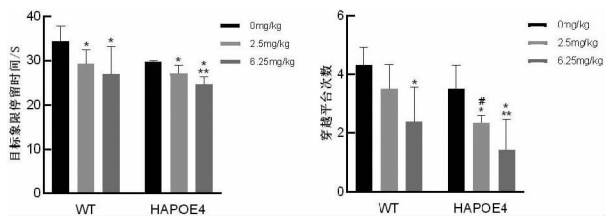


图 1 小鼠随训练天数增加逃避潜伏期变化情况

Fig. 1 The change of the escape latency of mice with the increase of the number of training days



注:*:与同基因型小鼠 0 mg/kg 组相比具有统计学意义;**:与同基因型小鼠 2.5 mg/kg 组相比具有统计学意义;#:与同等剂量染毒处理 WT 基因型小鼠相比具有统计学意义;下同。

图 2 小鼠第六条目标象限停留时间与穿越平台次数结果

Fig. 2 The results of the time that the mice stayed in the target quadrant and the number of times they crossed the platform on the sixth day

2.3 悬尾实验 结果显示染毒和 APOE4 基因均会导致小鼠静止不动时间增加且差异具有统计学意义。基因型与染毒处理之间有交互作用。(见图 3)

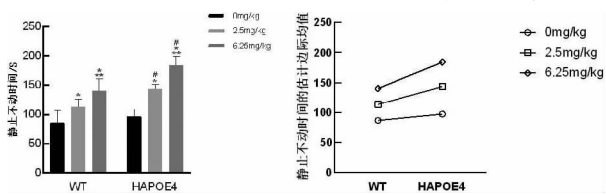


图 3 小鼠悬尾实验静止不动时间及其交互作用结果

Fig. 3 The immobility time of mice in the tail suspension test and the results of its interaction

2.4 甘氨酸镀银浸镀染色结果　甘氨酸银浸镀实验结果显示,镜下可观察到小鼠脑组织神经原纤维缠结(neurofibrillary tangles, NFTs)和部分树突被染成了黑色(见图 4)。通过观察各组小鼠海马区染色情况发现,染毒或 APOE4 基因均会导致小鼠染色明显加深增多。

2.5 APOE、LRP1 免疫组织化学结果　APOE、LRP1 免疫组织化学结果(见图 5),同基因型小鼠平均光密度(Mean optical density, MOD)值随染毒剂量增加而增加,差异具有统计学意义,同染毒处理时 HAPOE4 型小鼠 MOD 值高于 WT 型小鼠,差异具有统计学意义,基因型与染毒之间有交互作用。(见图 6)

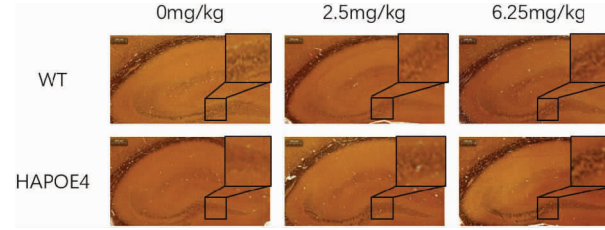


图 4　各组小鼠脑组织的甘氨酸银浸染色(甘氨酸银浸染色, ×4)

Fig. 4　Glycine silver impregnation stainingof the brain tissues of mice in each group (Glycine silver impregnation staining, ×4)

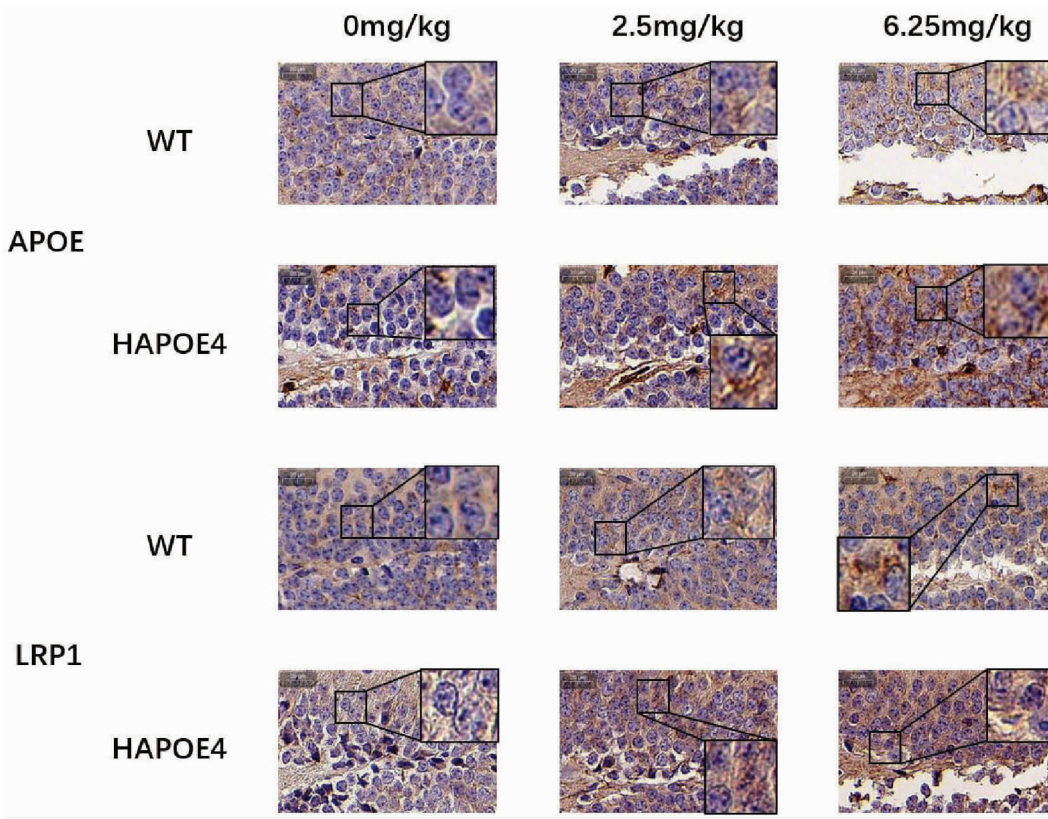


图 5　各组小鼠的 APOE、LRP1 免疫组织化学图像(DAB 染色, ×40)

Fig. 5　Immunohistochemical images of APOE and LRP1 in mice of each group (DAB staining, ×40)

2.6 tau、p－tau(ser199)、p－tau(ser396)免疫组织化学结果　Tau、p－tau(ser199)、p－tau(ser396)免疫组织化学结果(见图 7),同基因型小鼠 MOD 值随染毒剂量增加而增加,差异具有统计学意义,同染毒处理时 HAPOE4 型小鼠 MOD 值高于 WT 型小鼠,差异具有统计学意义,p－tau(ser199)、p－tau(ser396)基因型与染毒之间有交互作用。(见图 8)

3　讨　论

BaP 作为多环芳烃家族的代表性物质,在焦炉工业等行业中均有大量排放[14]。然而,已有研究证实 BaP 具有神经毒性。BaP 与多种神经退行性疾病有关[15]。APOE4 是 AD 的高度风险因素,与常见的 APOE2/3 等位基因相比,APOE4 等位基因增加了 AD 的发病风险[16]。本次实验结果显示,经过三个月的 BaP 亚慢性染毒处理,WT 型与 HAPOE4 型小鼠的学习记忆能力及抑郁状态均受到影响,并且这种影响具有一定剂量与基因型依赖性。具体表现为,随着染毒剂量的升高,小鼠的学习记忆能力进一步下降,抑郁状态进一步加重,并且 HAPOE4 型小鼠学习记忆能力

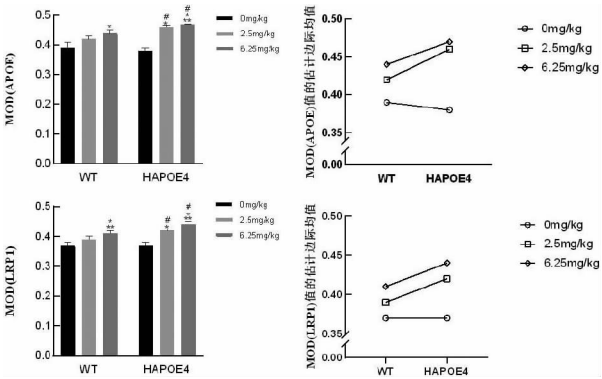


图 6　各组小鼠的 APOE、LRP1 免疫组织化学及其交互作用结果

Fig. 6　The results of immunohistochemistry of APOE and LRP1 in mice of each group and the interaction

下降与抑郁状态均更为严重。人群暴露 BaP 一般方式为口鼻吸入，本次实验采用腹腔注射方式染毒且染毒剂量不够全面，结论类推到人时存在一定局限性。

APOE 在大脑神经胶质 - 神经元交流中起着至关重要的作用，主要负责将胆固醇为主的脂质从神经胶质细胞转运至神经元中[17]，再经由神经元表面的 LRP1 等关键受体转运至神经元胞内并参与相关生理活动[18]。APOE4 与 APOE3 载体构成的不同是导致 Aβ 沉积以及 tau 蛋白异常磷酸化的关键因素[19]。LRP1 在脑的不同区域高度表达，并作为 APOE 的主要受体发挥作用[20]。研究表明，LRP1 可能通过抑制磷脂酰肌醇 3 - 激酶（phosphoinositide 3 - kinase，PI3K）/蛋白激酶 B（protein kinase B，Akt）信号通路从而激活介导 tau 磷酸化的糖原合成酶激酶 - 3（glycogen synthase kinase 3β，GSK - 3β）通路并最终促使 tau 蛋白异常磷酸化[11]。本次研究结果提示随着染毒剂量的增加，小鼠海马区 APOE 与 LRP1 均表达增加，并且 HAPOE4 型小鼠表达量更高，且染毒与基因型之间均具有交互作用，这提示 BaP 发挥毒性作用的过程中很可能参与调控了 APOE 基因的转录表达等过程，有待进一步实验证明。

tau 蛋白过度磷酸化是多种神经退行性疾病的共同病理表现和标志，多种神经毒物都可引起 tau 蛋白磷酸化水平升高[21]。p - tau（ser199）、p - tau（ser396）均是 tau 蛋白磷酸化的典型位点[22]，本次研究结果提示随着染毒剂量增加，小鼠海马区 tau、p - tau（ser199）、p - tau（ser396）均表达增加，并且 HAPOE4 型小鼠表达量更高，且染毒与基因型之间均

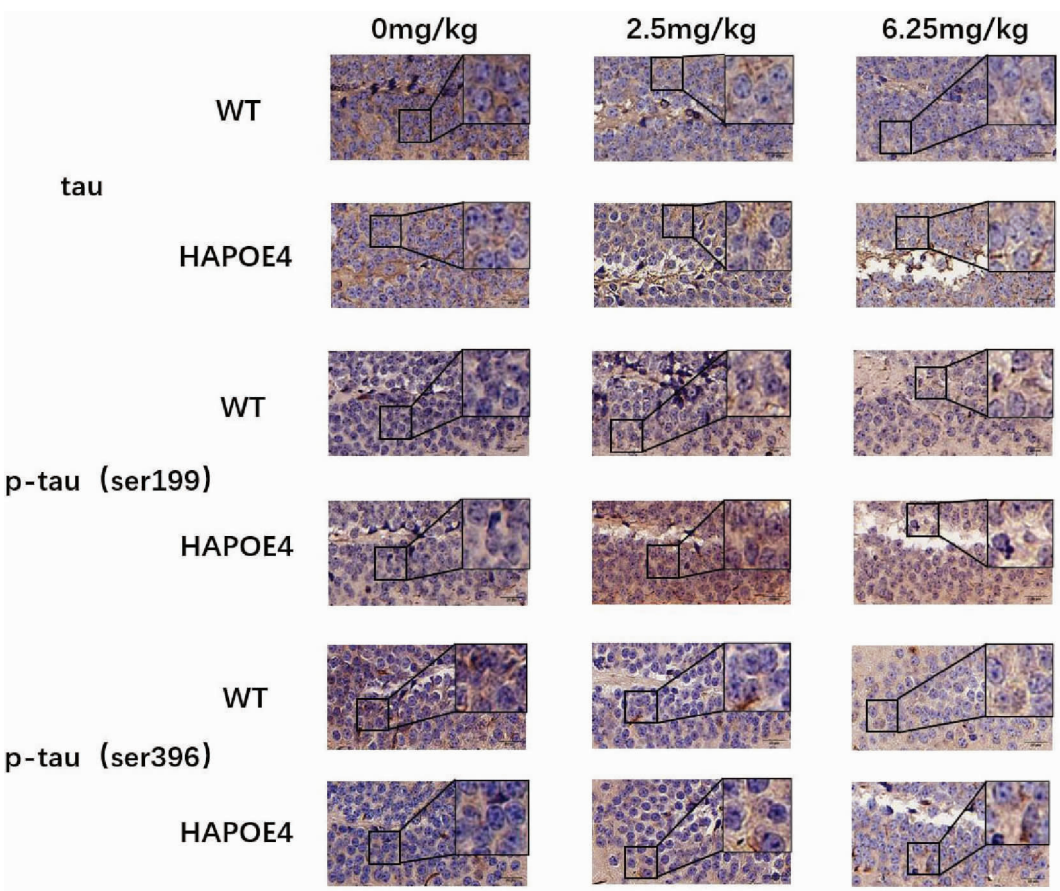


图 7　各组小鼠的 Tau、p - tau（ser199）、p - tau（ser396）免疫组织化学图像（DAB 染色，×40）

Fig. 7　Immunohistochemical images of Tau, p - tau (ser199), and p - tau (ser396) in mice of each group (DAB staining, ×40)

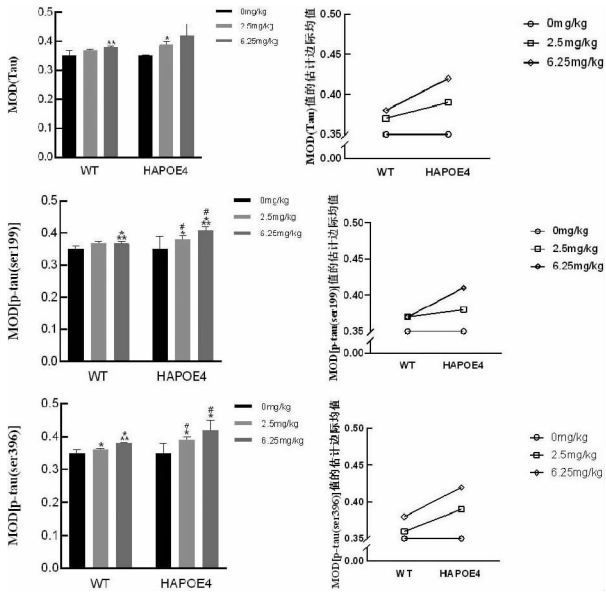


图 8　各组小鼠的 Tau、p－tau(ser199)、p－tau(ser396)免疫组织化学及其交互作用结果

Fig. 8　The results of immunohistochemistry of Tau, p－tau (ser199) and p－tau (ser396) in mice of each group and the interaction

具有交互作用,这提示 BaP 与 HAPOE4 基因型都促进了小鼠 tau 蛋白异常磷酸化的发生,交互作用的存在提示二者之间可能具有某种相互调控关系。免疫组织化学结果分析时可能因选取区域不同而造成主观差异,存在一定局限性。

神经原纤维缠结(NFTs)是指 AD 患者大脑皮质细胞的一种病理变化,出现 NFTs 提示神经退行性变处于较晚期状态[23－24]。甘氨酸银浸染色结果提示随着染毒剂量的增加,小鼠海马区染色明显变深变多,且 HAPOE4 型小鼠更为明显,提示出现了 NFTs,模型出现了晚期神经退行性变的典型病理改变。但本次实验仅用染色程度作为评判标准,存在局限性,应考虑在下一步设计中引入定量或半定量分析方法。

综上所述,亚慢性 BaP 染毒会促使 WT 型与 HAPOE4 型小鼠 tau 蛋白异常磷酸化、进一步形成 NFTs,最终导致小鼠出现学习记忆能力下降、抑郁状态等轻度认知功能障碍典型症状。本研究结果提示,在 BaP 亚慢性染毒与 APOE4 基因联合作用下,最终可造成小鼠轻度认知功能障碍,这提示 BaP 进入生物体内后可能通过某些途径参与了 APOE 的相关调控转录等过程并上调 APOE 与 LRP1 表达,进一步使小鼠出现 tau 蛋白异常磷酸化等病理改变,最终导致小鼠出现轻度认知功能障碍。

利益冲突声明　本研究不存在任何利益冲突

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