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在线全文

槲皮素通过抑制p38 MAPK/NOX4信号通路减轻H_2O_2诱导的人子宫内膜基质细胞氧化应激损伤*

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【摘要】 目的 探讨槲皮素对过氧化氢（hydrogen peroxide, H_2O_2）诱导人子宫内膜基质细胞（human endometrial stromal cells, HESCs）损伤的保护作用及其可能的作用机制。**方法** 体外培养HESCs，加入不同浓度的槲皮素（0、10、20和40 μmol/L）作用24 h，验证给予不同剂量的槲皮素对正常子宫内膜细胞没有毒性，随后采用250 μmol/L H_2O_2孵育细胞12 h，建立H_2O_2诱导的HESCs损伤模型。槲皮素预处理24 h，H_2O_2刺激HESCs后，CCK-8检测细胞活力，筛选有效的干预剂量，随后将HESCs分为空白组、H_2O_2模型组、H_2O_2+槲皮素组，采用DCFH-DA荧光探针检测细胞内活性氧（reactive oxygen species, ROS）水平；Annexin Ⅴ/PI双染，流式细胞术检测槲皮素对H_2O_2诱导HESCs凋亡的影响；JC-1染色法检测细胞线粒体膜电位；Western blot检测相关蛋白NADPH氧化酶4（NADPH oxidase 4, NOX4）、p38丝裂原活化蛋白激酶（p38 mitogen-activated protein kinase, p38 MAPK）及磷酸化p38 MAPK（p-p38 MAPK）表达。**结果** 根据CCK-8实验结果，选择槲皮素20 μmol/L为有效干预剂量。ROS检测显示，与空白组相比，H_2O_2模型组ROS的平均荧光强度升高（P<0.01），而与H_2O_2模型组相比槲皮素处理下调了ROS的平均荧光强度，减轻了氧化损伤（P<0.05）。细胞凋亡检测结果显示，H_2O_2模型组细胞凋亡率较空白组增加（P<0.01），而与槲皮素共同作用则逆转了细胞凋亡率的增加（P<0.05）。JC-1染色检测线粒体膜电位变化情况显示，与空白组相比，H_2O_2诱导所致的线粒体膜电位降低的细胞比例增加（P<0.01），而槲皮素处理后线粒体膜电位降低的细胞比例低于H_2O_2模型组（P<0.05）。Western blot结果显示，与空白组相比，H_2O_2模型组NOX4蛋白、p-p38 MAPK蛋白相对表达量升高（P<0.05）；而加入槲皮素后，与H_2O_2模型组相比，NOX4蛋白、p-p38 MAPK蛋白相对表达量降低（P<0.05）。**结论** 槲皮素预处理对H_2O_2诱导的HESCs氧化损伤具有保护作用，其机制可能是减少ROS过量累积以及抑制p38 MAPK/NOX4信号通路。

【关键词】 槲皮素 子宫内膜基质细胞 氧化应激 细胞凋亡 活性氧

Quercetin Alleviates H_2O_2-Induced Oxidative Stress Damage to Human Endometrial Stromal Cells by Inhibiting the p38 MAPK/NOX4 Signaling Pathway

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【Abstract】 Objective This study aims to systematically evaluate the protective role of quercetin (QCT), a naturally occurring flavonoid, against oxidative damage in human endometrial stromal cells (HESCs) induced by hydrogen peroxide (H_2O_2). Oxidative stress, such as that induced by H_2O_2, is known to contribute significantly to cellular damage and has been implicated in various reproductive health issues. The study is focused on investigating how QCT interacts with specific molecular pathways to mitigate this damage. Special attention was given to the p38 MAPK/NOX4 signaling pathway, which is crucial to the regulation of oxidative stress responses in cellular systems. By elucidating these mechanisms, the study seeks to confirm the potential of QCT not only as a protective agent against oxidative stress but also as a therapeutic agent that could be integrated in treatments of conditions characterized by heightened oxidative stress in endometrial cells. **Methods** *In vitro* cultures of HESCs were treated with QCT at different concentrations (0, 10, 20, and 40 μmol/L) for 24 h to verify the non-toxic effects of QCT on normal endometrial cells. Subsequently, 250 μmol/L H_2O_2 was used to incubate the cells for 12 h to establish an H_2O_2-induced HESCs injury model. HESCs were pretreated with QCT for 24 h, which was followed by stimulation with H_2O_2. Then, CCK-8 assay was performed to examine the cell viability and to screen for the effective intervention concentration. HESCs were divided into 3 groups, the control group, the H_2O_2 model group, and the H_2O_2+QCT group. Intracellular levels of reactive oxygen species (ROS) were precisely quantified using the DCFH-DA fluorescence assay, a method known for its accuracy in detecting and quantifying oxidative changes within the cell. The mitochondrial membrane potential was determined by JC-1 staining. Annexin Ⅴ/PI double staining and flow cytometry were performed to determine the effect of QCT on H_2O_2-induced apoptosis of HESCs. Furthermore, to delve deeper into the cellular mechanisms underlying the observed effects, Western blot analysis was conducted to measure the expression levels of the critical proteins involved in oxidative stress response, including NADPH oxidase 4 (NOX4), p38 mitogen-activated protein kinase (p38 MAPK), and phosphorylated p38

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MAPK (p-p38 MAPK). This analysis helps increase understanding of the specific intracellular signaling pathways affected by QCT treatment, giving special attention to its potential for modulation of the p38 MAPK/NOX4 pathway, which plays a significant role in cellular defense mechanisms against oxidative stress. **Results** In this study, we started off by assessing the toxicity of QCT on normal endometrial cells. Our findings revealed that QCT at various concentrations (0, 10, 20, and 40 μmol/L) did not exhibit any cytotoxic effects, which laid the foundation for further investigation into its protective roles. In the H_2O_2-induced HESCs injury model, a significant reduction in cell viability was observed, which was linked to the generation of ROS and the resultant oxidative damage. However, pretreatment with QCT (10 μmol/L and 20 μmol/L) significantly enhanced cell viability after 24 h (P<0.05), with the 20 μmol/L concentration showing the most substantial effect. This suggests that QCT can effectively reverse the cellular damage caused by H_2O_2. Furthermore, the apoptosis assays demonstrated a significant increase in the apoptosis rates in the H_2O_2 model group compared to those in the control group (P<0.01). However, co-treatment with QCT significantly reversed this trend (P<0.05), indicating QCT's potential protective role in mitigating cell apoptosis. ROS assays showed that, compared to that in the control group, the average fluorescence intensity of ROS in the H_2O_2 model group significantly increased (P<0.01). QCT treatment significantly reduced the ROS fluorescence intensity in the H_2O_2+QCT group compared to the that in the H_2O_2 model group, suggesting an effective alleviation of oxidative damage (P<0.05). JC-1 staining for mitochondrial membrane potential changes revealed that compared to that in the control, the proportion of cells with decreased mitochondrial membrane potential significantly increased in the H_2O_2 model group (P<0.01). However, this proportion was significantly reduced in the QCT-treated group compared to that of the H_2O_2 model group (P<0.05). Finally, Western blot analysis indicated that the expression levels of NOX4 and p-p38 MAPK proteins were elevated in the H_2O_2 model group compared to those of the control group (P<0.05). Following QCT treatment, these protein levels significantly decreased compared to those of the H_2O_2 model group (P<0.05). These results suggest that QCT may exert its protective effects against oxidative stress by modulating the p38 MAPK/NOX4 signaling pathway. **Conclusion** QCT has demonstrated significant protective effects against H_2O_2-induced oxidative damage in HESCs. This protection is primarily achieved through the effective reduction of ROS accumulation and the inhibition of critical signaling pathways involved in the oxidative stress response, notably the p38 MAPK/NOX4 pathway. The results of this study reveal that QCT's ability to modulate these pathways plays a key role in alleviating cellular damage associated with oxidative stress conditions. This indicates not only its potential as a protective agent against cellular oxidative stress, but also highlights its potential for therapeutic applications in treating conditions characterized by increased oxidative stress in the endometrium, thereby offering the prospect of enhancing reproductive health. Future studies should explore the long-term effects of QCT and its clinical efficacy *in vivo*, thereby providing a clear path toward its integration into therapeutic protocols.

【Key words】 Quercetin Endometrial stromal cells Oxidative stress Apoptosis Reactive oxygen species

子宫内膜是女性生殖系统的关键组成部分，由功能层和基底层组成，包括上皮细胞和间质细胞。诸多疾病与子宫内膜容受性异常相关，包括不孕、复发性流产和反复着床失败等。氧化应激是一种复杂的生理状态，源于细胞和组织内活性氧（reactive oxygen species, ROS）的积累与生物解毒清除能力的失衡[1]。目前，已有文献报道，氧化应激会导致子宫内膜细胞蜕膜化异常[2]，继而诱发细胞增殖受抑、衰老、凋亡；并且氧化应激在多囊卵巢综合征[3]、卵巢功能不全、子宫内膜异位症[4]等疾病导致的内膜功能受损中都充当了炎症进展的核心环节。因此，研究和开发能够抵抗和修复子宫内膜受氧化应激影响的药物，有望提高子宫内膜容受性，对于临床治疗具有重要的意义。

黄酮类化合物（flavonoids）是一类具有芳香环结构的羟基化酚类化合物，而槲皮素（quercetin, QCT）是其中的一个特殊亚型。槲皮素是一种强大的抗氧化剂，能够有效地提高植物在应激和非应激环境下的耐受性[5]。不仅如此，槲皮素在人体内也具有多种药理功效，包括抗菌[6]、抗病毒[7]、抗氧化[8]、调节免疫[9]、改善代谢综合征（降血糖、降血脂、降血压）[10]等诸多方面。本研究采用H_2O_2诱导人子宫内膜基质细胞（human endometrial stromal cells, HESCs）建立氧化应激损伤模型，通过细胞及分子生物学技术探讨槲皮素对HESCs氧化应激损伤的保护作用及其作用机制。

1　材料与方法

1.1　细胞

人子宫内膜基质细胞系，由厦门大学医学与生命科学学院王海滨教授馈赠。

1.2　主要药品及试剂

槲皮素（粉末）购自成都植标化纯公司，使用前用DMSO（D2650, SIGMA）进行溶解。DMEM/F12（11320033,

ThermoScientific)；澳大利亚胎牛血清(10099141C，美国Gibco)；BCA蛋白定量试剂盒(71285-3，北京中生华美)；DCFH-DA荧光探针ROS检测试剂盒(ab113851，Abcam)；一抗抗体β-actin、NADPH氧化酶4(NADPH oxidase 4，NOX4)和p38丝裂原活化蛋白激酶(p38 mitogen-activated protein kinase，p38 MAPK)(英国Abcam公司)；CCK-8试剂盒(C0038)、羊抗兔和兔抗鼠二抗(北京碧云天公司)；超敏ECL化学发光液(LD-8012，LDBIO)；细胞培养箱(德国Eppendorf公司)；JC-1检测试剂盒(Abcam公司)；Anexxin Ⅴ FITC细胞凋亡试剂盒(V13241)和多功能酶标仪(Multiskan GO)(美国Thermo Scientific公司)；全自动化学发光成像分析系统(Tanon-5200，上海天能公司)；电泳、转膜装置(美国 Bio-Rad 公司)；流式细胞仪(FACS Aria Ⅲ，美国BD公司)。

1.3 细胞活力检测

使用CCK8试剂盒，按照推荐方案评估细胞活力。将HESCs接种到96孔板中，每孔7 000个细胞，然后加入不同浓度的槲皮素(0、10、20和40 μmol/L)作用24 h[11-12]，评估细胞活力。将HESCs接种于96孔板中，采用250 μmol/L的H_2O_2孵育细胞12 h(关于H_2O_2剂量和处理时间的选择为本课题组前期研究成果，尚未发表)，建立H_2O_2刺激HESCs损伤模型；并分别用10、20、40 μmol/L槲皮素预孵育细胞24 h，再用250 μmol/L H_2O_2处理细胞12 h后，每孔加入CCK-8溶液(10 μL/孔)，37 ℃、体积分数为5%CO_2培养箱中继续孵育2 h，采用酶标仪于450 nm处检测各孔吸光度(A_{450})值，检测细胞活力，筛选有效的干预剂量作为后续实验的槲皮素药物处理组条件。

1.4 细胞内ROS的检测

细胞接种于6孔板，分为空白组(细胞未予H_2O_2和槲皮素处理)、H_2O_2模型组、H_2O_2+槲皮素组。其中，H_2O_2+槲皮素组用20 μmol/L槲皮素预处理24 h，随后H_2O_2模型组和H_2O_2+槲皮素组加入250 μmol/L H_2O_2作用12 h。收集各组细胞，加入终浓度为20 μmol/L的DCFH-DA并于37 ℃孵育30 min，以磷酸盐缓冲液(PBS)洗涤3次，利用流式细胞仪进行分析。

1.5 流式细胞术检测细胞凋亡比例

将HESCs接种于6孔板中，按照1.4的分组方法处理后收集各组细胞，以预冷的PBS洗涤2次，室温下与100 μL含PI(1 mg/L)的1×Annexin Ⅴ工作溶液避光孵育15 min。随后加入400 μL 1×结合缓冲液并迅速涡旋后，立即使用流式细胞仪分析晚期凋亡细胞比例。

1.6 线粒体膜电位评估

线粒体膜电位(mitochondrial membrane potential，MMP)用JC-1检测试剂盒按照说明书进行检测。将HESCs接种于6孔板中，按照1.4的分组方法处理后收集各组细胞，与3 μmol/L JC-1工作液在37 ℃避光孵育20 min。细胞用DPBS清洗3次后，用流式细胞仪进行分析。结果以线粒体膜电位较低的细胞比例表示。

1.7 Western blot检测HESCs NOX4、p38 MAPK及磷酸化p38 MAPK（p-p38 MAPK）相对表达量

将HESCs接种于6孔板中，按照1.4的分组方法处理后收集各组细胞，加入哺乳动物蛋白抽提剂超声提取1 min。裂解终止后，将细胞裂解液在4 ℃、12 000 r/min离心15 min，收集上清液用于蛋白表达分析。BCA法测定蛋白含量，电泳时加入15 μL样品；120 V电压电泳，转膜后1%BSA封闭2 h；加入一抗NOX4(1∶10 000)、p-p38 MAPK(1∶5 000)、p38 MAPK(1∶5 000)，4 ℃孵育过夜；TBST洗4次，每次5 min；加入二抗室温孵育2 h；TBST洗4次，每次5 min；ECL显影，以β-actin为对照，image J软件分析灰度值。目的蛋白相对表达量= 目的蛋白灰度值/内参蛋白灰度值×100%。

1.8 统计学方法

采用SPSS 22.0统计软件进行分析，计量资料以$\bar{x} \pm s$表示，多组间比较采用单因素方差分析(One-Way ANOVA)，组间多重比较采用LSD-t法，$P<0.05$为差异有统计学意义。

2 结果

2.1 槲皮素预处理改善H_2O_2诱导的HESCs活力下降和凋亡

本研究首先验证了给予不同剂量的槲皮素对正常子宫内膜细胞没有毒性(图1A)。采用CCK-8法检测槲皮素对H_2O_2诱导的细胞损伤的保护作用(图1B)，与空白组相比，H_2O_2处理导致HESCs活力下降($P<0.01$)；与H_2O_2模型组相比，槲皮素(10，20 μmol/L)预处理组在给药24 h后，HESCs活力增强($P<0.05$)，其中20 μmol/L效果最为明显。因此，20 μmol/L槲皮素用于后续实验。细胞凋亡检测结果显示(图1C、1D)，H_2O_2模型组细胞凋亡率较空白组增加($P<0.01$)，而与槲皮素共同作用则逆转了细胞凋亡率的增加($P<0.05$)。

2.2 槲皮素减轻H_2O_2诱导的HESCs氧化应激

结果显示(图2)，与空白组相比，H_2O_2模型组ROS的平均荧光强度升高($P<0.01$)，而与H_2O_2模型组相比，槲皮素预处理下调了ROS的平均荧光强度，减轻了氧化损伤($P<0.05$)。

2.3 槲皮素对H_2O_2诱导的HESCs线粒体损伤的保护作用

结果显示(图3)，与空白组相比，H_2O_2诱导所致的线

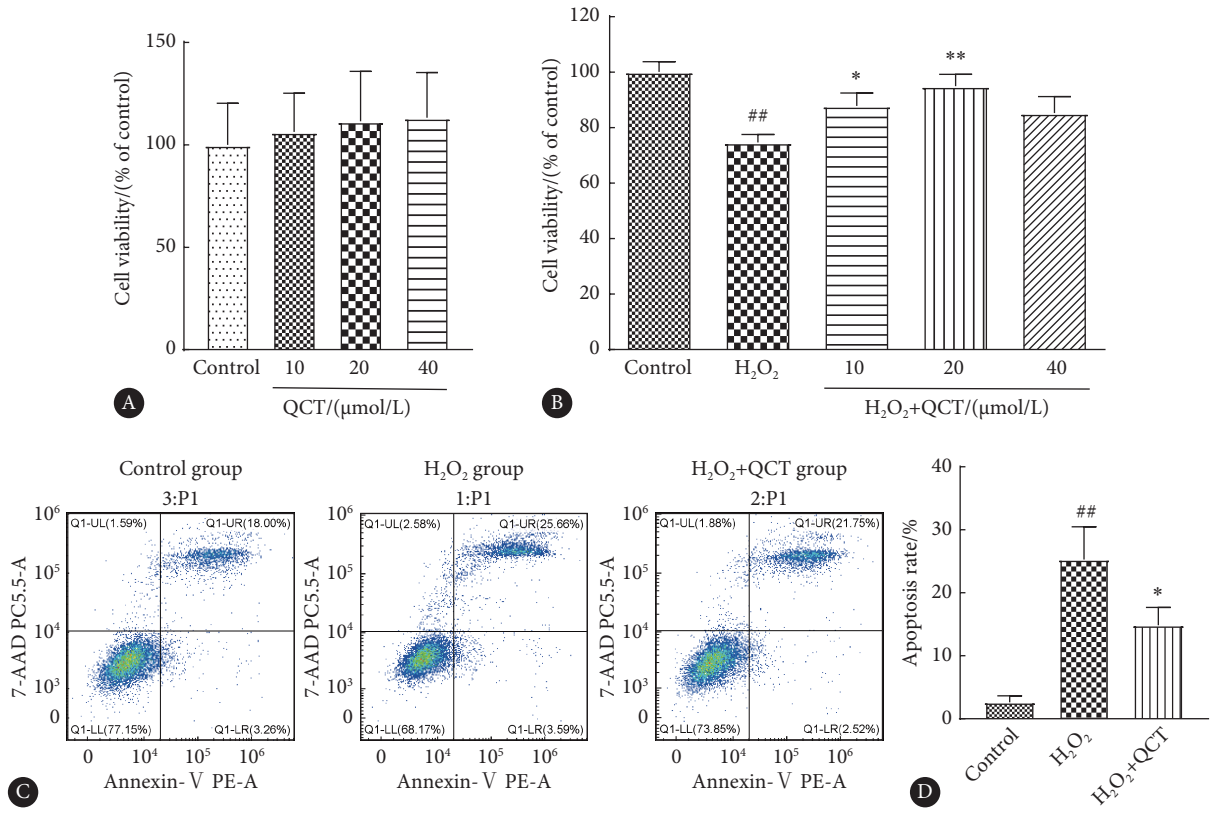

图 1 槲皮素对细胞活力和细胞凋亡的影响

Fig 1 The effect of QCT on cell viability and cell apoptosis

A, Assessment of the viability of HESCs treated with different doses of QCT; B, cell viability determined by CCK-8 assay; C, apoptosis in HESCs as determined by flow cytometry (the concentration of QCT is 20 μmol/L); D, quantitative analysis of the ratios of apoptotic cells (the concentration of QCT is 20 μmol/L). $^{\#\#}P<0.01$, vs. control group; $^{*}P<0.05$, $^{**}P<0.01$, vs. H_2O_2 group.

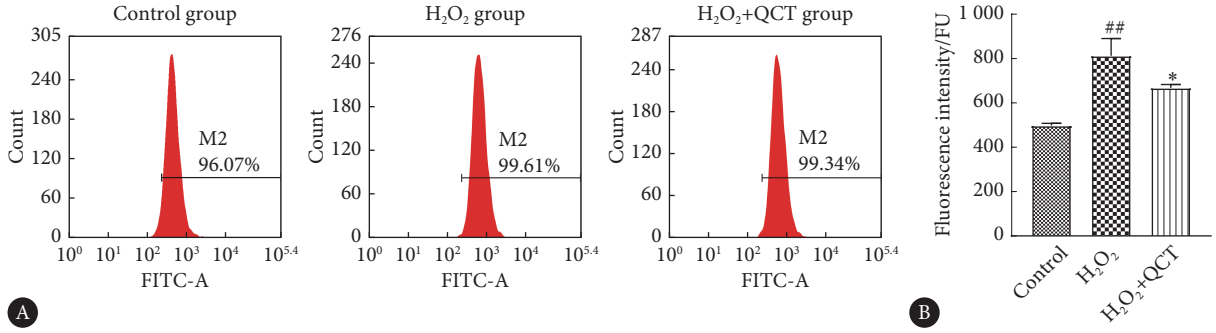

图 2 槲皮素对H_2O_2诱导HESCs细胞ROS的影响

Fig 2 The effect of QCT on H_2O_2-induced ROS in HESCs

A, The ROS levels expressed in HESCs as determined by flow cytometry; B, quantitative analysis of the mean ROS fluorescence intensity. The concentration of QCT is 20 μmol/L. $^{*}P<0.05$, vs. H_2O_2 group; $^{\#\#}P<0.01$, vs. control group.

粒体膜电位降低的细胞比例增加($P<0.01$)，而槲皮素预处理后线粒体膜电位降低的细胞比例与H_2O_2模型组相比下降($P<0.05$)。

2.4　槲皮素降低HESCs中NOX4、p38 MAPK及p-p38 MAPK蛋白的相对表达量

Western blot结果显示(图4)，与空白组相比，H_2O_2模型组NOX4蛋白、p-p38 MAPK蛋白相对表达量升高($P<0.05$)；而加入槲皮素后，与H_2O_2模型组相比，NOX4蛋白、p-p38 MAPK蛋白相对表达量降低($P<0.05$)。

3　讨论

子宫内膜是胚胎着床和妊娠维持的关键组织，其健康状况直接影响女性的生育能力。随着女性生育年龄的推迟，子宫内膜衰老及容受性下降，导致生育能力降低。

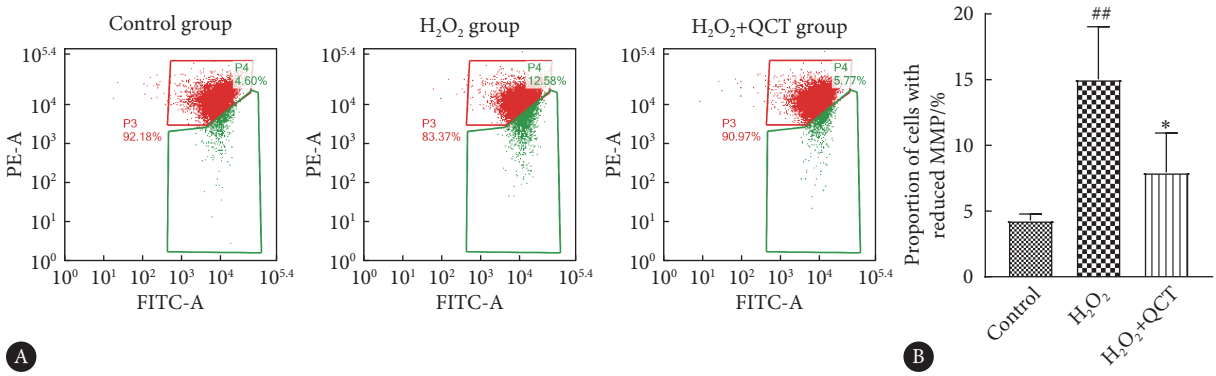

图 3 通过JC-1染色和流式细胞仪测量的线粒体膜电位

Fig 3 Mitochondrial membrane potential (MMP) as measured by JC-1 staining and flowcytometry

A, MMP as measured by JC-1 staining and flowcytometry; B, the proportion of cells with decreased MMP. The concentration of QCT is 20 μmol/L. * P<0.05, vs. H_2O_2 group; ## P<0.01, vs. control group.

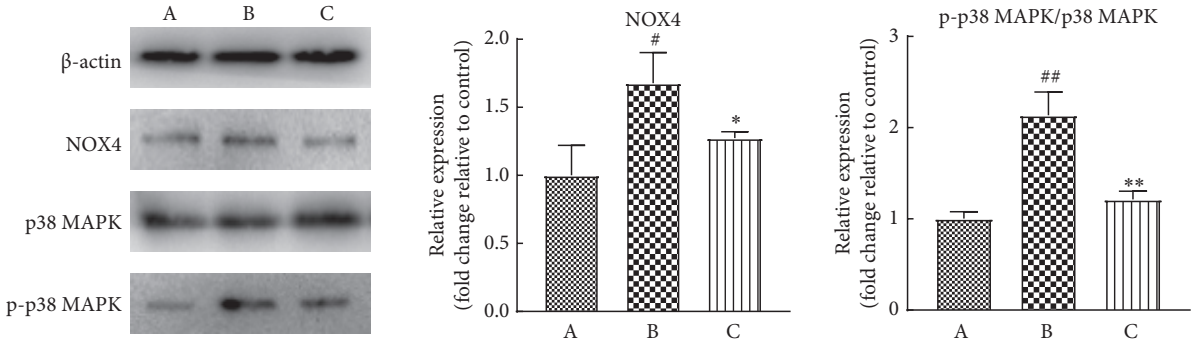

图 4 Western blot检测各组NOX4、p38 MAPK及p-p38 MAPK蛋白相对表达量

Fig 4 Western blot was performed to determine the protein expressions of NOX4, p38 MAPK, and p-p38 MAPK in the three groups

A: Blank group; B: H_2O_2 group; C: H_2O_2+QCT (20 μmol/L) group. * P<0.05, ** P<0.01, vs. H_2O_2 group; # P<0.05, ## P<0.01, vs. control group.

子宫衰老主要由子宫内膜干细胞衰老和子宫内膜基质细胞蜕膜化受损来介导[13],包括氧化应激、炎症、纤维化和DNA损伤等过程[14]。虽然ROS在细胞内信号传递方面发挥重要作用,但是当细胞内ROS浓度过高时,细胞被诱导至氧化应激状态,甚至触发程序性死亡。在生理条件下,随着年龄的增长,ROS失衡也会促使子宫内膜细胞凋亡[15]。因此,研发新药逆转子宫内膜细胞氧化应激损伤,在改善子宫内膜容受性及延缓子宫衰老等方面具有重要意义。

截至目前,槲皮素被认为是自然界中最强大的类黄酮抗氧化剂,由双键和酚羟基提供相应的抗氧化活性[16],在治疗氧化应激相关疾病中应用广泛,包括心血管疾病、神经炎症性疾病、癌症、2型糖尿病、非酒精性脂肪肝、肥胖等。具体作用机制为:槲皮素能够抵抗癌症代谢,通过调节 PI3K/Akt/mTOR、Wnt-β-catenin和MAPK/ERK1/2通路,促进细胞活力丧失、细胞凋亡和自噬[17];缓解动脉粥样硬化,通过抑制p38 MAPK/p16通路,减慢巨噬细胞中被氧化的低密度脂蛋白诱导的衰老[18];通过调节SIRT1/FOXO3A和p38 MAPK信号通路,减轻视网膜缺血再灌注损伤(retinal ischemia-reperfusion, RIR),而ROS的过量产生被认为是RIR损伤的主要原因[19]。受上述研究思路的启发,本文拟深挖槲皮素在子宫内膜基质细胞中的抗氧化机制,研究其能否有效逆转H_2O_2诱导的子宫内膜基质细胞氧化应激损伤。

近年来,有关槲皮素在女性生殖系统中作用的研究相对较少,主要聚焦于子宫内膜纤维化方面。目前已证实槲皮素能够通过多条途径缓解子宫内膜纤维化进程:包括上调miR-145,抑制TGF-β1/Smad2/Smad3通路的激活[20];与NR4A1直接结合,抑制mTOR信号传导[21]。但是,目前在应用槲皮素逆转子宫内膜氧化应激损伤中的研究尚存空白,H_2O_2诱导的细胞损伤可能导致线粒体内膜上线粒体渗透性转换孔开放,导致线粒体膜的去极化,并促使凋亡细胞因子的释放[22]。因此本研究通过构建H_2O_2刺激的HESCs损伤模型,证明了槲皮素可以提高子宫内膜基质细胞的活力,同时槲皮素(20 μmol/L)预给药24 h能够明显改善H_2O_2诱导的子宫内膜基质细胞的凋亡,减少ROS的过度产生,维持线粒体膜电位的稳定性,减少线粒

体通透性转换孔的开放, 减缓氧化应激损伤。

上述结果证明, 槲皮素同样可以在子宫内膜基质细胞中发挥相似的活性, 但其下游发挥关键作用的通路仍未知。NOX4是一种NADPH氧化酶, 通过催化电子从NAPDH转移到分子氧而产生超氧阴离子[23], 进而影响线粒体代谢[24]。而MAPK家族中p38 MAPK表达的变化对氧化应激损伤尤为重要, 其在缓解心肌氧化应激纤维化中的作用已被证实, 经由TGFβ/NOX4/ROS/p38 MAPK通路有望提供新的治疗靶点[25]。目前, 已有诸多研究证明靶向p38 MAPK/NOX4通路在疾病治疗中的优势。全身炎症通过TGFβ/NOX4/ROS/p38 MAPK通路导致心肌氧化应激纤维化, 有望提供新的治疗靶点[26]。多巴胺D4受体通过降低p38 MAPK/NOX4轴相关氧化应激来减慢腹主动脉瘤的生长趋势[27]。然而, 目前尚无此通路在保护子宫内膜损伤中的作用的相关研究。本研究证实, H_2O_2刺激的HESCs损伤状态下其p38 MAPK和NOX4表达明显上调, 而通过给予槲皮素治疗后, 可明显降低p38 MAPK和NOX4的表达。这提示槲皮素在一定程度上可以抑制p38 MAPK和NOX4的表达, 进而通过抗氧化作用保护H_2O_2诱导的人子宫内膜基质细胞氧化应激损伤。

综上所述, 本研究明确了槲皮素在保护子宫内膜基质细胞免受氧化损伤中的作用, 其机制可能与减少ROS过量累积以及通过抑制p38 MAPK/NOX4信号通路有关。因此, 槲皮素可能成为子宫内膜基质细胞的保护药物, 并有望为子宫内膜损伤及其相关疾病的临床治疗提供新的途径。但本研究主要集中于槲皮素对氧化应激损伤的保护作用, 而对于槲皮素在子宫内膜基质细胞中的全面作用了解可能存在不足, 需要进一步探讨。

*　　*　　*

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Author Contribution　CHEN Xiunan is responsible for formal analysis, writing--original draft, and writing--review and editing. WANG Ruiqi is responsible for formal analysis, visualization, and writing--original draft. SHAN Hongying is responsible for resources and supervision. ZHOU Ping is responsible for data curation, methodology, resources, and writing--review and editing. LI Rong is responsible for conceptualization, funding acquisition, supervision, and writing--review and editing. All authors consented to the submission of the article to the Journal. All authors approved the final version to be published and agreed to take responsibility for all aspects of the work.

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