·基础研究·

EGCG阻断HDAC6-PI3K/AKT/mTOR轴以激活自噬促进小胶质细胞清除Aβ

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摘　要：【目的】为明确表没食子儿茶素没食子酸酯（EGCG）是否参与小胶质细胞对β-淀粉样蛋白（Aβ）的清除和自噬诱导，以探讨EGCG在防治阿尔茨海默病（AD）的潜在机制。【方法】将6月龄APP/PS1小鼠随机分为模型组和EGCG组，另将野生型小鼠作为对照组，每组15只，EGCG组持续灌胃给药[5 mg/(kg·d)]8周后，进行旷场实验及Y迷宫检测小鼠学习记忆能力，硫磺素-S染色评价小鼠脑实质中Aβ的含量及分布，免疫荧光检测小鼠海马组织$Aβ_{1-42}$、胶质纤维酸性蛋白（GFAP）、离子钙结合适配器分子1（Iba1）表达水平；同时予20 μmol/L $Aβ_{1-42}$诱导N9小鼠胶质细胞模型，检测不同浓度EGCG（5 μmol/L、10 μmol/L、20 μmol/L）处理后的细胞活力，Western blot检测$Aβ_{1-42}$、低密度脂蛋白受体相关蛋白1（LRP1）、晚期糖基化终末产物受体（RAGE）、淀粉样前体蛋白（APP）、胰岛素降解酶（IDE）、脑啡肽酶（NEP）、微管相关蛋白1轻链3（LC3）-Ⅱ/LC3-Ⅰ、磷脂酰肌醇-3-羟激酶（PI3K）、p-PI3K、蛋白激酶B（AKT）、p-AKT、哺乳动物雷帕霉素靶蛋白（mTOR）、p-mTOR、组蛋白去乙酰化酶6（HDAC6）水平；最后通过小胶质细胞与神经元SH-SY5Y细胞共培养，检测细胞活力及Caspase-3水平，验证EGCG介导Aβ清除对神经元的保护作用。【结果】EGCG增加APP/PS1小鼠在旷场中央区域活动时间及次数（$P<0.05$），提高Y迷宫测试交替百分比（$P<0.01$）；EGCG减少APP/PS1小鼠海马组织中Aβ沉积，增加小胶质细胞数量；体外实验显示EGCG可提高Aβ诱导N9细胞的存活率（$P<0.01$），并上调RAGE活性（$P<0.05$），促进Aβ的内化吞噬（$P<0.01$），通过下调HDAC6水平（$P<0.05$），抑制PI3K、AKT、mTOR的磷酸化（$P<0.001$）而增加LC3-Ⅱ/LC3-Ⅰ比值（$P<0.001$）激活小胶质细胞自噬；EGCG通过小胶质细胞对$Aβ_{1-42}$的清除，提高SH-SY5Y细胞存活率（$P<0.05$），降低Caspase-3的活性（$P<0.01$），对神经元具有保护作用。【结论】EGCG通过靶向阻断HDAC6-PI3K/AKT/mTOR轴，激活小胶质细胞自噬清除Aβ。

关键词：表没食子儿茶素没食子酸酯；阿尔茨海默病；β-淀粉样蛋白；小胶质细胞；自噬；组蛋白去乙酰化酶6
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EGCG Promotes Aβ Clearance of Microglia Through Blockage of the HDAC6-PI3K/AKT/mTOR Signalling Axis Followed by Autophagy Activation

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Abstract:【Objective】To clarify whether epigallocatechin gallate (EGCG) is involved in the clearance of amyloid β-protein (Aβ) and autophagy induction by microglia, so as to explore the potential mechanisms of EGCG in the prevention

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and treatment of Alzheimer's disease (AD).【Methods】Six-month-old APP/PS1 mice were randomly divided into model and EGCG groups, with some additional wild type (WT) mice as the control group, each group consisting of 15 mice. The EGCG group received continuous gavage administration[5 mg/(kg·d)] for 8 weeks, followed by the open field test and Y-maze to assess the learning and memory abilities of the mice. Thioflavin-S staining was used to evaluate the content and distribution of amyloid β-protein (Aβ) in the brain parenchyma of the mice, and immunofluorescence was employed to detect the expression levels of $Aβ_{1-42}$, glial fibrillary acidic protein (GFAP), and ionized calcium-binding adapter molecule 1 (Iba1) in the hippocampal tissue of the mice. Additionally, N9 mouse microglial cells were induced with 20 μmol/L $Aβ_{1-42}$, and the cell viability was measured after treatment with different concentrations of EGCG (5 μmol/L, 10 μmol/L, 20 μmol/L). Western blotting was used to detect the levels of $Aβ_{1-42}$, low density lipoprotein receptor-related protein 1 (LRP1), receptor for advanced glycation endproducts (RAGE), amyloid precursor protein (APP), insulin degrading enzyme (IDE), neprilysin (NEP), microtubule associated protein 1 hydrogen chain 3 (LC3)-Ⅱ/LC3-Ⅰ, phosphatidylinositol 3-hydroxy kinase (PI3K), p-PI3K, protein kinase B (AKT), p-AKT, mammalian target of rapamycin (mTOR), p-mTOR, and histone deacetylase 6 (HDAC6). Finally, through the co-culture of microglial cells and neuronal SH-SY5Y cells, cell viability and Caspase-3 levels were measured to verify the protective effect of EGCG-mediated Aβ clearance on neurons.【Results】EGCG increased the activity time and frequency of APP/PS1 mice in the central area of the open field (P<0.05), and enhanced the percentage of alternation in the Y-maze test (P<0.01); EGCG reduced Aβ deposition in the hippocampal tissue of APP/PS1 mice and increased the number of microglia; *in vitro* experiments showed that EGCG improved the survival rate of Aβ-induced N9 cells (P<0.01), upregulated RAGE activity (P<0.05), and promoted the internalization and phagocytosis of Aβ (P<0.01). ECGC activated microglial autophagy by downregulating the level of HDAC6 (P<0.05), inhibiting the phosphorylation of PI3K, AKT, mTOR (P<0.001), and increasing the LC3-Ⅱ/LC3-I ratio (P<0.001); EGCG improved the survival rate of SH-SY5Y cells (P<0.05) and reduced the activity of Caspase-3 (P<0.01) by clearing $Aβ_{1-42}$ through microglia, and had a protective effect on neurons.【Conclusion】EGCG activates microglial autophagy to clear Aβ by targeting and inhibiting the HDAC6-PI3K/AKT/mTOR axis.

Key words: epigallocatechin gallate; Alzheimer's disease; amyloid β-protein; microglia; autophagy; histone deacetylase 6

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β-淀粉样蛋白(amyloid β-protein,Aβ)由淀粉样前体蛋白(amyloid precursor protein,APP)经酶的特定切割生成,其在脑内的异常沉积形成斑块是阿尔茨海默病(Alzheimer's disease,AD)的核心病理特征之一[1-3]。其中$Aβ_{1-42}$因强聚集倾向和高神经毒性成为关键致病因子,不仅直接损害神经元功能,更能触发神经炎症反应和氧化应激级联,形成恶性循环加速神经退行进程[4-6]。因此清除$Aβ_{1-42}$及其聚合物成为治疗AD的研究热点[7-8]。小胶质细胞作为中枢神经系统内固有免疫细胞,在受到$Aβ_{1-42}$的神经毒性刺激后,会激活并迅速增殖,同时增强吞噬活性,以清除有害物质,保护神经元免受损害[9]。然而近几年研究揭示,在AD病理环境下,激活的小胶质细胞一方面可以维持对Aβ的吞噬清除能力,另一方面又可过度释放炎症因子导致神经微毒性环境形成,这种功能双重性提示精准调控小胶质细胞活性可能成为Aβ清除治疗的关键节点[10-11]。自噬作为维持细胞内稳态的核心机制,不仅参与神经元内异常蛋白的清除,更通过调节小胶质细胞的吞噬功能影响Aβ代谢过程[12-13]。表没食子儿茶素没食子酸酯(epigallocatechin gallate,EGCG)已知具有抗癌[14-15]、抗炎和神经保护作用等广泛的药理特性,在多种神经退行性疾病中具有潜在治疗能力[16-17]。我们先前研究表明,EGCG可通过上调神经元细胞中脑啡肽酶(neprilysin,NEP)的表达,减轻AD患者的认知功能衰退[18]。然而,EGCG对AD进展中小胶质细胞生物学功能的影响以及相关的细胞机制仍不清楚。在本研究中,我们试图确定EGCG是否参与小胶质细胞对Aβ的清除和自噬诱导,并研究其在阿尔茨海默病中的神经保护特性。

1　材料与方法

1.1　实验动物

APP/PS1双转基因小鼠和野生型雄性小鼠(3月龄)购自北京维通利华公司(中国北京)。小鼠饲

养于广州医科大学按照机构动物护理和使用委员会(IACUC)批准的方案,并依据机构指南进行饲养,饲养至6月龄时开始实验。在小鼠6月龄时,将APP/PS1小鼠随机分为模型组和EGCG组,野生型小鼠作为对照组,每组15只。EGCG组每天灌胃给予EGCG(5 mg/kg,Sigma-Aldrich,989-51-5),对照组和模型组予同体积的生理盐水,持续给药8周。

1.2 旷场实验

灌胃给药结束后,在安静和暗光环境下,将小鼠置于50 cm×50 cm×50 cm规格的隔音暗箱内中央,观察并记录30 min内小鼠自由活动的轨迹。每次测试结束后,使用体积分数75%酒精清洁旷场箱,避免气味对下一只小鼠的影响。通过欣软软件(上海欣软信息科技有限公司)将旷场箱分为25个象限,测量活动总距离(cm)和在9个中央象限停留时间(sec)评估小鼠在新环境中的自主行为、探索行为和焦虑状态。

1.3 Y迷宫实验

安静环境下将小鼠置于臂长35 cm、宽5 cm、高15 cm规格的Y迷宫中,任其自由探索5 min,记录小鼠的活动路径、总移动距离及进入各个开放臂探索的次数,以评估小鼠的学习记忆能力。

1.4 硫磺素S染色

小鼠行为学测试结束后禁食禁水12 h,麻醉后取出全脑,置于40 g/L多聚甲醛固定液中固定后脱水透明,石蜡包埋后切片风干,脱蜡后使用硫磺素S(TH-S)染色。利用TH-S自带绿色荧光,且与成熟Aβ特异性结合的能力,评价脑实质中淀粉样蛋白的含量及分布。

1.5 免疫荧光检测

固定的大脑切片脱蜡水化后,使用PBS清洗5 min,室温下使用含体积分数0.2%Triton X-100(Sigma-Aldrich,T8787)的5%山羊血清封闭30 min,4 ℃下一抗孵育过夜,次日PBS洗涤后与荧光二抗室温孵育1 h,使用DAPI对细胞核染色,封片后使用荧光显微镜获取图像。

1.6 细胞培养

N9小鼠小胶质细胞和SH-SY5Y细胞系由广州中医药大学科技创新中心提供。使用含10%胎牛血清和1%青霉素-链霉素的DMEM培养基,于37 ℃、体积分数5%CO_2、90%湿度细胞培养箱中常规培养,隔天换液一次。为研究EGCG对小胶质细胞清除Aβ的影响,使用20 μmol/L $Aβ_{1-42}$和不同浓度的ECGC(5 μmol/L、10 μmol/L、20 μmol/L)处理N9细胞24 h。为验证EGCG是否通过小胶质细胞清除Aβ来保护神经元,收集经20 μmol/L $Aβ_{1-42}$和20 μmol/L ECGC处理N9细胞24 h后的上清液,用以培养SH-SY5Y细胞。

1.7 MTT法检测细胞活力

将N9细胞或SH-SY5Y细胞以每孔5 000个细胞的密度接种于96孔板中,使用含体积分数10%胎牛血清的DMEM培养基培养24 h后,弃去原培养液,按各实验需求予相应药物处理24 h,随后每孔加入0.5 mg/mL浓度的MTT溶液孵育4 h,弃上清后每孔加入150 μL DMSO溶液,室温低速震荡10 min以充分溶解结晶物。使用酶标仪测量570 nm处的OD值,并换算细胞活力。

1.8 免疫印迹实验检测相关蛋白表达量

免疫印迹(Western blot)实验操作参考已发表文章[19]。用含蛋白酶抑制剂和磷酸酶抑制剂的RIPA裂解液对脑组织或细胞样品进行裂解并提取蛋白质。BCA定量试剂盒检测蛋白浓度。根据目的蛋白的分子量,选用适当浓度的聚丙烯酰胺凝胶进行蛋白电泳,然后转移到PVDF膜中,置于5%脱脂牛奶封闭2 h,一抗4 ℃孵育过夜后二抗室温孵育1 h,洗膜后进行ECL显影,并用Image J分析灰度值。

1.9 统计分析

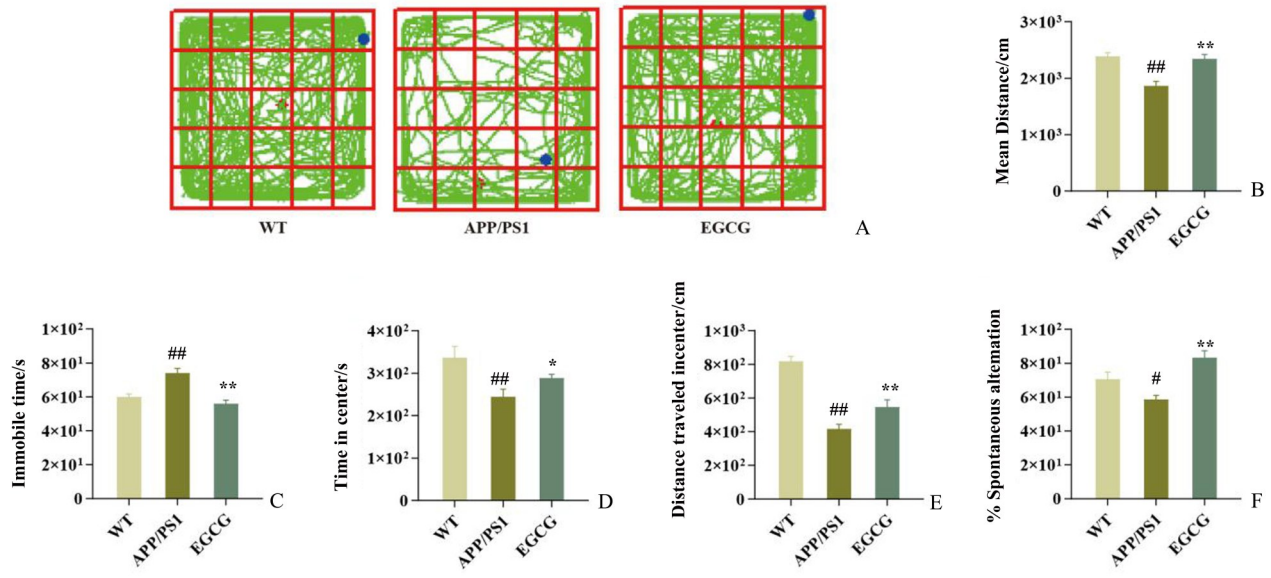
实验数据使用GraphPad Prism软件(版本10.1.2)进行统计,以均值±标准差的形式呈现。数据分布特性通过Shapiro-Wilk正态性检验评估,符合正态分布的数据采用单因素方差分析(ANOVA)模型配合Tukey多重比较法进行组间差异显著性判定;若数据偏离正态性假设,采用Kruskal-Wallis非参数秩和检验进行统计。$P<0.05$被视为具有统计学意义。

2 结果

2.1 EGCG减轻APP/PS1小鼠的认知障碍

为评估EGCG对APP/PS1小鼠认知障碍的影响,在EGCG处理后进行旷场实验和Y迷宫行为测试。旷场实验结果显示,与野生型小鼠相比,APP/PS1组小鼠的平均移动距离明显下降($P<0.01$),静止时间增加($P<0.01$),而EGCG处理显著逆转了

这些影响（$P<0.01$；图 1A-1C）。APP/PS1 组小鼠在中央区域活动的时间及次数也明显减少（$P<0.01$），EGCG 处理能够消除这些影响（图 1D-1E）。因此，EGCG 可增加 APP/PS1 小鼠在陌生环境中的自主和探索行为。在随后的 Y 迷宫测试中，APP/PS1 小鼠的交替百分比低于野生型小鼠（$P<0.05$），经 EGCG 干预可显著提高（$P<0.01$；图 1F）。以上结果共同表明，EGCG 可以改善 APP/PS1 小鼠的焦虑状态和学习记忆认知障碍。



A-E: The open field test evaluated the spontaneous behaviors of wild type (WT) mice, APP/PS1 mice, and Epigallocatechin gallate (EGCG)-treated APP/PS1 mice in a novel environment. A: Movement trajectory diagrams of mice in each group. Mean distance (B) and immobile time (C) of mice (F_B=8.102, P_B=0.0011, F_C=7.766, P_C=0.001 3). Locomotor time (D) and distance travelled (E) in centre area numbers (F_D=7.018, P_D=0.002 3, F_E=7.557, P_E=0.001 6). F: Alternation percentage of mice in Y-maze test (F=7.344, P=0.002 0). n=15, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* WT group. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* APP/PS1 group.

图 1　EGCG 缓解 APP/PS1 小鼠的认知缺陷

Fig. 1　EGCG alleviated the cognitive deficit in APP/PS1 mice

2.2　EGCG 促进小胶质细胞增殖及其对 $A\beta_{1-42}$ 的内化吞噬

如图 2A 所示，与野生型小鼠相比，APP/PS1 组小鼠 TH-S 染色（绿色）及 $A\beta_{1-42}$（红色）荧光强度明显增加，EGCG 干预明显减弱，表明 ECGC 可减少 APP/PS1 小鼠脑实质中的 Aβ 沉积。此外，使用胶质纤维酸性蛋白（glial fibrillary acidic protein，GFAP）特异性标记星形胶质细胞（洋红色），离子钙结合适配器分子 1（ionized calcium-binding adapter molecule 1，Iba1）特异性标记小胶质细胞（黄色）发现，EGCG 干预可明显增加 APP/PS1 小鼠海马组织中小胶质细胞的数量，而星形胶质细胞数量未见明显改变。与体内实验结果一致，EGCG 通过保护 N9 小胶质细胞免受 $A\beta_{1-42}$ 诱导的毒性，恢复了细胞的活力（图 2B），提示 EGCG 可促进小胶质细胞增殖。随后我们分别检测 N9 细胞内外 $A\beta_{1-42}$ 的表达水平，发现 EGCG 处理可显著降低 Aβ 诱导下小胶质细胞上清液中的 $A\beta_{1-42}$ 的含量（$P<0.001$），同时增加胞内 $A\beta_{1-42}$ 水平（$P<0.01$；图 2C，第 4 列与第 5 列对比）。为评估 EGCG 在小胶质细胞内化 Aβ 过程中的影响，检测了 $A\beta_{1-42}$ 两个关键转运体低密度脂蛋白受体相关蛋白 1（low density lipoprotein receptor-related protein 1，LRP1）[20-21] 和晚期糖基化终末产物受体（receptor for advanced glycation endproducts，RAGE）[20-23] 表达水平。实验结果表明，EGCG 显著增加了 Aβ 刺激下 N9 细胞的 RAGE 水平（$P<0.05$），但 LRP1 表达未见明显改变。同时，经 siRAGE 处理后，EGCG 无法降低 Aβ 毒性刺激下的胞外 $A\beta_{1-42}$ 水平（图 2F-2I），说明 siRAGE 可消除 EGCG 对胞外 Aβ 的清除作用。以上数据意味着在 Aβ 的毒性刺激下，EGCG 不仅促进小胶质细胞的增殖，还通过提高 RAGE 受体活性，促进小胶质细胞

对$A\beta_{1-42}$吞噬。

2.3 EGCG抑制PI3K/AKT/mTOR轴以诱导小胶质细胞自噬,促进胞内$A\beta_{1-42}$降解

如图3A-3B所示,20 μmol/L Aβ1-42的诱导可增加N9细胞内APP[12, 24-26]水平($P<0.001$),但EGCG治疗未见明显变化,提示EGCG未调控小胶质细胞中$A\beta_{1-42}$的产生。结合体外实验中EGCG处理改变了Aβ诱导小胶质细胞内外$A\beta_{1-42}$水平(图2C-2E),我们推测小胶质细胞吞噬Aβ后,通过降解途径清除Aβ[27]。出乎意料的是,$A\beta_{1-42}$相关降解酶NEP[28]和胰岛素降解酶(insulin degrading enzyme, IDE)[27, 29-30]的水平未明显受到EGCG干预的影响(图3A-3D)。通过文献调研,小胶质细胞自噬在淀粉样斑块的降解中起调节作用[27, 31-33],自噬功能障碍可导致疾病进展。体内外实验结果证实,EGCG可显著增加APP/PS1小鼠脑组织及Aβ诱导N9细胞自噬活性指标微管相关蛋白1轻链3(microtubule associated protein 1 hydrogen chain 3, LC3)中LC3-Ⅱ/LC3-I的比值($P<0.001$;图3E-3H),提示EGCG干预后自噬体形成增加,激活了小胶质细胞的自噬。细胞自噬受多种信号通路调节,其中磷脂酰肌醇-3-羟激酶(phosphatidylinositol 3-hydroxy kinase, PI3K)/蛋白激酶B(protein kinase B, AKT)/哺乳动物雷帕霉素靶蛋白(mammalian target of rapamycin, mTOR)信号通路是调控自噬的经典途径[34-36],实验结果发现EGCG干预可明显减少Aβ毒性诱导下N9细胞p-PI3K、p-AKT、p-mTOR蛋白表达($P<0.001$;图3I-3O),各组间PI3K、AKT、mTOR蛋白质水平差异无明显统计学意义。提示EGCG处理后该通路受到了显著抑制。以上结果表明,EGCG通过阻断PI3K/AKT/mTOR轴并激活自噬,促进了小胶质细胞对$A\beta_{1-42}$的降解。

2.4 EGCG通过下调HDAC6促进Aβ内化和自噬,促进小胶质细胞清除$A\beta_{1-42}$

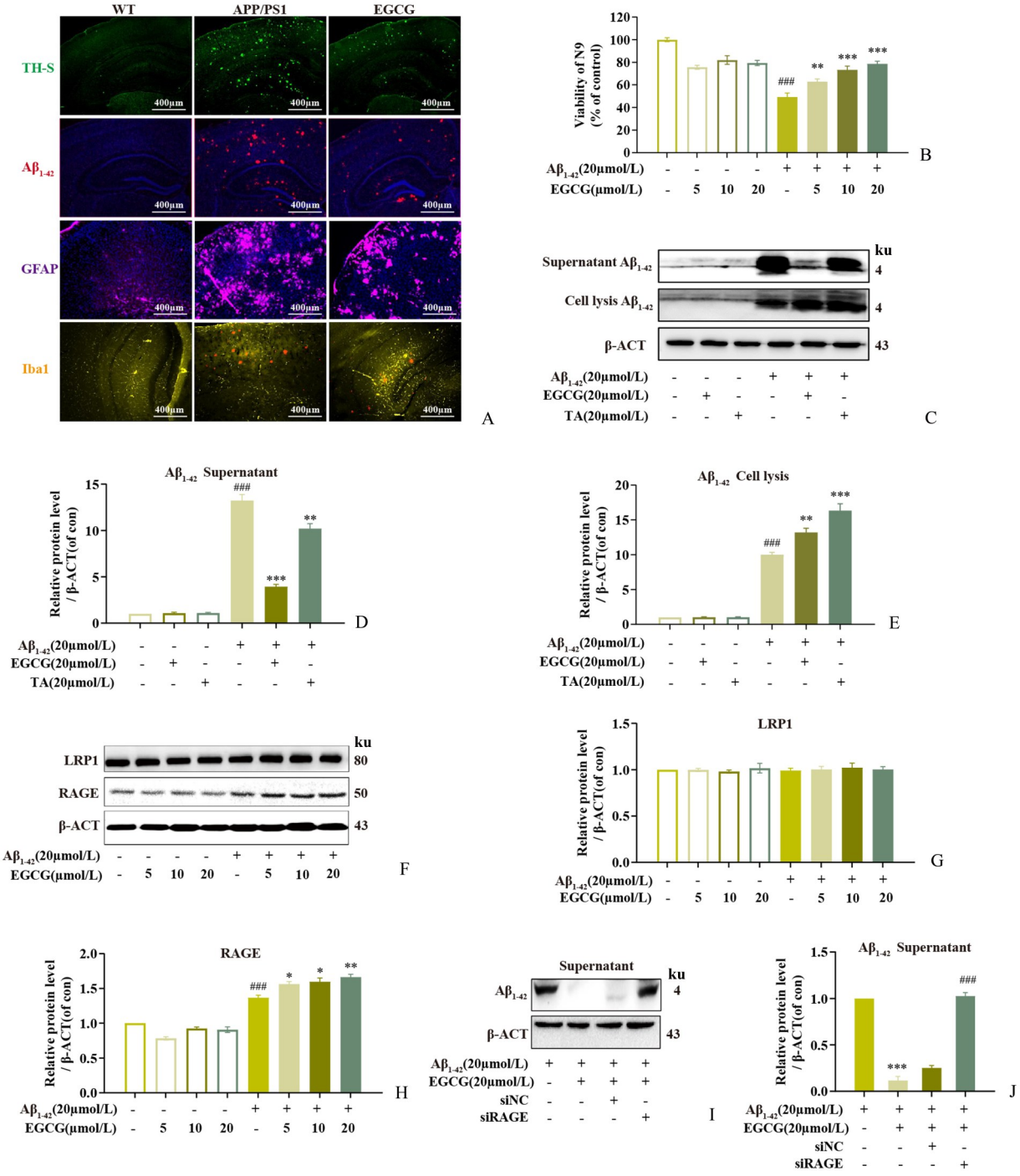
近年研究指出,抑制组蛋白去乙酰化酶6(histone deacetylase 6, HDAC6)可减少小胶质细胞极化或降低Aβ负荷[37-40],提高小胶质细胞对Aβ沉淀的内化摄取能力,改善认知障碍。实验结果如图4A-4B所示,EGCG干预可下调胶质细胞中HDAC6蛋白的表达($P<0.05$)。此外,使用HDAC6抑制剂tubastatin-A(TA)处理后,小胶质细胞培养上清液中$A\beta_{1-42}$水平降低,而在细胞内增加(图2C,第4列与第6列)。并且,与EGCG一致,TA显著激活了小胶质细胞的自噬显著提高LC3-Ⅱ/LC3-I的比值($P<0.001$;图4C-4D),激活了小胶质细胞的自噬。以上结果表明,EGCG通过抑制HDAC6表达,不仅促进小胶质细胞对$A\beta_{1-42}$的内化吞噬,还刺激细胞自噬,促进AD中$A\beta_{1-42}$的消除。随后我们验证了AKT/mTOR通路在TA诱导自噬中的参与,结果证实HDAC6阻断显著减少了AKT、mTOR的磷酸化($P<0.01$;图4E-4I)。综合来看,我们展示了EGCG通过下调HDAC6表达以促进$A\beta_{1-42}$的内化吞噬、激活自噬以及随后小胶质细胞清除$A\beta_{1-42}$的过程。

2.5 EGCG通过促进小胶质细胞清除$A\beta_{1-42}$,减少神经元损伤

为验证EGCG是否通过小胶质细胞清除Aβ来保护神经元,我们收集经20 μmol/L $A\beta_{1-42}$和20 μmol/L ECGC处理N9细胞24 h后的上清液,用以培养SH-SY5Y细胞。结果表明,当与N9条件培养基共培养时,EGCG消除了$A\beta_{1-42}$对SH-SY5Y细胞的毒性($P<0.05$;图5A)。此外与EGCG作用一致,TA对HDAC6的阻断也保护了SH-SY5Y细胞免受$A\beta_{1-42}$介导的毒性($P<0.05$)。此外,EGCG显著降低了Aβ诱导N9细胞中天冬氨酸特异性的半胱氨酸蛋白水解酶-3(cysteine-aspartate-specific protease-3, Caspase-3)的活性($P<0.01$;图5B,第4列与第5列对比),表明它通过小胶质细胞介导的清除作用,保护SH-SY5Y细胞免受$A\beta_{1-42}$毒性的侵害。HDAC6抑制剂TA在共培养中也降低了Caspase-3的活性($P<0.001$;图5B,第4列与第6列对比)。综上所述,EGCG通过小胶质细胞对$A\beta_{1-42}$的清除,保护了神经元免受$A\beta_{1-42}$毒性的侵害。

3 讨论

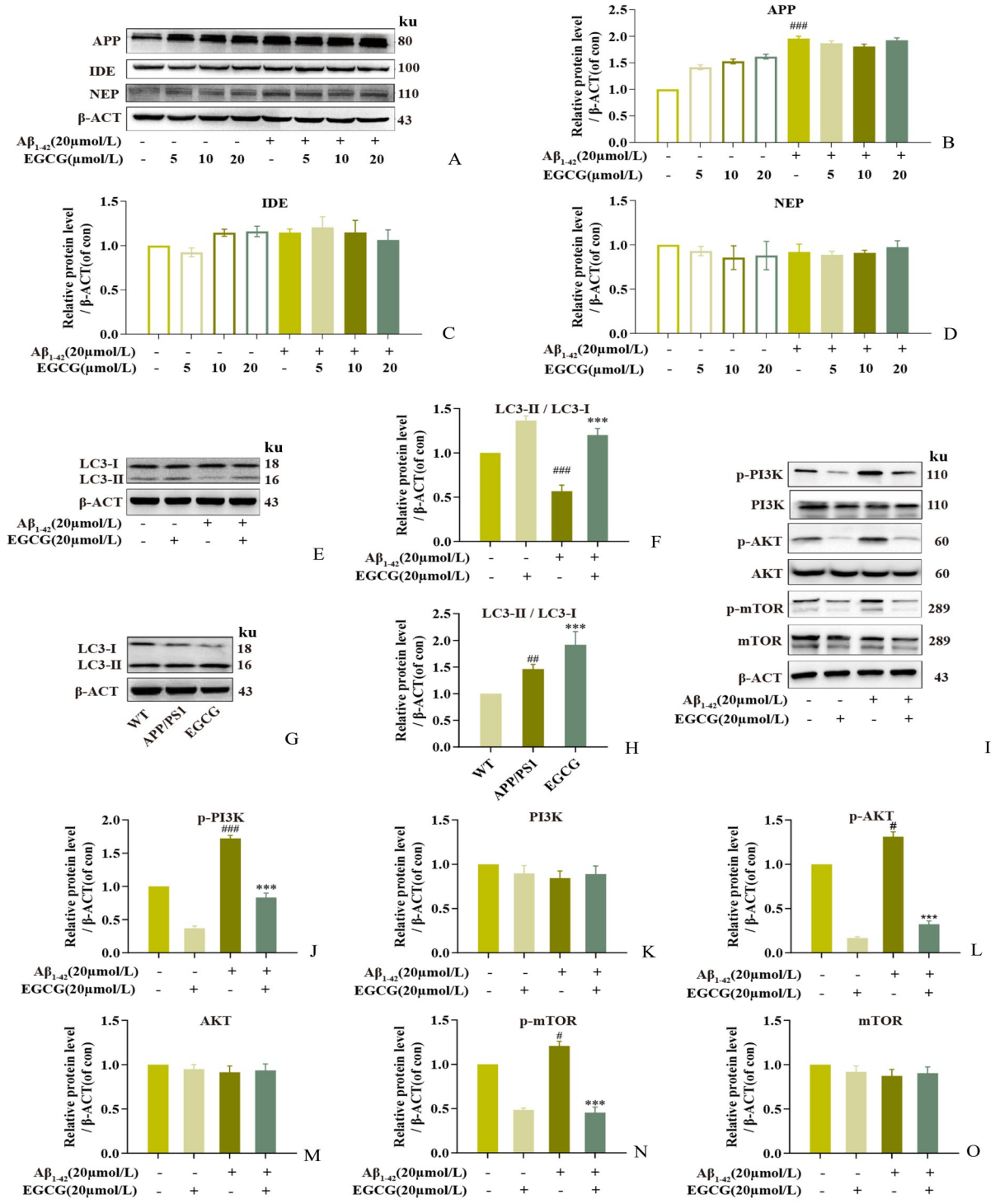
作为AD的核心致病因子,Aβ的病理生成源于APP经β-和γ-分泌酶异常切割的代谢失衡[1-3]。值得注意的是,Aβ在脑内的病理沉积不仅与其过度生成相关,更与溶酶体-蛋白酶体-自噬系统等蛋白清除机制的代偿性衰退形成恶性循环[12-13, 32]。相较于Aβ生成速率的改变,清除系统功能障碍已被证实是晚发型AD进展的主要驱动因素[41-44],这使得增强Aβ清除效能的治疗策略成为当前药物研发的重要方向。在众多潜在治疗靶点中,基于自噬调控的清除机制修复显示出独特优势。我们最新



A: Immunofluorescence and TH-S staining of the hippocampus tissue of APP/PS1 mice treated with EGCG. Mice hippocampus tissues were collected for immunofluorescence and TH-S staining to detect $A\beta_{1-42}$ deposition, astrocytes, and microglia. GFAP (Dyed Magenta) and Ibal (Dyed Yellow) were biomarkers of astrocytes and microglia, respectively. Scale bar: 400μm. B: The cell viability of N9 microglial cells after treatment with EGCG and $A\beta_{1-42}$ for 24 hours (F=29.82, P < 0.000 1). C-E: The cell culture supernatant and lysate of microglia cells treated with EGCG and HDAC6 inhibitor (tubastatin-A, TA) were collected for western blotting to detect $A\beta_{1-42}$ levels. Representative of 3 independent experiments (F_D=64.97, P_D < 0.000 1, F_E=198.0, P_E < 0.000 1). F-H: LRP1 and RAGE levels in EGCG-treated microglia (F_G=0.171 9, P_G=0.987 5, F_H=117.6, P_H < 0.000 1). I-J: $A\beta_{1-42}$ levels in EGCG-treated of siRAGE microglia culture supernatant (F=189.2, P < 0.000 1). n=3, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 *vs.* Control group. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs.* $A\beta_{1-42}$ group.

图2　EGCG促进小胶质细胞增殖并上调对$A\beta_{1-42}$的内化摄取

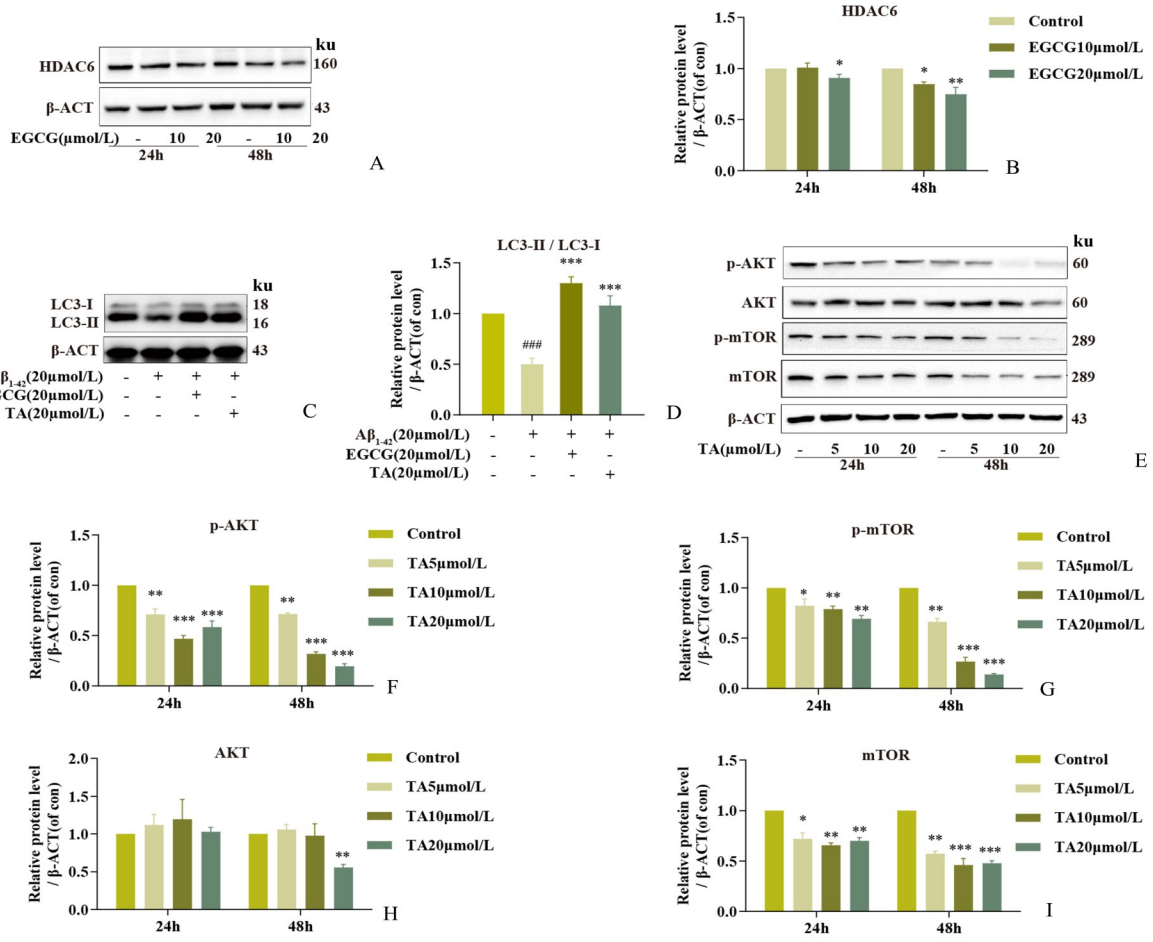
Fig. 2　EGCG promoted the proliferation of microglia and upregulated extracellular $A\beta_{1-42}$ uptake



A-D: APP, neprilysin, and insulysin levels in microglial cells treated with EGCG and $A\beta_{1-42}$ for 24 h were detected by Western blotting (F_B=58.96, P_B < 0.000 1, F_C=1.311, P_C=0.307 2, F_D=0.304 1, P_D=0.941 7). E-H: LC3 Ⅱ/Ⅰ levels in microglia and APP/PS1 mice after EGCG treatmentEGCG promoted autophagy of microglia (F_F=50.77, P_F < 0.000 1, F_H=83.53, P_H < 0.000 1). I-O: The PI3K/AKT/mTOR pathway was blocked in microglial cells treated with EGCG (F_J=160.1, P_J < 0.000 1, F_K=0.748 6, P_K=0.553 0, F_L=104.0, P_L < 0.000 1, F_M=0.403 7, P_M=0.754 5, F_N=79.91, P_N < 0.000 1, F_O= 0.797 8, P_O=0.528 9). Representative of 3 independent experiments. n=3, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 *vs.* Control group or WT group. $^{***}P$<0.001 *vs.* $A\beta_{1-42}$ group or APP/PS1 group.

图3 EGCG诱导小胶质细胞自噬并阻断PI3K/AKT/mTOR通路

Fig. 3 EGCG induced autophagy of microglia and blockage of the PI3K/AKT/mTOR pathway



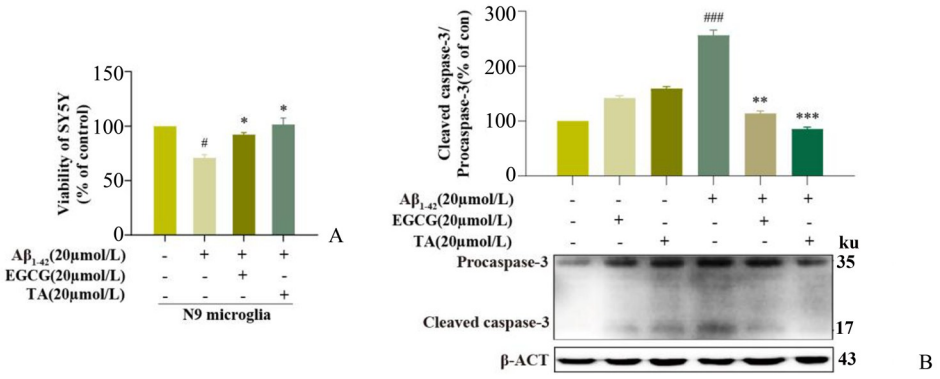
A–B: HDAC6 level significantly decreased in microglia treated with EGCG for 24 and 48 h (F_{24h}=8.194, P_{24h}=0.019 3, F_{48h}=13.73, P_{48h}=0.005 8). C–D: LC3 Ⅱ/Ⅰ level in microglial cells treated with tubastatin-A for 24 h (F=28.10, P=0.000 1). n=3, $^{###}P$<0.001 *vs*. Control group. $^{***}P$<0.001 *vs*. $Aβ_{1-42}$ group. E–I: AKT/mTOR pathway was downregulated in microglia treated with tubastatin-A for 24 and 48 h. Representative of 3 independent experiments (F_{F24h}=36.33, P_{F24h} < 0.000 1, F_{F48h}=182.8, P_{F48h} < 0.000 1, F_{G24h}=12.38, P_{G24h}=0.002 2, F_{G48h}=117.7, P_{G48h} < 0.000 1, F_{H24h}=1.056, P_{H24h}= 0.419 7, F_{H48h}=11.34, P_{H48h}=0.003 0, F_{I24h}=8.137, P_{I24h}=0.008 2, F_{I48h}=20.87, P_{I48h}=0.000 4). n=3, $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs*. Control group.

图4　EGCG下调HDAC6促进小胶质细胞内化$Aβ_{1-42}$及自噬清除

Fig. 4　EGCG promoted $Aβ_{1-42}$ uptake and autophagy to mediate clearance of $Aβ_{1-42}$ in microglia through HDAC6 downregulation

研究揭示了EGCG-HDAC6-PI3K/AKT/mTOR信号轴对小胶质细胞自噬活性的特异性调控作用，并表明靶向这一信号通路对Aβ清除具有积极意义。

EGCG是一种天然的黄烷醇，因其对癌症、血管系统、心血管疾病和神经系统的潜在影响而被广泛研究[45-48]。实验研究证实，EGCG可改善AD患者的空间认知学习能力，并减少脑淀粉样变性[49-51]，这与我们的研究结果一致（图1）；然而，对于EGCG在小胶质细胞中的作用却知之甚少。小胶质细胞是中枢神经系统中的常驻免疫细胞，起源于胚胎卵黄囊中的髓系祖细胞[9]，由于其与淀粉样斑块的密切关联，在AD的背景下具有不同的功能。许多研究人员认为，在AD的各个阶段，Aβ被小胶质细胞吞噬以维持体内平衡；另一方面，Aβ也会引发小胶质细胞的炎症反应，从而影响AD的进展[11]。然而，只有少数研究关注EGCG介导的对Aβ诱导的小胶质细胞炎症和神经毒性的减轻作用，以及对神经元损伤的保护作用[52-55]。目前尚无研究报道EGCG对小胶质细胞清除Aβ的调节作用。本研究增进了我们对EGCG与小胶质细胞清除Aβ之间关系的了解，但未涉及EGCG对Aβ诱导的小胶质细胞炎症的影响。我们的数据不仅揭示了EGCG在促进小胶质细胞增殖以及激活Aβ清除方面的作用，还表明HDAC6-PI3K/AKT/mTOR信号轴是治疗小胶质



A: The viability of neurons. The conditioned medium from N9 cells treated with $A\beta_{1-42}$ and EGCG was collected, centrifuged, and used for culturing SH-SY5Y cells, which were then subjected to the MTT assay (F=5.456, P=0.024 5). B: The Caspase-3 activity in SH-SY5Y cells cultured with the conditioned medium from N9 cells treated with $A\beta_{1-42}$ and EGCG for 24 h was detected by western blotting (F=9.718, P=0.000 7). Representative of 3 independent experiments. n=3, $^{\#}P$<0.05, $^{\#\#\#}P$<0.001 *vs*. Control group. $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs*. $A\beta_{1-42}$ group.

图5 EGCG促使小胶质细胞清除$A\beta_{1-42}$保护SH-SY5Y细胞免受Aβ毒性损害

Fig. 5 EGCG protected SH-SY5Y cells from Aβ-mediated toxicity via clearance of $A\beta_{1-42}$ by microglia

细胞以清除阿尔茨海默病中Aβ的潜在靶点。

吞噬作用是一种在进化上保守的机制,细胞通过该机制识别、吞噬并在溶酶体中降解细胞外物质,在某种程度上,这一途径与自噬类似[56-59]。自噬是一种细胞质自我降解过程,它可循环利用细胞内成分从而生成生物能量。吞噬作用和自噬通过降解有害的细胞内外物质,在维持细胞和组织的内环境稳定方面都发挥着重要作用。许多研究都强调了自噬和吞噬作用在小胶质细胞清除Aβ过程中的作用[12, 13]。有趣的是,最近的证据表明,在外周巨噬细胞中,自噬和吞噬作用之间存在功能性的相互作用[60]。然而,尚无研究深入评估自噬在小胶质细胞的吞噬摄取和/或降解过程中的作用。本研究强调了EGCG在小胶质细胞培养基中减少Aβ的作用。RAGE作为跨膜受体,其稳定性受泛素-蛋白酶体系统调控。EGCG可通过直接与细胞外Aβ结合形成RAGE-Aβ复合物,依赖内吞作用进入细胞,或激活信号通路及炎症调节等多途径促进小胶质细胞摄取Aβ[22]。EGCG是绿茶中一种主要儿茶素,具有抗氧化和其他生物活性,可能通过减少氧化应激介导RAGE的降解[23],延长其半衰期,从而提高RAGE水平,促进小胶质细对Aβ的摄取,以清除胞外毒性蛋白,维持细胞内环境稳态,保护神经元(图2C-2I,图5)。

此外,研究还发现EGCG激活了小胶质细胞的自噬(图3B-3C),这表明自噬和吞噬作用之间的功能性相互作用也可能发生在小胶质细胞中。在EGCG处理后,小胶质细胞中的Aβ是仅通过自噬降解,还是通过自噬和吞噬作用的协同机制降解,目前尚不清楚。PI3K/AKT/mTOR轴作为自噬调控的经典信号通路,我们的研究结果证实了EGCG可减少Aβ毒性诱导下小胶质细胞PI3K、AKT、mTOR蛋白的磷酸化,通过下调PI3K/AKT/mTOR轴诱导小胶质细胞自噬(图3)。EGCG的酚羟基结构可直接与PI3K的ATP位点结合,抑制其催化活性,减少第二信使磷脂酰肌醇-3, 4, 5-三磷酸(phosphatidylinositol 3, 4, 5-trisphosphate, PIP3)的生成,从而阻断AKT的膜定位和激活[61]。同时,EGCG还可阻断磷酸肌醇依赖性激酶1(phosphoinositide dependent protein kinase 1, PDK1)对AKT的Thr308位点磷酸化[62],以及哺乳动物雷帕霉素靶蛋白复合物2(mTORC2)[63]对AKT的Ser473位点磷酸化,降低AKT的活性,进而抑制其下游效应分子。还有研究表明,EGCG下调哺乳动物雷帕霉素靶蛋白复合物1(mTORC1)的活性[63],并抑制其下游靶点如S6K的磷酸化,从而抑制蛋白质合成和细胞增殖。活性氧(reactive oxygen species, ROS)是PI3K/AKT/mTOR通路的潜在激活因子之一[64],具有强抗氧化能力的EGCG可减少ROS的堆积,调节细胞内氧化还原状态。此外,EGCG还可通过抑制DNA甲基转移酶(DNA methyltransferase, DNMTs)、抑制组蛋白去乙酰化酶(histone deacetylase, HDAC)等方式影响表观遗传修饰[15, 65],阻断PI3K/AKT/mTOR信号通路。

随着年龄增长，小胶质细胞的染色质可及性发生变化，导致基因表达调控受限。HDAC是作为一类对染色体的结构修饰和基因表达调控的具有重要作用的蛋白酶，其表达上调促使组蛋白启动子去乙酰化，可造成炎症通路抑制因子的沉默[37]，促使小胶质细胞分泌炎症介质。在哺乳动物中，有18种HDAC酶，近年研究发现小胶质细胞中的HDAC1/2是调控衰老小鼠髓鞘再生能力的关键，条件性删除HDAC1/2可恢复髓鞘再生[37, 66]。此外研究还揭示了HDAC6与AD的关联[39]，HDAC6是主要的微管去乙酰化酶，其抑制可增加微管乙酰化，促进自噬体和溶酶体的融合；并且HDAC6可与热休克蛋白90（heat shock protein 90，HSP90）[40]形成复合物，调控其客户蛋白（如AKT）的稳定性；抑制小胶质细胞中的HDAC6会导致小胶质细胞极化或淀粉样蛋白负荷降低，提高小胶质细胞对Aβ沉淀的内化摄取能力以改善认知障碍[66-69]。我们发现，EGCG下调了HDAC6的表达（图4A），且HDAC6抑制剂与EGCG作用一致，均能抑制PI3K/AKT/mTOR信号通路（图3I，图4E），激活了自噬（图3E-3H，图4C），并促进了小胶质细胞对Aβ的清除（图2C）。同时，EGCG可能通过抑制HDAC6活性，增强组蛋白乙酰化，从而开放染色质结构，促进RAGE基因的转录。这意味着EGCG通过抑制HDAC6活性来促进小胶质细胞的自噬激活和Aβ清除。

综上所述，本研究不仅阐述了EGCG促进小胶质细胞自噬和Aβ清除、有助于神经元保护并改善认知功能障碍，还揭示了一种靶向EGCG-HDAC6-PI3K/AKT/mTOR信号轴来治疗AD的方法。这种非神经元依赖的作用模式增进了我们对EGCG与小胶质细胞清除Aβ之间关系的了解。

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