

SIRT1-cAMP在白藜芦醇改善小鼠卵巢储备中的作用

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【摘要】 目的 探索组蛋白去乙酰化酶(Sirtuin1, SIRT1)激动剂对氧化应激损伤卵巢功能低下小鼠卵巢储备影响及SIRT1-cAMP通路的可能作用。方法 通过SIRT1激动剂白藜芦醇(resveratrol, Res)及H₂O₂干预人卵巢黄素化颗粒细胞,流式细胞学检测颗粒细胞凋亡及线粒体膜电位(mitochondrial membrane potential, MMP),ELISA测定颗粒细胞雌激素分泌能力,Western blot及RT-PCR检测颗粒细胞功能性受体及雌激素合成相关基因表达;最后通过Res预处理3-硝基丙酸(3-nitropropionic acid, 3-NPA)诱导建立的氧化应激损伤早发性卵巢功能不全(premature ovarian insufficiency, POI)模型小鼠,从动情周期、卵巢指数、激素水平、卵泡计数等方面评估Res对卵巢功能的保护作用,PCR检测小鼠卵巢组织环腺苷酸(cyclic adenosine monophosphate, cAMP)信号通路关键基因表达。结果 Res可以通过SIRT1改善氧化应激损伤人颗粒细胞雌激素合成能力、功能性受体表达及雌激素合成相关基因表达,改善氧化应激损伤POI小鼠卵巢功能。卵巢组织内cAMP信号通路关键基因EPAC、RAP1b、RAP1a、PLCβ3、CREB1、CAMK2α及SIRT1表达升高。结论 Res可以改善氧化应激损伤小鼠卵巢储备功能,SIRT1-cAMP信号通路可能是其潜在作用机制,两者之间可能存在正反馈作用。

【关键词】 卵巢储备; 早发性卵巢功能不全(POI); 氧化应激; 组蛋白去乙酰化酶(SIRT1); 小鼠

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Role of SIRT1-cAMP in the improvement of ovarian reserve by resveratrol in mice

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【Abstract】 Objective To explore the effects of Sirtuin1 (SIRT1) agonist on ovarian reserve and the possible role of SIRT1-cAMP pathway in oxidative stress induced ovarian dysfunction in mice. **Methods** Resveratrol (Res) and H₂O₂ were used to intervene human ovarian luteinized granulosa cells, and the apoptosis and mitochondrial membrane potential of granulosa cells were detected by flow cytometry, and the estrogen secretion capacity of granulosa cells was determined by enzyme-linked immunosorbent assay. Western blot and RT-PCR were used to detect the expression of functional receptors and genes related to estrogen synthesis in granulosa cells. Finally, oxidative stress injured premature ovarian insufficiency (POI) model mice induced by 3-nitropropionic acid were pretreated with Res, and its protective function on ovarian reserve was evaluated from estrous cycle, ovarian index, hormone level and follicular counting. PCR was used to detect the expression of key genes in the cyclic adenosine monophosphate (cAMP)

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signaling pathway in mouse ovarian tissues. **Results** Res could improve the estrogen synthesis ability of human granulosa cells, the expression of functional receptors and genes related to estrogen synthesis in oxidative-stress damaged granulosa cells through SIRT1. Meanwhile, Res could improve the ovarian function of oxidative-stress damaged POI mice and increase the ovarian expression of key genes in cAMP signaling pathway including *EPAC*, *RAP1b*, *RAP1a*, *PLCb3*, *CREB1*, *CAMK2α* and *SIRT1*. **Conclusion** Res can improve the ovarian reserve of oxidative-stress injured mice, and SIRT1-cAMP signaling pathway may be the potential mechanism, and positive feedback may function between them.

【Key words】 ovarian reserve; premature ovarian insufficiency (POI); oxidative stress; Sirtuin1 (SIRT1); mouse

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随着女性生育时间的延迟以及三孩政策的开放,生育力保存及卵巢功能保护越来越受到女性关注。早发性卵巢功能不全(premature ovarian insufficiency, POI)病因复杂,约50%以上的患者病因不明确,近年来研究发现氧化应激在始基卵泡形成、卵泡发育、成熟、闭锁等多个环节中均发挥作用,可能对卵巢储备功能维持具有重要影响^[1]。研究表明,组蛋白去乙酰化酶(Sirtuin1, SIRT1)参与调节细胞应激、衰老等多个生理病理过程^[2]。SIRT1可通过激活PI3K/AKT/mTOR通路减少POI大鼠模型颗粒细胞氧化应激,抑制细胞凋亡,参与调控始基卵泡募集过程^[3-4]。本课题组前期研究发现POI患者血清氧化水平升高,抗氧化水平降低,并且SIRT1含量及活性降低。因此,我们推测氧化应激可能通过SIRT1影响颗粒细胞发育及功能,进而参与POI发生,但其具体机制不详^[5]。SIRT1特异激动剂白藜芦醇(resveratrol, Res)预处理可以改善环磷酰胺所致大鼠卵巢颗粒细胞损伤,而SIRT1选择性抑制剂EX527可以逆转Res的保护作用,表明SIRT1可能是Res作用的重要中介^[6]。而Res在卵母细胞减数分裂过程中可以上调胞质环腺苷酸(cyclic adenosine monophosphate, cAMP),激活Epac1/CaMKKβ/AMPK/SIRT1/PGC-1α通路,进而促进脂肪酸氧化、线粒体呼吸等生理活动^[7-9]。但Res对氧化应激诱导POI的改善作用及机制尚不清楚。

因此,本研究拟从细胞水平检测Res对氧化应激损伤人黄素化颗粒细胞功能作用,并从体内实验验证Res对氧化应激损伤POI模型小鼠卵巢作用,最后探索SIRT1-cAMP通路是否参与其作用过程,寻找POI潜在致病机制及可能治疗方向。

资 料 和 方 法

人原代颗粒细胞培养 2019年11月至2020年5月于上海集爱遗传与不孕诊疗中心收集人原代颗粒细胞15例,并经过复旦大学附属妇产科医院伦理委员会批准(批准号:2019-47)。入选标准:(1)在诊疗中心就诊的女性患者,年龄<40岁,未绝经,平素月经规律,经量正常,血FSH水平、AMH水平正常。(2)无化疗、放疗、自身免疫性疾病、卵巢原发或转移肿瘤、盆腔及卵巢炎症性疾病、盆腔或卵巢手术史。(3)无生殖相关家族病史,母亲月经规律且停经年龄≥40岁,若有子女,则子女青春期正常发育、月经规律。(4)行第二代试管婴儿,即卵胞浆内单精子显微注射技术(intracytoplasmic sperm injection, ICSI)。

将含有卵泡液的颗粒细胞移入刻度离心管,223.8×g离心5 min,弃去上清液。沉淀中加入3倍体积的红细胞裂解液,混匀,静置3 min,223.8×g离心5 min,沉淀中加入15%胎牛血清的DMEM/F12不含酚红培养基,接种于6孔板中。

细胞实验分组及处理 实验前1天颗粒细胞6孔板铺板(5×10^5 /孔),随机分为3组:H₂O₂组、H₂O₂+Res组和正常对照组,分别予100 μmol/L H₂O₂, 10 μmol/L Res+100 μmol/L H₂O₂培养液干预24 h。

动物实验分组及处理 6周龄雌性SPF级C57小鼠(上海捷思杰公司),共24只,均经阴道涂片筛查,性周期正常,适应性喂养1周。随机分为对照组、3-NPA组^[10]、3-NPA+Res组及Res组,每组6只。对照组小鼠第1~3天予0.5%甲基纤维素灌胃,第4~24天予0.5%甲基纤维素灌胃+生理盐水腹腔注射;3-NPA组小鼠第1~3天予0.5%甲基纤

维素灌胃,第4~24天予0.5%甲基纤维素灌胃+40 mg/kg 3-NPA腹腔注射;3-NPA+Res组第1~3天予40 mg/kg Res灌胃,第4~24天予40 mg/kg Res灌胃+40 mg/kg 3-NPA腹腔注射;Res组第1~3天予40 mg/kg Res灌胃,第4~24天予40 mg/kg Res灌胃+等量生理盐水腹腔注射^[11];从动情周期、卵巢指数、卵泡计数及激素水平评估小鼠卵巢功能^[12]。每天早10时行阴道分泌物涂片,观察动情周期。每周称量体重,于停药后处死。小鼠双侧卵巢称重并计算卵巢指数。卵巢指数=卵巢重量(mg)/小鼠体质量(g)×100%。

检测指标与方法 (1)细胞凋亡检测:胰酶消化,4℃223.8×g离心5 min,加入5 μL的Annexin V-PE及5 μL 7-AAD,避光孵育15 min,使用流式细胞仪检测细胞凋亡。(2)线粒体膜电位(mitochondrial membrane potential, MMP)检测:加入罗丹明123染液10 g/mL,37℃细胞培养箱孵育30 min,流式细胞仪检测MMP水平。(3)E₂、FSH及AMH检测:收集细胞上清液或血清,4℃223.8×g离心10 min,取上清,ELISA检测E₂(25~2 000 pg/mL)、FSH(4~140 U/L)及AMH(0.156~10 ng/mL)浓度。(4)颗粒细胞免疫荧光FSHR检测:1×10⁵/mL颗粒细胞爬片,PBS冲洗3次,4%多聚甲醛固定15 min,PBS冲洗3次,0.5% Triton X-100室温通透20 min,PBS冲洗3次,室温封闭60 min,FSHR抗体(1:500)4℃湿盒孵育过夜。PBS冲洗3次,荧光二抗室温孵育60 min,DAPI避光孵育5 min,PBS冲洗,在共聚焦显微镜下观察并拍照。

实时定量荧光聚合酶链反应(RT-PCR) Trizol冰上裂解细胞或组织15 min,提取RNA,配置逆转录体系,应用Prime Script™ RT Master Mix逆转录试剂盒,37℃15 min,80℃5 s,4℃10 min曲线扩增,收集cDNA。应用SYBR Premix Ex Taq PCR试剂盒,配置扩增体系,每孔10 μL。每个样品设置3复孔,384孔板上样,利用荧光定量PCR仪扩增,设置扩增及溶解曲线,2^{-ΔΔCT}用于作图及计算统计学差异。

Western blot法检测卵巢组织或细胞中SIRT1、AMHR2、FSHR、LHR、SOD2、GSH-Px、MDA5表达 制备蛋白样品,BCA检测蛋白浓度,30 μg蛋白样品上样,60 V电压跑浓缩胶,120 V分离胶电泳。330 mA恒电流转膜90 min,冰水混合物降温。牛奶

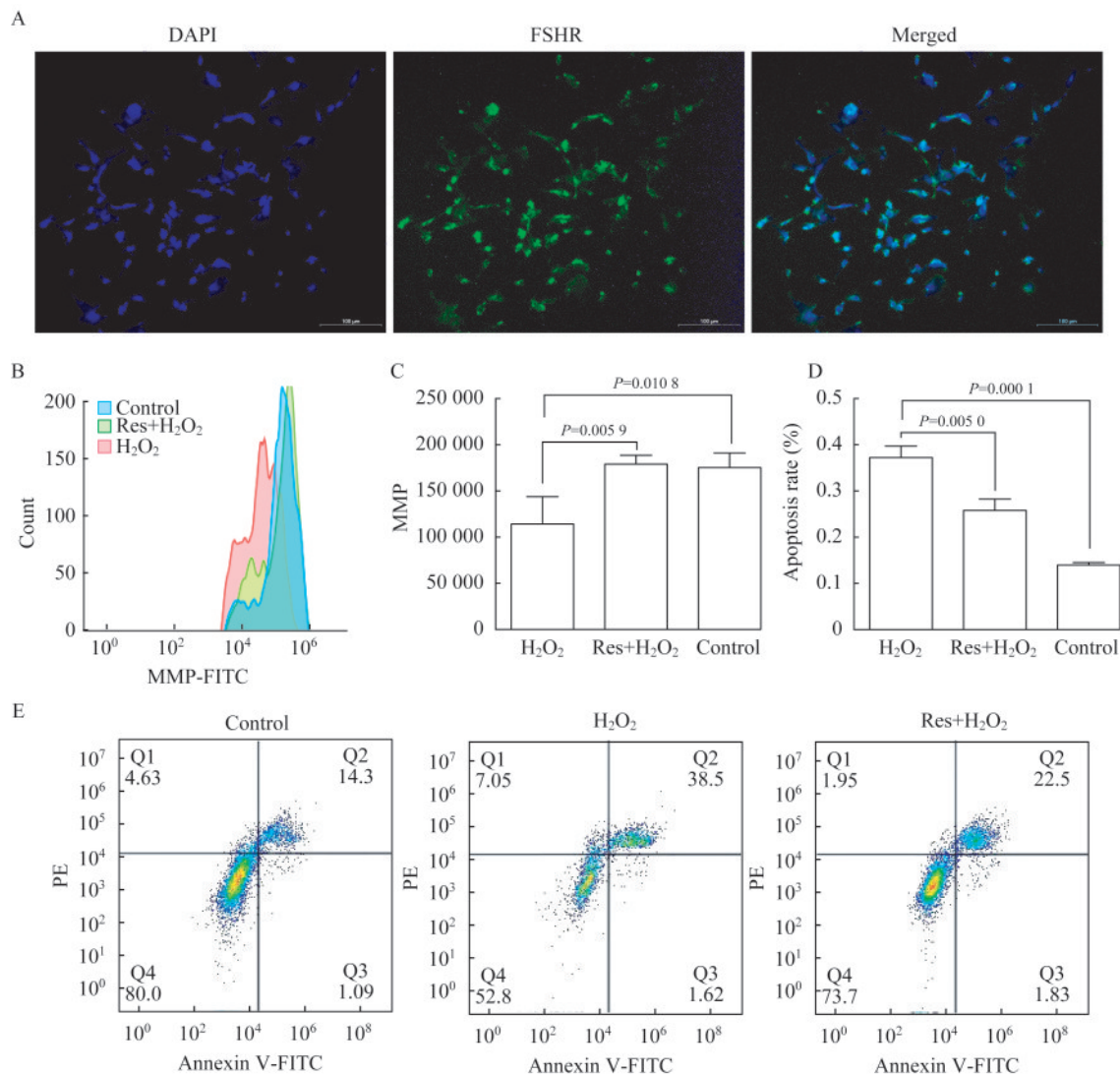
封闭60 min,一抗稀释液SIRT1(1:2 000)、FSHR(1:2 000)、AMHR2(1:2 000)、LHR(1:2 000)、SOD2(1:1 000)、MDA5(4 μg/mL)、GSH-Px(1 μg/mL)、GAPDH(1:2 000)4℃孵育过夜。第二天室温摇床孵育二抗60 min,应用ECL超敏发光液试剂盒配置发光液,放入发光仪曝光条带。

统计学分析 所有实验均重复3次。所有数据用 $\bar{x} \pm s$ 表示,采用GraphPad Prism 6.0软件分析,两组间比较用独立样本 t 检验。 $P < 0.05$ 为差异有统计学意义。

结 果

SIRT1激动剂Res可改善氧化应激导致的颗粒细胞损伤 免疫荧光检测显示人黄素化颗粒细胞表达颗粒细胞特异性受体FSHR。通过H₂O₂诱导氧化应激损伤颗粒细胞模型,发现H₂O₂组线粒体膜电位降低,细胞凋亡显著增加,成功建立氧化应激损伤颗粒细胞模型。Res+H₂O₂干预组细胞线粒体膜电位增加,细胞凋亡显著减少。此外,Res+H₂O₂干预组抗氧化指标SOD₂及GSH-Px表达明显升高,氧化指标MDA5表达降低,颗粒细胞表面功能性受体FSHR、LHR表达部分增加,雌激素合成能力增加,雌激素合成及调控基因CYP19A1、CYP17A1、AR、NR5A1、STAR以及颗粒细胞功能受体基因FSHR、LHR、AMHR2、ESR1、ESR2 mRNA水平均显著升高。以上结果表明Res可能通过激活SIRT1增加颗粒细胞激素合成能力及其表面受体表达,改善氧化应激导致的颗粒细胞损伤(图1、2)。

Res可以改善POI小鼠卵巢功能 4组小鼠体重未见显著差异,3-NPA组小鼠平均动情间期延长,平均动情期缩短,卵巢指数下降,血清FSH水平升高,AMH水平降低,始基卵泡数量降低,与POI表型相似。而3-NPA+Res组平均动情间期缩短,平均动情期延长,卵巢指数上升,血清FSH水平降低,AMH水平升高,始基卵泡数量增加,说明Res具有改善小鼠POI表型的作用。进一步检测发现,3-NPA组小鼠血清SOD、GSH-Px活性降低,MDA活性升高,而3-NPA+Res组小鼠血清SOD、GSH-Px活性升高,MDA活性降低。以上结果表明SIRT1激动剂Res具有改善小鼠POI表型的作用(图3)。



A: Immunofluorescence indicated that granulosa cells expressed specific receptor FSHR ($\times 100$). B-C: MMP of the H₂O₂ group was decreased, while that of the Res+H₂O₂ group was increased. D-E: Apoptosis increased in the H₂O₂ group, and significantly decreased in the Res + H₂O₂ group.

图1 Res抑制氧化应激损伤颗粒细胞凋亡

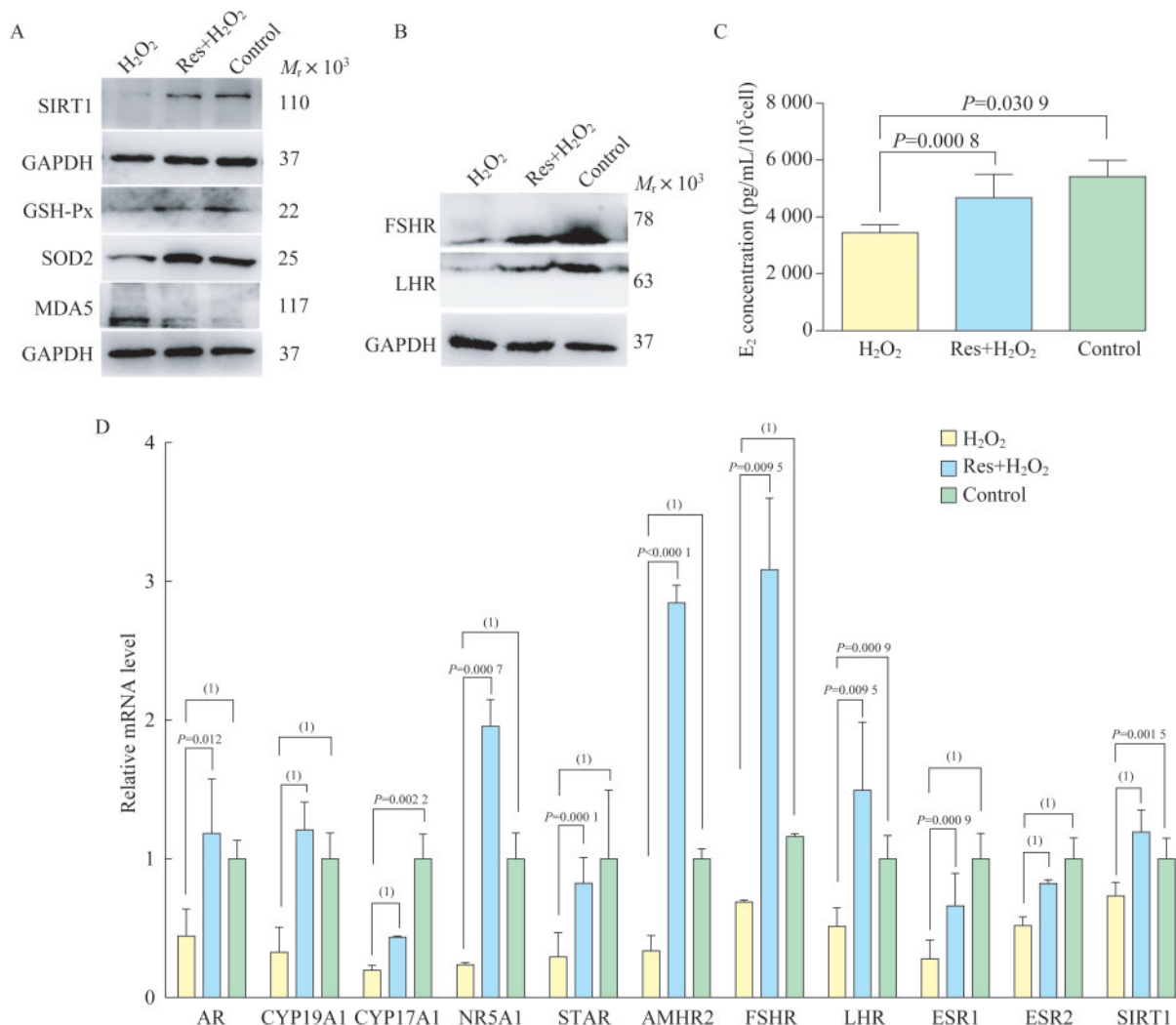
Fig 1 Resveratrol inhibits the apoptosis of oxidative-stress injured granulosa cells

SIRT1-cAMP 信号通路可能参与 Res 改善氧化应激 POI 小鼠卵巢功能过程 通过 PCR 检测对照组、3-NPA 组、3-NPA+Res 组、Res 组小鼠卵巢组织中 cAMP 信号通路关键基因,结果发现 3-NPA 组小鼠卵巢组织 *EPAC*、*RAP1a*、*RAP1b*、*PLCb3*、*CREB1*、*CAMK2α* 及 *SIRT1* 基因表达降低,*SIRT1* 活性降低,说明 cAMP 信号通路可能参与氧化应激 POI 小鼠的发生,而 3-NPA+Res 组卵巢组织中 *EPAC*、*RAP1a*、*RAP1b*、*PLCb3*、*CREB1*、*CAMK2α* 及 *SIRT1* 基因表达升高,以上结果进一步说明 cAMP-EPAC-Rap-CAMK 信号通路可能参与氧化应

激 POI 小鼠的发生,Res 通过激活 cAMP-EPAC-Rap-CAMK 信号通路改善小鼠 POI 表型作用,cAMP 信号通路与 *SIRT1* 之间可能存在正反馈作用(图 4)。

讨 论

POI 发病率逐年增加,卵巢功能下降所带来的一系列低雌激素症状严重影响女性身心健康和生活方式。POI 病因复杂,其发生机制更是不详。氧化应激在 POI 疾病中的作用目前已得到认可,POI 患者血清抗氧化活性降低,氧化活性升高^[13-14]。氧



A-B: The expressions of SIRT1, SOD2, GSH-Px, FSHR and LHR were decreased and the expression of MDA5 was increased in the H₂O₂ group, which was reversed in Res+H₂O₂ group. C: The synthesis ability of E₂ was increased in granulosa cells of Res+ H₂O₂ group; D: PCR showed that the expressions of AR, CYP19A1, CYP17A1, NR5A1, STAR, AMHR2, FSHR, LHR, ESR1, ESR2 and SIRT1 were increased in Res+ H₂O₂ group. ⁽¹⁾P<0.0001.

图2 Res可以改善氧化应激损伤颗粒细胞功能

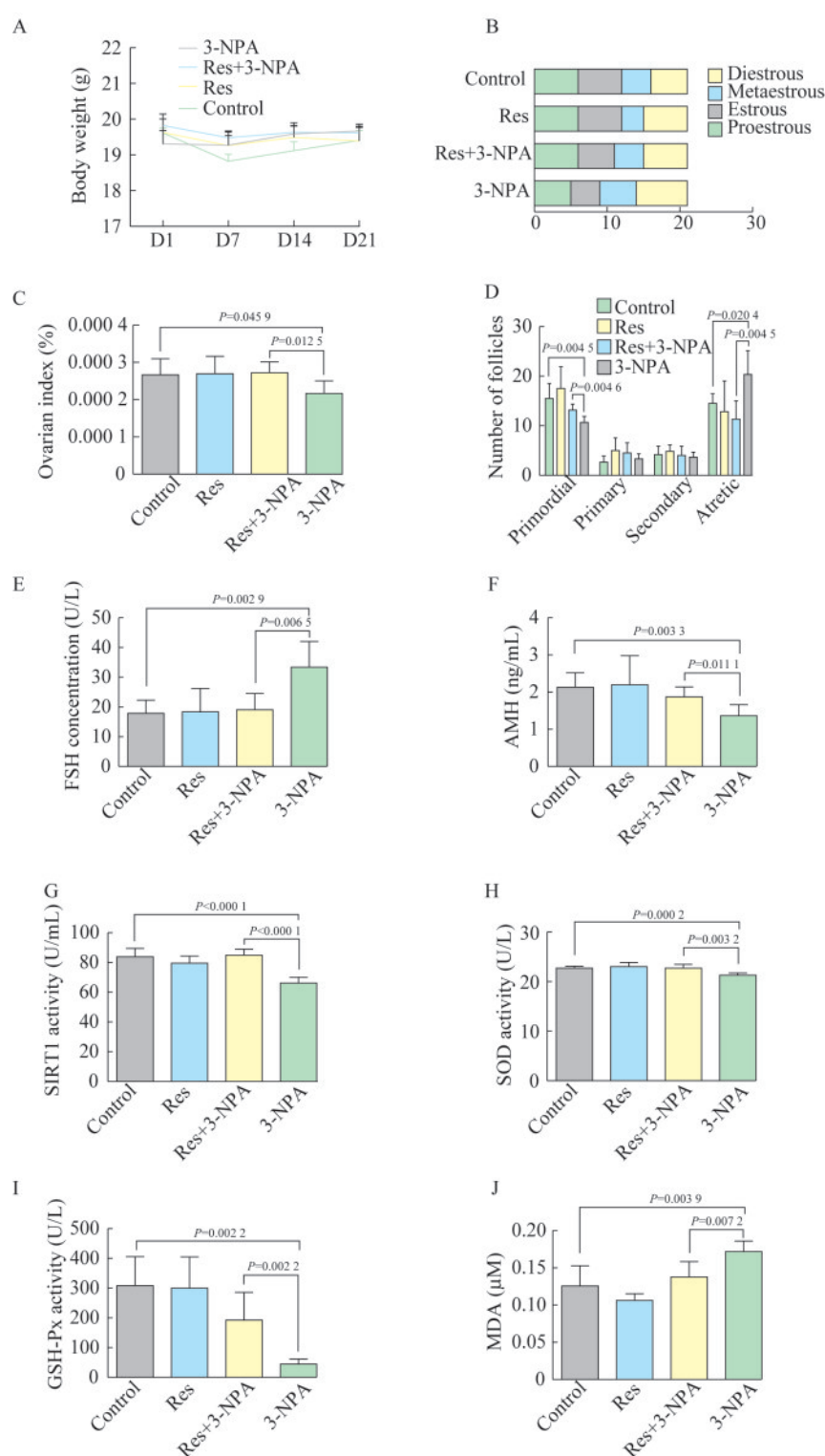
Fig 2 Resveratrol can improve function of oxidative-stress damaged granulosa cells

化应激通过抑制卵细胞成熟,促进细胞凋亡,诱导或加速POI发生^[15-16]。

近年来,多项研究提示SIRT1参与氧化应激过程^[17-18]。SIRT1激活可以促进PI3K-AKT通路介导的FOXO1去乙酰化修饰,抑制氧化应激诱导的卵巢颗粒细胞自噬^[19]。白藜芦醇(Res)是SIRT1特异激动剂,实验结果提示Res具有改善氧化应激损伤颗粒细胞及POI小鼠卵巢功能,进一步说明Res可以通过激活SIRT1改善POI小鼠低雌激素状态。

cAMP是细胞内参与调节物质代谢和生物学功

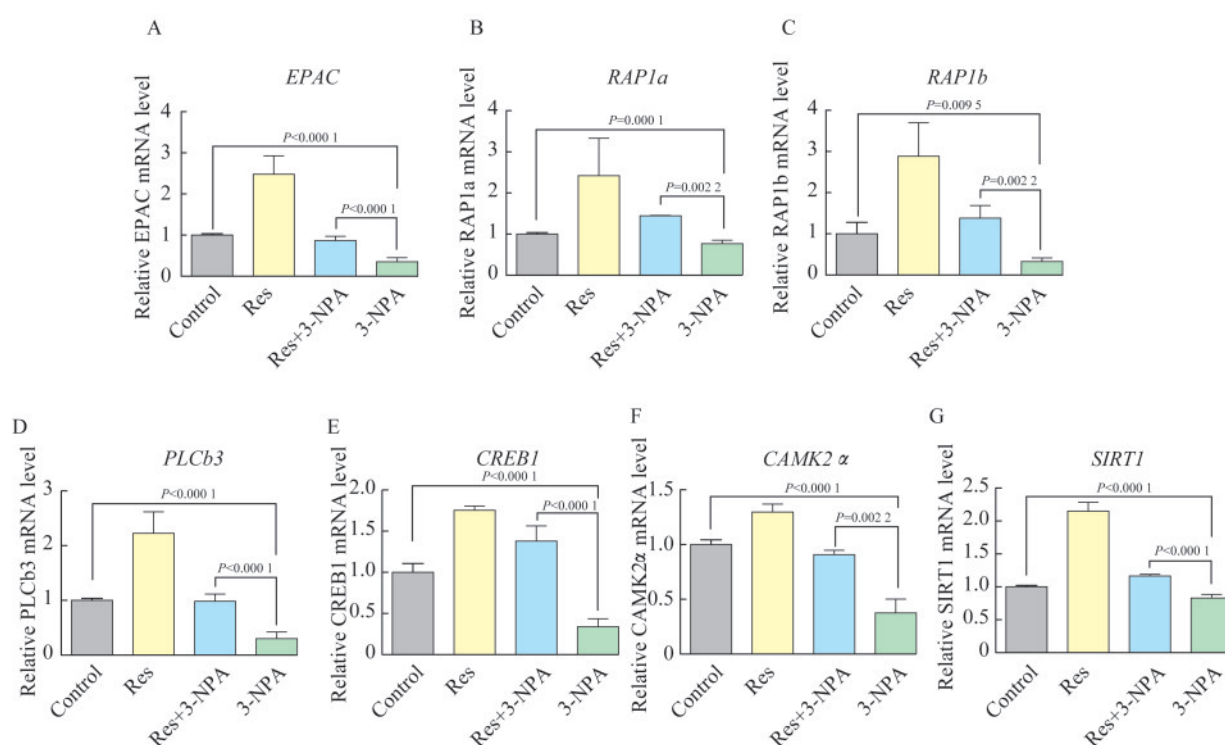
能的重要物质,是细胞内信号传导过程中的“第二信使”。cAMP贯穿卵泡发育全过程,卵泡细胞内高水平的cAMP维持卵母细胞处于减数分裂静止期^[20]。Res可以增强cAMP信号,在黄素化人颗粒细胞中,cAMP与SIRT1表达之间的正反馈机制维持SIRT1在高水平状态^[21-22]。cAMP信号通过激活SIRT1改善阿霉素诱导的氧化应激损伤、细胞凋亡及心肌毒性^[23]。本研究发现3-NPA组cAMP信号通路关键基因EPAC、RAP1b、RAP1a、PLCb3、CREB1、CAMK2α及SIRT1基因表达降低,SIRT1



A: No significant difference in body weight among the four groups; B: The mean length of the diestrous phase was prolonged and the mean length of estrous phase was shortened in 3-NPA group, while Res reversed this trend; C: Ovarian index decreased in 3-NPA group and increased in the Res+3-NPA group; D: The number of primordial follicles decreased in 3-NPA group, while increased in Res+3-NPA group; E-F: Serum FSH level increased and AMH level decreased in 3-NPA group, while reversed in Res+3-NPA group; G-J: The activities of SIRT1, SOD, GSH-Px decreased and the activities of MDA increased in 3-NPA group, while reversed in Res+3-NPA group.

图3 Res对小鼠POI表型的作用

Fig 3 Effect of resveratrol on POI phenotype of mice



A-G: The expressions of *EPAC*, *RAP1a*, *RAP1b*, *PLCb3*, *CREB1*, *CAMK2α* and *SIRT1* in ovarian tissues of 3-NPA group decreased, while increased in Res+3-NPA group.

图4 cAMP信号通路参与氧化应激POI小鼠发生

Fig 4 The cAMP signaling was involved in oxidative-stress induced POI mice

活性降低,而3-NPA+Res组小鼠卵巢组织 *EPAC*、*RAP1b*、*RAP1a*、*PLCb3*、*CREB1*、*CAMK2α* 及 *SIRT1* 基因表达升高, *SIRT1* 活性升高,以上结果进一步说明 cAMP 信号通路参与氧化应激 POI 的发生, Res 可以增强 cAMP 信号通路关键基因表达,两者之间可能存在正反馈机制。

综上所述, Res 可以通过 *SIRT1*-cAMP 通路抗氧化作用改善 POI 表型, 本研究有助于寻找 POI 治疗潜在靶点, 改善 POI 患者低雌激素状态及维持卵泡发育及功能。但是本实验存在一定的局限性, 首先, 氧化应激细胞模型采用 H_2O_2 , 以动物模型是 3-NPA, 后续应该增加 3-NPA 细胞实验以保持一致性; 其次, 近年来的研究发现 Res 在激活 *SIRT1* 的同时, *SIRT3* 也参与起调控作用, 后续实验须进一步排除 *SIRT3* 的干扰; 最后, 本文机制研究较浅, *SIRT1* 与 cAMP 信号通路之间的具体调控机制尚不清楚, 仍需后续的实验研究来阐明。

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利益冲突声明 所有作者均声明不存在利益冲突。

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