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汉黄芩苷通过调节抗氧化基因表达和机体代谢延长果蝇寿命

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摘要 为探究汉黄芩苷是否具有抗衰老作用,本研究以果蝇为模型,考察汉黄芩苷对果蝇自然寿命的影响。采用 RT-PCR 和 UPLC-MS/MS 代谢组学技术,探索汉黄芩苷发挥抗衰老作用的潜在机制。结果显示,0.02 和 0.5 mg/mL 汉黄芩苷均可整体延长果蝇寿命,并能够分别延长果蝇平均寿命 5.64% 和 5.39%,延长最高寿命 2.74% 和 5.12%;与 30 d 组相比,汉黄芩苷能够显著上调果蝇体内抗氧化酶基因 *SOD*1、*SOD*2 和 *CAT* 的表达水平,下调 *MTH* 的表达水平。果蝇代谢组学分析共找到 17 个潜在生物标志物,主要参与氨基酸代谢(D-谷氨酰胺和 D-谷氨酸代谢,丙氨酸、天冬氨酸和谷氨酸代谢,精氨酸和脯氨酸代谢,缬氨酸、亮氨酸和异亮氨酸代谢)和能量代谢(氮代谢)。该结果表明,汉黄芩苷延缓衰老与上调抗氧化基因表达和调控不同代谢途径有关。

关键词 汉黄芩苷;黑腹果蝇;衰老;基因表达;UPLC-MS/MS 代谢组学

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Wogonoside Extends the Lifespan of *Drosophila melanogaster* by Regulating the Expression of Antioxidant Genes and Body Metabolism

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Abstract To investigate whether wogonoside has an anti-aging role, we studied its effect on the natural lifespan of *Drosophila melanogaster*. Metabonomics techniques, such as RT-PCR and UPLC-MS/MS, were used to explore the potential anti-aging effect of wogonoside. The results showed that 0.02 mg/mL and 0.5 mg/mL wogonoside could significantly prolong the overall longevity of fruit flies and improve the mean lifespan by 5.64% and 5.39%, and the maximum lifespan by 2.74% and 5.12%, respectively. Dietary supplement of wogonoside significantly up-regulated the expression of antioxidant genes *SOD*1, *SOD*2 and *CAT*, and down-regulated the expression of *MTH* in fruit flies, compared with the 30 d group. Seventeen potential biomarkers regulated by wogonoside treatment were found. The metabolic pathway

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analysis suggested that wogonoside antagonizes senescence mainly through modulating amino acid metabolism (D-glutamine and D-glutamate metabolism; alanine, aspartate and glutamate metabolism; arginine and proline metabolism; valine, leucine and isoleucine biosynthesis) and energy metabolism (nitrogen metabolism). The results suggest that the anti-aging role of wogonoside is associated with increasing the expression of antioxidant genes and regulating different metabolic pathways.

Key words wogonoside; *Drosophila melanogaster*; aging; gene expression; UPLC-MS/MS metabonomics

衰老是机体成熟后,各器官功能普遍的、逐渐降低的过程。涉及衰老的机制学说有多种,包括:自由基学说、免疫学说、端粒酶学说、神经内分泌学说和DNA 损伤修复学说等。现代药理研究表明,自由基累积引起的氧化损伤是导致衰老及相关疾病的一个主要因素[1-3]。

中药黄芩为唇形科植物黄芩(*Scutellaria baicalensis* Georgi)的干燥根,是我国的大宗药材之一,具有清热燥湿、泻火解毒、凉血安胎之功效。其活性成分主要是黄酮类,包括黄芩素、黄芩苷、汉黄芩素和汉黄芩苷等,它们的药理作用广泛[4],主要包括:清除自由基和抗氧化[5-7]、抗炎[7]、神经保护[8]、抗病毒以及抗肿瘤作用等。据文献报道,黄芩素[9-10]、黄芩苷[11]和汉黄芩素[12]均具有改善衰老相关特征,如寿命、学习和记忆能力的作用。然而,汉黄芩苷作为黄芩的主要成分之一,目前关于其是否具有抗衰老活性及其可能的作用机制的研究却鲜有报道。

代谢组学是研究在外部刺激或内部干扰,如药物、饮食治疗或病态情况下,机体生物体系(细胞、体液或组织)产生的所有代谢产物种类和数量变化的一门科学。通过对疾病引起的代谢产物变化进行代谢组学分析,有助于人们更好地理解病变过程及机体内物质的代谢途径[13-14]。液相-质谱联用(liquid chromatography-mass spectrometry, LC-MS)技术被广泛用于代谢组学分析,其具有分离效能高、分析速度快以及检测灵敏度高等特点。另外,LC-MS 技术对代谢物的检测比较全面,可以同时检测出数百种化合物,包括氨基酸、脂肪酸、糖类和有机酸等,能够比较完整地展现出整个代谢轮廓[15-16]。因此,基于 LC-MS 技术的代谢组学越来越多地被用于药物活性评价及其作用机制的研究。

本研究以果蝇(*Drosophila melanogaster*)为模式生物,考察汉黄芩苷的抗衰老活性。通过测定果蝇体内铜锌超氧化物歧化酶基因(copper-zinc superoxide dismutase, *SOD*1)、锰超氧化物歧化酶基因(manganese superoxide dismutase, *SOD*2)、过氧化氢酶基因(catalase, *CAT*)以及玛士撒拉基因(methuselah, *MTH*)的表达水平,评价汉黄芩苷的抗氧化水平;并通过结合超高效液相色谱串联质谱(ultra performance liquid chromatography tandem mass spectrometry, UPLC-MS/MS)代谢组学技术,研究汉黄芩苷对果蝇内源性代谢产物的影响,进一步探索汉黄芩苷延缓衰老的作用机制。

1　材料与方法

1.1　实验动物

野生型 W^{1118} 品系黑腹果蝇由山西大学应用生物学研究所提供,饲养于温度为 25 ℃,湿度为 65%,自然昼夜节律光照的智能人工气候箱中。

1.2　试剂

汉黄芩苷(质量分数 >98%)购自成都瑞芬思科技有限公司;引物 *SOD*1(NM_057387.3)、*SOD*2(NM_057577.3)、*CAT*(NM_080483.3)、*MTH*(AF_109308.1)和 *Rp*49(NM_079843.2)由生工生物工程(上海)股份有限公司合成;0.1% 焦碳酸二乙酯(diethylpyrocarbonate, DEPC)水溶液购自生工生物工程(上海)股份有限公司;RNAiso Plus, PrimeScript™ RT Master Mix(Perfect Real Time),和 SYBR Premix Ex Taq™ Ⅱ(Tli RNaseH Plus)均购自宝生物工程(大连)有限公司;分析纯的无水乙醇、氯仿、异丙醇和丙酸均购自天津市风船化学试剂科技有限公司;色谱纯的甲醇和乙腈购自美国赛默飞世尔科技公司。

1.3　仪器

MGC-350HP-2 人工气候箱,购自 上海一恒科学仪器有限公司;玻璃匀浆器(1 mL、2 mL),江苏盐城泓宇玻璃仪器厂;T960 热循环仪、CG-02 实时荧光定量 PCR 仪和 Neofuge 13R 高速冷冻离心机,力康生物医疗科技控股有限公司;NanoDrop 2000 Spectrophotometer 和 Dionex UltiMate 3000 UHPLC-Q Exactive Orbitrap-MS,美国赛默飞世尔科技公司;Acquity UPLC HSS T3 column(2.1 mm × 100 mm, 1.8 μm),美国沃特世科技(上海)有限公司。

1.4　培养基的制备

将琼脂 10 g、玉米 85 g、蔗糖 70 g 和酵母粉 18

g,加水至1 L加热煮沸。然后,加入丙酸5 mL配制成空白培养基。在空白培养基中,分别添加浓度为0.02和0.5 mg/mL的汉黄芩苷(Wogonoside,Ws)制备成含药培养基。

1.5 果蝇寿命试验

收集3 d内羽化的雄性果蝇成虫,随机分为3组(空白对照组、汉黄芩苷0.02 mg/mL组与0.5 mg/mL组),每组200只,于温度25 ℃、湿度65%的恒温恒湿智能人工气候箱中培养,每隔2 d更换1次相应的新鲜培养基。每天统计死亡的果蝇数量,直至果蝇全部死亡,绘制生存曲线,计算果蝇的平均寿命、中位寿命和最高寿命。

1.6 果蝇体内基因表达水平测定

1.6.1 样本收集 收集3 d内羽化的雄性果蝇成虫,随机分为4组(空白对照2组:3 d组和30 d组、30 d + 0.02 mg/mL Ws和30 d + 0.5 mg/mL Ws组),其中3 d组和30 d组是以空白培养基喂养,30 d + 0.02 mg/mL Ws组和30 d + 0.5 mg/mL Ws组分别以含汉黄芩苷浓度0.02 mg/mL和0.5 mg/mL的培养基喂养,分别于第3 d和第30 d,饥饿2 h后收集果蝇样本。收集的所有果蝇样本以液氮速冻后,转移至-80 ℃保存,备用。

1.6.2 实时定量荧光 PCR(quantitative real-time PCR,qRT-PCR) 取15只果蝇置于预冷的玻璃匀浆器中,加入800 μL RNAiso Plus试剂后,冰浴下快速匀浆至完全均质。转入1.5 mL EP管中,于4 ℃,12 000 g,离心10 min。吸取上清液于新的EP管中,加入200 μL氯仿,充分震荡混匀15 s,室温下静置5 min,4 ℃,12 000 g,离心15 min。吸取上层清液200 μL于新的EP管中,加入等体积异丙醇,轻柔混匀后,室温下静置10 min,4 ℃,12 000 g,离心10 min。弃去上清液,加入1 mL 75%乙醇洗涤沉淀,4 ℃,12 000 g,离心5 min,弃去上清液,重复1次。沉淀自然晾干,加入20 μL 0.1%的DEPC水溶解总RNA沉淀,测定RNA的浓度与纯度。按照试剂盒说明,将RNA反转录成cDNA,采用Real-time PCR法,以*Rp*49为管家基因,测定抗氧化相关基因*SOD*1、*SOD*2、*CAT*与长寿基因*MTH*的mRNA表达水平[17-18]。

1.7 果蝇代谢组学分析

1.7.1 样本制备 样本收集同1.6.1。取10只果蝇,加入200 μL提取溶剂(甲醇:水=4:1),在冰浴下匀浆至完全均质,涡旋30 s,4 ℃,13 000 g,离心20 min,吸取上清液转移入新离心管中,4 ℃,13 000 g,离心10 min,上清液移入带有衬管的液相小瓶中,从每个待测样本中吸取10 μL,混合后,制备成QC样本。所有样本均置于-80 ℃保存。分析前,样本于4 ℃解冻。

1.7.2 液质联用方法 色谱条件 色谱柱:Acquity UPLC HSS T3 column(2.1 mm × 100 mm,1.8 μm),流动相A:0.1%甲酸水溶液,流动相B:0.1%甲酸乙腈溶液;洗脱程序:0 min:3% B,1.5 min:3% B,5 min:49% B,15 min:98% B,17 min:98% B,18 min:3% B,21 min:3% B;柱温:40 ℃;自动进样器温度10 ℃;进样量10 μL;流速0.25 mL/min。质谱条件 采用加热电喷雾离子源(HESI),喷雾电压:3 500 V(+)、2 500 V(-),毛细管温度:320 ℃(±),鞘气流速:35 arb(±),辅气流速:10 arb(±);采用正负离子切换采集模式,扫描模式:Full Scan/dd-MS2;Full MS分辨率:70 000 FWHM,AGC target:1×10^6;dd-MS2分辨率:17 500 FWHM,AGC target:1×10^5;碰撞能量(Stepped NCE):10%,30%,50%;质量扫描范围:m/z 100 ~ 1 000。每8个样本,插入1个QC样本。

1.7.3 液质数据处理 将Xcalibur工作站采集到的所有数据导入Compound Discoverer 2.0软件,该软件可自动完成谱峰识别、滤噪等前处理。具体处理参数设置如下:质量范围:100 ~ 1 000 Da,质量偏差:5 ppm,信噪比阈值:3,最少元素数目:C、H,最多元素数目:C、H、O、N、P、S,分配阈值:70。

将上述处理所得数据导入Excel 2007中进行峰面积归一化后,再导入Simca-P 13.0中,进行多元统计分析。采用偏最小二乘判别分析(partial least-squares discriminant analysis,PLS-DA)和正交偏最小方差判别分析(orthogonal partial least-squares discriminant analysis,OPLS-DA)考察3 d组与30 d组的分离情况,同时结合S-plot载荷图找出对分类起主要作用的变量,以*VIP* > 1以及T检验($P < 0.05$)找出差异代谢物。采用PLS-DA考察4组样本的分离情况,对已找到的差异代谢物的峰面积进行4组样本间的单因素方差分析,找出与30 d组相比,汉黄芩苷能够显著回调的潜在生物标志物。将找到的潜在生物标志物的峰面积导入在线数据库MetaboAnalyst 3.0(http://www.metaboanalyst.ca)中,进行皮尔森相关性分析。使用在线数据库:Metlin(http://metlin.scripps.edu),HMDB(http://www.hmdb.ca),Massbank(http://www.massbank.jp),Lipid Maps(http://www.lipidmaps.org),KEGG

(http://www.kegg.jp), Biocyc (https://biocyc.org), Pubchem (https://pubchem.ncbi.nlm.nih.gov/)对代谢物进行指认。

1.8 统计分析

使用 SPSS 16.0 和 Graphpad prism 5 软件对实验数据进行统计分析,实验结果采用平均值 ± 标准误($\bar{x} \pm SEM$)表示。汉黄芩苷给药组与空白对照组间生存率比较采用 Kaplan-Meier 检验,平均值与最高寿命比较应用组间样本方差分析。$P < 0.05$ 表示具有统计学意义。

2 结果

2.1 汉黄芩苷延长果蝇自然寿命

与空白对照组相比,在培养基中添加 0.02 mg/mL 和 0.5 mg/mL 汉黄芩苷均能显著延长果蝇寿命(Fig. 1A, B)。如 Table 1 所示,0.02 mg/mL 和 0.5 mg/mL 的汉黄芩苷可分别提高果蝇平均寿命 5.64% 和 5.39%($P < 0.01$);0.02 和 0.5 mg/mL 汉黄芩苷可分别提高果蝇最高寿命 2.74% 和 5.12%($P < 0.05$ 或 $P < 0.001$)。

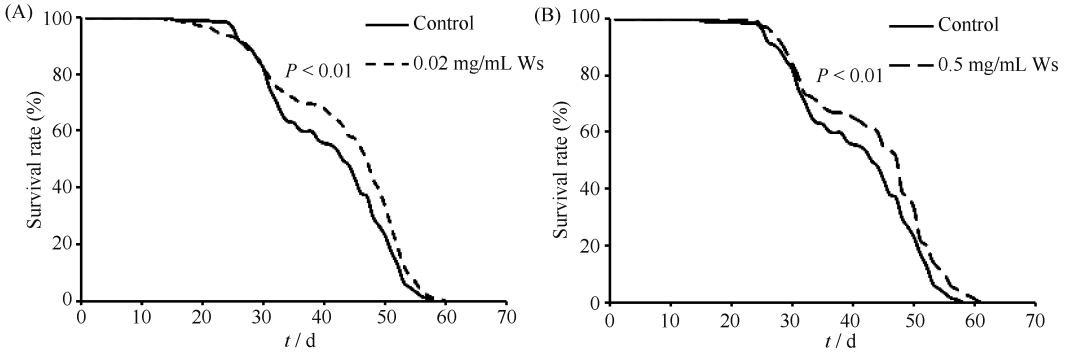

Fig. 1 Survival curves of *Drosophila melanogaster* treated with wogonoside (A) (B) After fruit flies were treated with different concentrations of wogonoside (0, 0.02 and 0.5 mg/mL Ws), the survival rate was examined by the Kaplan-Meier method and Log-rank test. $P < 0.01$, compared with the control group (0 mg/mL Ws)

Table 1 Effects of wogonoside on the lifespan of *Drosophila melanogaster* in different groups

Group	Mean lifespan (days)	Median lifespan (days)	*P*-value (Log-rank test)	Maximum lifespan (days)
Control	40.8 ± 0.7	43.0 ± 1.6	—	54.7 ± 0.4
0.02 mg/mL Ws	43.1 ± 0.8 **	47.0 ± 0.8 **	< 0.01	56.2 ± 0.4 *
0.50 mg/mL Ws	43.0 ± 0.8 **	47.0 ± 0.7 **	< 0.01	57.5 ± 0.5 ***

Values are the mean ± SEM of 200 fruit flies from every group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with the control group

2.2 汉黄芩苷上调果蝇体内抗氧化酶基因 *SOD*1、*SOD*2、*CAT* 的表达并下调 MTH 水平

RT-PCR 的结果(Fig. 2)显示,与 3 d 组相比,30 d 组的果蝇体内的抗氧化基因 *SOD*1、*SOD*2 和 *CAT* 的表达水平均显著降低($P < 0.01$, $P < 0.001$),长寿基因 *MTH* 的表达水平显著升高($P < 0.05$)。与 30 d 组相比,在果蝇膳食中添加 0.02 mg/mL 汉黄芩苷,能够显著上调果蝇体内 *SOD*1、*SOD*2 和 *CAT* 基因的表达水平($P < 0.05$),并显著下调 *MTH* 基因表达水平($P < 0.001$);0.5 mg/mL 汉黄芩苷能够显著回调 *SOD*1 和 *MTH* 基因,对 *SOD*2 和 *CAT* 基因的表达也有轻微回调作用。

2.3 代谢组学分析

2.3.1 UPLC-MS/MS 数据多元统计分析　为探究随年龄增长果蝇体内代谢物变化,采用 PLS-DA 方法对 3 d 组与 30 d 组果蝇样本获得的复杂数据进行降维处理(Fig. 3A)。结果显示,3 d 组与 30 d 组果蝇明显分开,表明伴随着年龄的增加,果蝇的代谢轮廓会发生一定的变化。采用排列实验(permutation test)对 PLS-DA 模型进行验证(Fig. 3B)。结果显示,Q2 点的回归线与纵轴的截距小于 0,左侧的所有 Q2 值和 R2 值均低于右侧的原始点,表明 PLS-DA 模型质量较好。为确定果蝇随年龄增加而发生明显变化的内源性代谢物,采用 OPLS-DA 方法对 3 d 和 30 d 两组果蝇样本重建模型(Fig. 3C)。结果表明,30 d 龄果蝇体内内源性代谢产物与 3 d 龄果蝇存在明显差异。在 OPLS-DA 模型的基础上,结合 S-plot 载荷图(Fig. 3D),*VIP* 值(>1)及 *t*-检验($P < 0.05$),共确定 27 个差异代谢物并通过在线数据库进行指认,结果见 Fig. 4, Table 2 和 Table 3。为

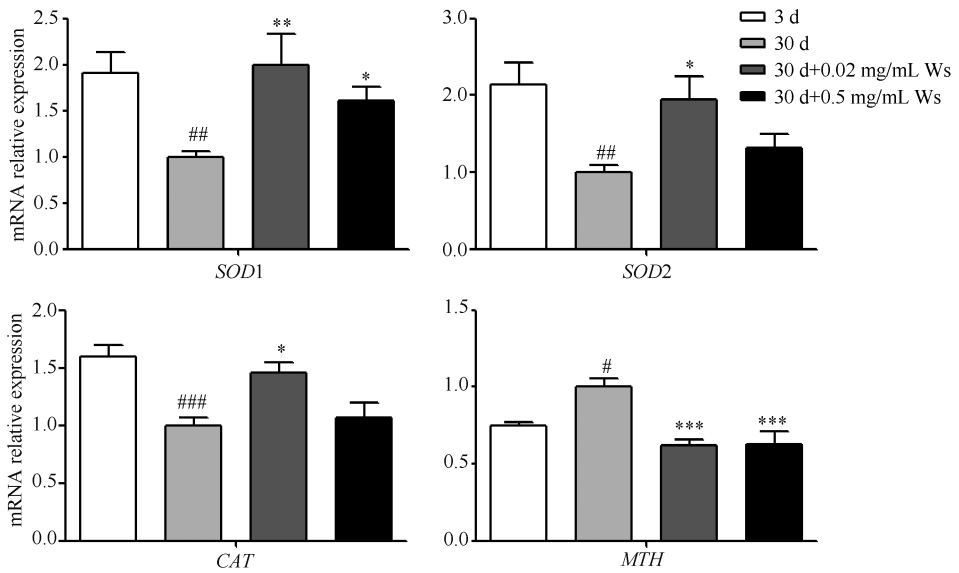

Fig. 2 Effects of wogonoside on the antioxidant genes and *MTH* gene levels of *Drosophila melanogaster* in different groups The expression levels of genes were obtained by the RT-PCR experiment. Data are expressed as mean ± SEM. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, compared with the corresponding 3-day group without Ws; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the corresponding 30-day group

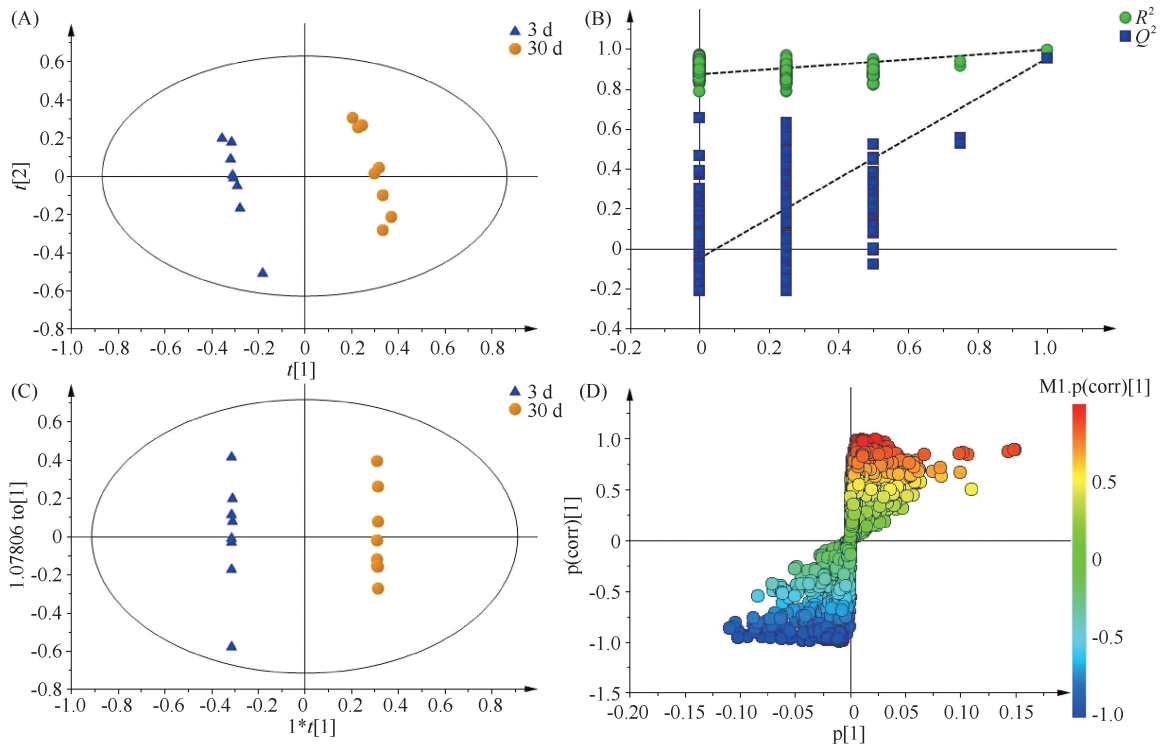

Fig. 3 Multivariate statistical analysis of UPLC-MS/MS data for *Drosophila melanogaster* samples in the 3-day and 30-day groups PLS-DA score plots ($R^2X=0.502$, $R^2Y=1$, $Q^2=0.969$) (A): the horizontal axis indicates the scores of every sample point on the first principal component and the vertical axis indicates the scores of the every sample point on the second principal component; Permutation test (B): the PLS-DA model was validated using the response of the permutation test through 200 permutations, the regression line of the Q^2-points intersected the vertical axis (on the left) below zero, and all Q^2-values and R^2-values to the left were lower than the original points to the right, indicating a low risk of overfitting; OPLS-DA score plots ($R^2Y=1$, $Q^2=0.931$) (C) combines the quadrature signal correction method and the PLS-DA to modify the PLS-DA; S-plots (D) provides visualization of the OPLS-DA predictive component loading to facilitate model interpretation and reveal the relevant changes of endogenous metabolites responsible for the score plot ($n=8$)

进一步探究汉黄芩苷对年龄增长引起的果蝇体内代谢紊乱的调节作用,对所有组别的果蝇样本进行 PLS-DA 分析(Fig. 5A),结果显示 3 d 组、30 d 组和给药组均明显分开,表明在果蝇膳食中添加浓度为 0.02 或 0.5 mg/mL 的汉黄芩苷,均能够对 30 d 龄果蝇的代谢轮廓产生一定的回调作用。同样采用排列实验对 PLS-DA 模型进行验证(Fig. 5B),结果显示,PLS-DA 模型质量较好。另外,系统的稳定性由 QC 样本监测,PCA 中 QC 样本紧密聚集在一起,表明本研究建立的分析方法适应于该代谢组学研究。为明确汉黄芩苷能够调节的代谢物,对 3 d、30 d、0.02 mg/mL Ws 和 0.5 mg/mL Ws 的 4 组 27 个差异代谢物进行组间差异分析,共找到 17 个能够被汉黄芩苷回调的潜在生物标志物(Fig. 6)。为明确各潜在生物标志物之间的关联性,对其进行皮尔森相关性分析(Fig. 7),图中横轴和纵轴均代表变量的信息,颜色的深浅代表皮尔森相关系数的大小,红色代表正相关,蓝色代表负相关。从横轴来看,差异代谢物被分为两支,在同一分支下的代谢物的正相关性最强,即某一个物质的含量升高或降低,则与之相关性强的物质会随之升高或降低。结合 Fig. 6,可以看出,溶血磷脂酰乙醇胺(18∶3、16∶1、18∶1)、溶血磷脂酰胆碱(18∶3、16∶1、16∶0、18∶1)之间正相关性较强,但与其他差异代谢物之间的负相关性较强,表明各潜在生物标志物之间相互关联,相互影响。

Table 2　Differential metabolites associated with aging identified in *Drosophila melanogaster*

No.	Metabolite	RT (min)	*m/z* Measured	*m/z* Calculated	Adduction	Formula	*VIP*	Fold change[1]	Fold change[2]	Fold change[3]
1	L-Arginine	1.00	175.1189	175.1190	M + H$^+$	$C_6H_{14}N_4O_2$	3.65	0.76$^{\#}$	1.64**	1.83***
2	Histamine	1.01	112.0871	112.0869	M + H$^+$	$C_5H_9N_3$	1.90	0.66$^{\#\#}$	1.26	1.17
3	Pyroglutamic acid	1.03	130.0499	130.0499	M + H$^+$	$C_5H_7NO_3$	4.64	0.76$^{\#\#\#}$	1.21**	1.22**
4	Choline	1.05	104.1073	104.1070	M + H$^+$	$C_5H_{13}NO$	2.15	0.65$^{\#\#}$	1.35	1.40
5	Proline	1.07	116.0707	116.0706	M + H$^+$	$C_5H_9NO_2$	8.92	1.62$^{\#\#\#}$	1.01	1.12
6	L-Acetylcarnitine	1.10	204.1229	204.1230	M + H$^+$	$C_9H_{17}NO_4$	3.92	0.69$^{\#\#}$	1.34*	1.21
7	L-Glutamic acid	1.16	148.0604	148.0603	M + H$^+$	$C_5H_9NO_4$	1.83	1.22$^{\#\#}$	1.04	1.04
8	L-Glutamine	1.19	147.0762	147.0764	M + H$^+$	$C_5H_{10}N_2O_3$	4.78	0.62$^{\#\#\#}$	1.51**	1.66***
9	Taurine	1.20	126.0219	126.0219	M + H$^+$	$C_2H_7NO_3S$	3.18	0.70$^{\#}$	1.07	0.69
10	Gluconic acid	1.20	195.0505	195.0499	M − H$^-$	$C_6H_{12}O_7$	2.19	0.39$^{\#\#}$	0.99	1.69
11	Adenosine 5′-monophosphate	1.67	348.0703	348.0704	M + H$^+$	$C_{10}H_{14}N_5O_7P$	3.42	0.84$^{\#}$	0.98	0.91
12	UDP-N-acetylglucosamine	1.68	606.0745	606.0729	M − H$^-$	$C_{20}H_{18}N_9O_{12}P$	1.98	0.67$^{\#\#\#}$	1.42***	1.46***
13	L-Methionine	1.70	150.0581	150.0583	M + H$^+$	$C_5H_{11}NO_2S$	2.33	0.34$^{\#\#\#}$	1.26	1.02
14	L-Isoleucine	2.21	132.1019	132.1019	M + H$^+$	$C_6H_{13}NO_2$	5.79	0.38$^{\#\#\#}$	1.42**	1.04
15	Adenosine	2.33	268.1039	268.1040	M + H$^+$	$C_{10}H_{13}N_5O_4$	3.54	0.37$^{\#\#}$	0.91	0.77
16	L-Phenylalanine	4.54	166.0861	166.0863	M + H$^+$	$C_9H_{11}NO_2$	1.28	0.68$^{\#}$	1.14	0.84
17	3-Hydroxydecanoic acid	8.49	187.1334	187.1334	M − H$^-$	$C_{10}H_{20}O_3$	2.12	0.55$^{\#\#\#}$	1.52**	1.50**
18	LysoPE(18∶3)	9.93	476.2771	476.2772	M + H$^+$	$C_{23}H_{42}NO_7P$	3.20	2.91$^{\#\#\#}$	0.39***	0.33***
19	LysoPC(18∶3)	10.03	518.3246	518.3241	M + H$^+$	$C_{26}H_{48}NO_7P$	1.75	2.68$^{\#\#\#}$	0.51***	0.47***
20	LysoPE(16∶1)	10.21	452.2769	452.2772	M + H$^+$	$C_{21}H_{42}NO_7P$	3.87	1.90$^{\#\#\#}$	0.39***	0.35***
21	LysoPC(16∶1)	10.26	494.3240	494.3241	M + H$^+$	$C_{24}H_{48}NO_7P$	3.76	2.07$^{\#\#\#}$	0.46***	0.43***
22	LysoPC(16∶0)	11.34	496.3391	496.3398	M + H$^+$	$C_{24}H_{50}NO_7P$	1.73	1.89$^{\#\#\#}$	0.46***	0.51***
23	Linoleyl carnitine	11.38	424.3415	424.3421	M + H$^+$	$C_{25}H_{45}NO_4$	6.33	0.07$^{\#\#\#}$	5.09	5.51*
24	LysoPE(18∶1)	11.80	480.3083	480.3085	M + H$^+$	$C_{23}H_{46}NO_7P$	1.62	2.00$^{\#\#\#}$	0.49***	0.44***
25	LysoPC(18∶1)	11.86	522.3551	522.3554	M + H$^+$	$C_{26}H_{52}NO_7P$	4.15	2.73$^{\#\#\#}$	0.43***	0.40***
26	L-Palmitoylcarnitine	11.90	400.3418	400.3421	M + H$^+$	$C_{23}H_{45}NO_4$	6.58	0.09$^{\#\#\#}$	5.10*	7.56***
27	Elaidic carnitine	12.17	426.3575	426.3578	M + H$^+$	$C_{25}H_{47}NO_4$	2.69	0.12$^{\#\#\#}$	5.35***	7.86***

Fold change[1] represents the variation of different metabolites in 30 days group comparing with 3 days group; Fold change[2] and Fold change[3] represent the variation of different metabolites in 30 days + 0.02 mg/mL Ws group and 30 days + 0.5 mg/mL Ws group comparing with 30 days group, respectively; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs* 3 days group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* 30 days group

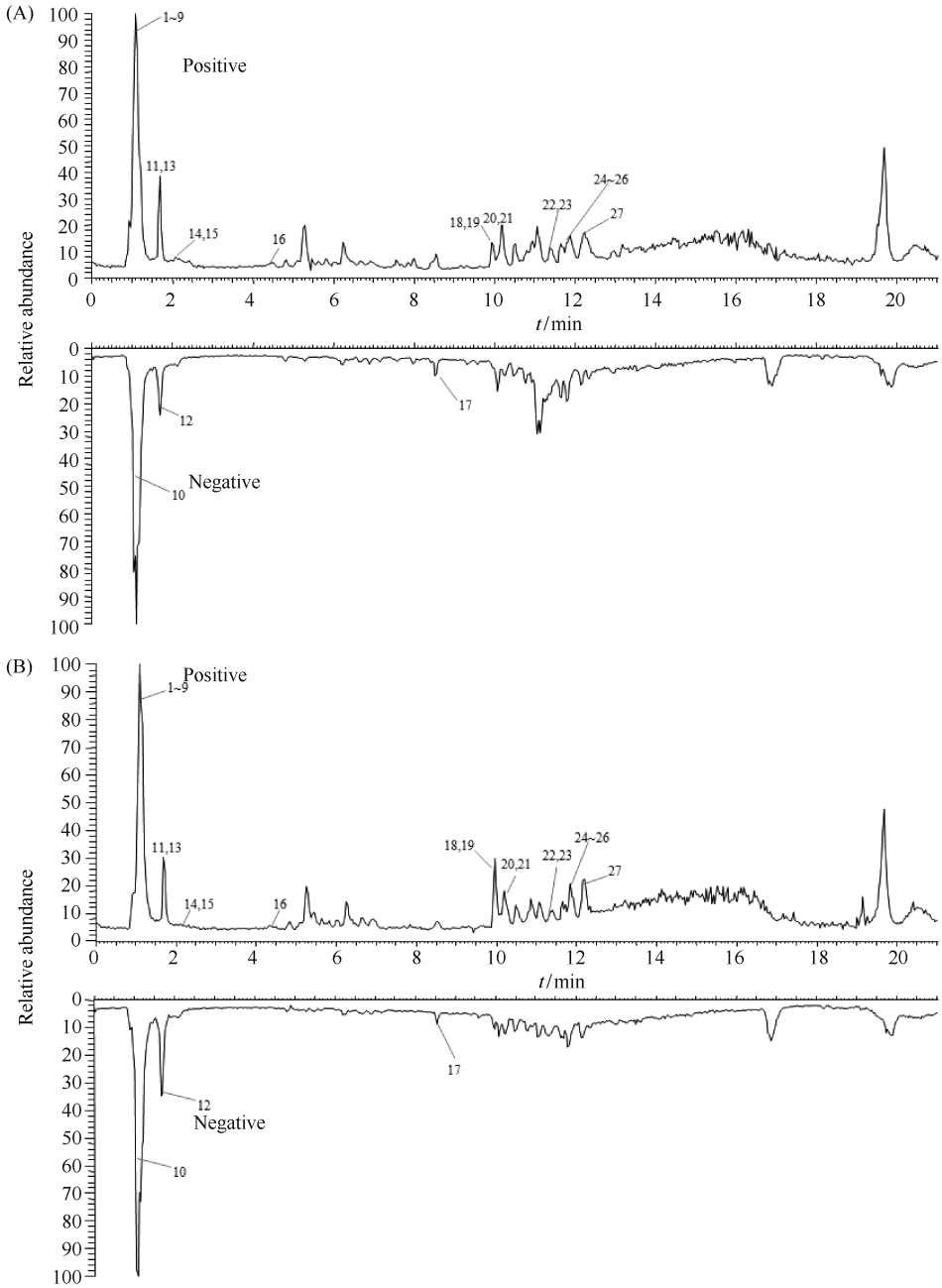

Fig. 4 Total positive ion and negative ion chromatograms of UPLC-MS/MS: the 3-day group (A), the 30-day group (B) Numbers represent differential metabolites in fruit flies between 3-day and 30-days group, which were identified by online database: Metlin (http://metlin.scripps.edu), HMDB (http://www.hmdb.ca), and Pubchem (https://pubchem.ncbi.nlm.nih.gov/). Specific metabolites' information is listed in Table 2

2.3.2 代谢通路分析 为研究汉黄芩苷能够调节的内源性代谢物所涉及到的代谢通路,以及各代谢物间的相互作用关系,将 17 个潜在生物标志物输入 MetaboAnalyst 3.0(http://www.metaboanalyst.ca)数据库中,进行通路富集分析,筛选出汉黄芩苷干预果蝇衰老最相关的 5 条代谢途径(pathway impact > 0.1):① D-谷氨酰胺和 D-谷氨酸代谢;② 氮代谢;③ 丙氨酸,天冬氨酸和谷氨酸代谢;④ 精氨酸和脯氨酸代谢;⑤ 缬氨酸、亮氨酸和异亮氨酸合成(Fig. 8)。结果表明,汉黄芩苷主要通过调节氨基酸的代谢和合成从而干预果蝇衰老过程。另外,参考 KEGG 数据库(http://www.kegg.jp),将所有潜在生物标志物进行关联,构建衰老相关代谢网络图(Fig. 9)。

Table 3　Mass fragment information of the differential metabolites in *D. melanogaster* between 3-day and 30-day groups were obtained by UPLC-MS/MS

No.	Metabolite	RT (min)	Scan mode	MS	MS/MS
1	L-Arginine	1.00	+	175.1189	158.0924, 130.0975, 116.0708, 112.0871, 70.0657, 60.0563
2	Histamine	1.01	+	112.0871	95.0607, 83.0608
3	Pyroglutamic Acid	1.03	+	130.0499	84.0449
4	Choline	1.05	+	104.1073	60.0814, 58.0657
5	Proline	1.07	+	116.0707	70.0657
6	L-Acetylcarnitine	1.10	+	204.1229	145.0495, 85.0289, 60.0814
7	L-Glutamic acid	1.16	+	148.0604	130.0499, 102.0552, 84.0448
8	L-Glutamine	1.19	+	147.0762	130.0499, 84.0448
9	Taurine	1.20	+	126.0219	108.0115
10	Gluconic acid	1.20	−	195.0505	177.0401, 159.0290, 129.0186, 101.0240, 99.0078, 89.0235, 87.0079, 85.0284, 75.0078, 71.0128, 59.0129
11	Adenosine 5′-monophosphate	1.67	+	348.0703	136.0618, 97.02873
12	UDP-N-acetylglucosamine	1.68	−	606.0745	402.9964, 384.9834, 323.0285, 282.0389, 176.9349, 158.9249, 96.9686, 78.9580
13	L-Methionine	1.70	+	150.0581	133.0319, 104.0532, 102.0553, 87.0267, 74.0607, 61.0113, 56.0502
14	L-Isoleucine	2.21	+	132.1019	86.0970, 69.0705
15	Adenosine	2.33	+	268.1039	136.0620
16	L-Phenylalanine	4.54	+	166.0861	131.0491, 120.0809, 103.0545
17	3-Hydroxydecanoic acid	8.49	−	187.1334	59.01289
18	LysoPE(18:3)	9.93	+	476.2771	335.2580, 261.2209
19	LysoPC(18:3)	10.03	+	518.3246	500.3149, 335.2571, 184.0735, 104.1074, 86.0968, 60.0814
20	LysoPE(16:1)	10.21	+	452.2769	434.2663, 311.2581, 280.2633, 237.2210
21	LysoPC(16:1)	10.26	+	494.3240	476.3139, 311.2582, 258.1112, 184.0735, 104.1073, 86.0969, 60.0814
22	LysoPC(16:0)	11.34	+	496.3391	478.3291, 313.2745, 258.1097, 184.0735, 104.1073, 86.0969, 60.0814
23	Linoleyl carnitine	11.38	+	424.3415	85.02879
24	LysoPE(18:1)	11.80	+	480.3083	462.2976, 339.2895, 265.2530
25	LysoPC(18:1)	11.86	+	522.3551	504.3449, 339.2901, 258.1067, 184.0735, 104.1073, 86.0969, 60.0814
26	L-Palmitoylcarnitine	11.90	+	400.3418	341.2689, 239.2366, 85.0288, 60.0814
27	Elaidic carnitine	12.17	+	426.3575	265.2519, 144.1021, 85.0290

The metabolites were identified by online database: Metlin (http://metlin.scripps.edu), HMDB (http://www.hmdb.ca), and Pubchem (https://pubchem.ncbi.nlm.nih.gov/)

3　讨论

果蝇因其生命周期短,易饲养,繁殖能力强以及具有大约 75% 与人类疾病同源的基因而作为研究衰老和衰老相关疾病的模式生物[19-20]。文献报道,中药延缓果蝇衰老是通过调节果蝇体内抗氧化酶及抗氧化基因表达水平实现的:黑豆皮提取物[21]、铁皮石斛原球茎[22]等通过上调果蝇体内抗氧化酶 SOD 和 CAT 活性延缓衰老;紫山药多糖[23]通过提高抗氧化基因的表达实现延长果蝇寿命的作用。另有研究表明,果蝇在衰老时,体内氨基酸和能量代谢会发生紊乱[24]。本研究以果蝇为模型,考察汉黄芩苷对其自然寿命的影响,并结合 RT-PCR 技术和代谢组学方法,从基因表达水平和内源性代谢产物角度,对衰老展开系统地研究,从氧化应激和代谢组学两方面阐明汉黄芩苷抗衰老的作用机制。

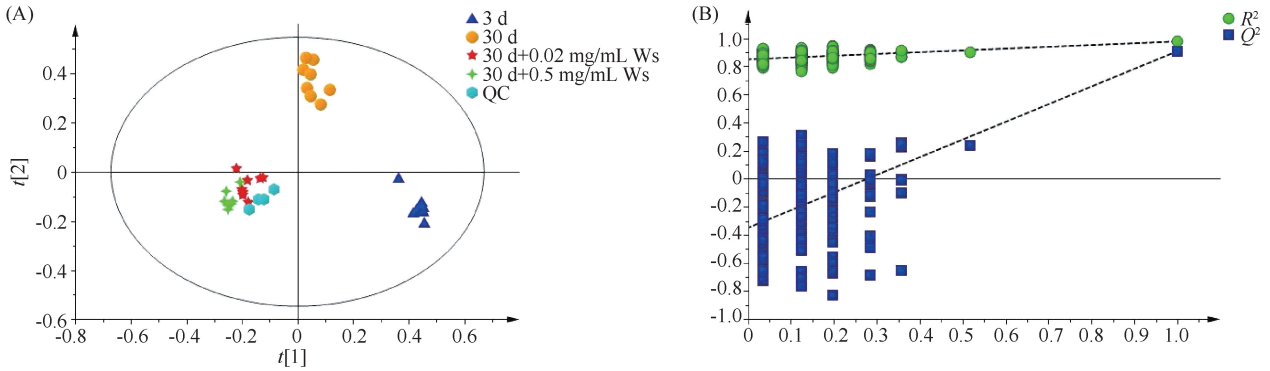

Fig. 5 PLS-DA score plots (A) and the Permutation test (B) of *D. melanogaster* samples collected from different groups Different colors and shapes in the figure indicate different groups: blue triangle, the 3-day group ($n=8$); orange circle, the 30-day group ($n=8$); red five-point star, 30-day + 0.02 mg/mL Ws group ($n=8$); green four-point star, 30-day + 0.5 mg/mL Ws group ($n=8$); light blue hexagon, QC samples group ($n=4$). The permutation test was obtained through 200 permutations ($R^2X=0.588$, $R^2Y=0.983$, $Q^2=0.852$)

自由基理论认为，活性氧（reactive oxygen species，ROS）的堆积是导致衰老以及与衰老相关疾病的一个重要原因。ROS 是指生物体正常代谢产生的含氧自由基和易形成自由基的过氧化物的总称，包括羟自由基、超氧阴离子和过氧化氢等[25]。真核细胞中的线粒体是细胞进行生物氧化的主要场所[26]。当机体内产生的活性氧超过了内源性抗氧化酶，如超氧化物歧化酶（superoxide dismutase，SOD）、过氧化氢酶、谷胱甘肽过氧化物酶（glutathione peroxidase，GPx）等，剩余的自由基会攻击线粒体，破坏细胞内的蛋白质、脂质及 DNA 等，引发氧化损伤，最终导致衰老和疾病[27-29]。真核细胞中的 SOD 主要有 SOD1 和 SOD2 两种亚型，其中 SOD2 存在于线粒体中。体外补充抗氧化剂，如维生素 E、虾青素等可以减弱氧化损伤[30]。据文献报道，SOD 与灵长类物种的寿命和特定代谢率相关[31]；超表达 SOD1 和 CAT 的转基因果蝇的寿命比野生型长 34%[32]；另外，通过靶向线粒体内 CAT 的过表达也可以延长鼠科动物的寿命[33]。*MTH* 基因位于果蝇 3 号染色体上，与果蝇寿命相关，当其发生突变后，成年果蝇的平均寿命会延长约 35%，并且对一系列外界胁迫因素如饥饿、高温以及百草枯引起的氧化应激的耐受性会显著增强。因此，*MTH* 又被称为长寿基因[34-36]。高中洪等的研究表明，黄芩的 4 种黄酮-黄芩苷、黄芩素、汉黄芩素和汉黄芩苷对脑线粒体的氧化损伤具有保护作用[37]。众所周知，酶是由其相对应的基因编码的，基因水平的高低一般表示酶含量的高低。本研究中，汉黄芩苷能够延长果蝇寿命，并上调果蝇体内抗氧化酶基因 *SOD*1、*SOD*2 和 *CAT* 以及下调 *MTH* 的表达水平，表明汉黄芩苷是通过清除体内自由基，改善线粒体氧化损伤的方式发挥抗衰老作用的。

与 3 d 龄果蝇相比，30 d 龄果蝇体内代谢物发生变化，表明机体的衰老可能伴随着代谢紊乱。谷氨酰胺是动物血液和游离氨基酸库中含量最丰富的氨基酸，是动物蛋白质合成的能源和前体，参与能量代谢、抗氧化防御、生物合成以及细胞信号转导等重要生理过程[38-39]。研究表明，在食物中添加谷氨酰胺能够改善老龄大鼠急性心肌梗死时肠屏障功能的损伤[39]，提高杂交鲟抗氧化防御系统的活性和血清非特异性免疫[40]。精氨酸是目前发现的动物细胞内功能最多的氨基酸，在免疫调节、激素分泌、蛋白质合成、细胞分裂和伤口复原等各种生理过程中，都发挥重要的作用。精氨酸是多胺和 NO 等生物活性物质的合成前体，具有提高机体免疫力的作用；参与鸟氨酸循环，有助于将血液中的氨转变为尿素而排泄出去；对于心脑血管疾病等老年慢性疾病具有显著地改善作用[41-42]。异亮氨酸与亮氨酸、缬氨酸同为支链氨基酸，其在调节机体应激、蛋白质的合成与代谢、糖代谢、能量代谢和肌肉代谢平衡中都起重要作用[43-44]。30 d 龄果蝇体内谷氨酰胺、精氨酸和异亮氨酸水平降低，这与闫明亮等[45]通过核磁技术发现衰老果蝇体内谷氨酰胺和异亮氨酸水平降低的结果是一致的，表明机体衰老过程中伴随着能量代谢紊乱和免疫调节能力减弱。给予果蝇汉黄芩苷后，17 个差异代谢物均被显著回调，说明汉黄芩苷可能通过调节机体免疫力，减弱炎症和氧化应激反应来改善衰老引起的代谢紊乱。

机体经新陈代谢不断与外界环境进行物质交换，以维持生物体的繁殖、生长、发育和自我更新。

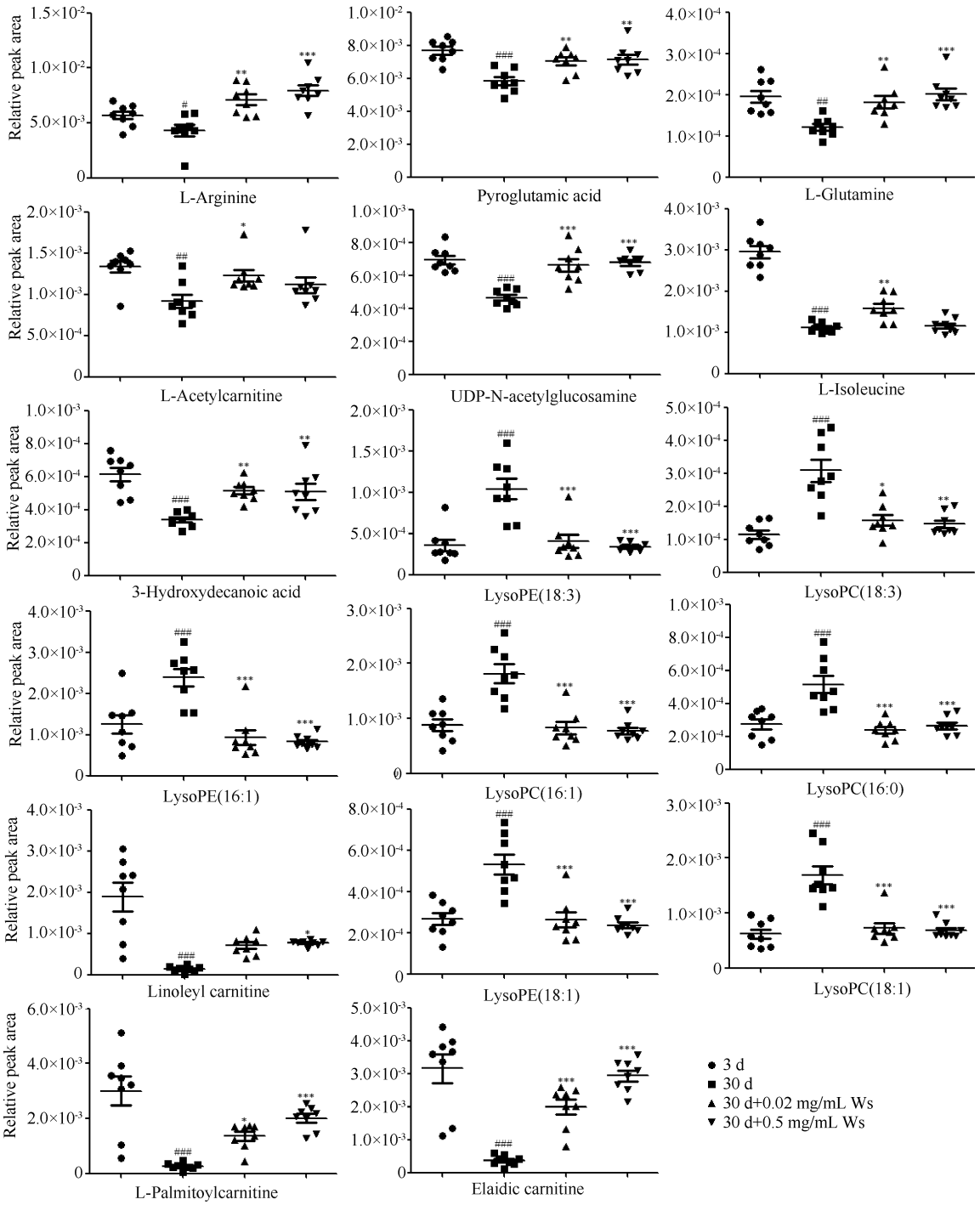

Fig. 6　Comparison on relative peak areas of the potential biomarkers associated with aging, which could be regulated by wogonoside in the tissues of *D. melanogaster* The relative peak areas of metabolites were obtained from UPLC-MS/MS analysis of *Drosophila melanogaster* samples. One-way ANOVA was used to compare the differences in metabolites between different groups. Data are expressed as mean ± SEM. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ compared with the 3-day group; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ compared with the 30-day group

新陈代谢包括物质代谢和能量代谢,物质代谢伴随着能量代谢。有研究报道,黄芩提取物能够干预机体氨基酸代谢、能量代谢等而发挥延缓果蝇衰老的作用[24]。本研究通过对汉黄芩苷延长果蝇寿命所干预的 17 个差异代谢物进行代谢通路分析,发现汉黄芩苷发挥抗衰老作用亦主要通过调节果蝇体内氨基酸代谢(D-谷氨酰胺和 D-谷氨酸代谢;丙氨酸,天冬氨酸和谷氨酸代谢;精氨酸和脯氨酸代谢;缬氨

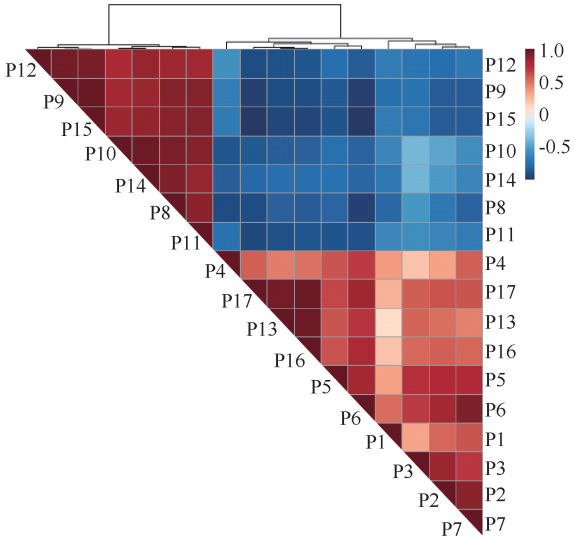

Fig. 7 Pearson correlation analysis of the potential biomarkers Pearson correlation analysis was performed on seventeen potential biomarkers by using the MetaboAnalyst 3.0 (http://www.metaboanalyst.ca). The horizontal and vertical axes represent variables: P1. Arginine, P2. Pyroglutamic Acid, P3. L-Glutamine, P4. L-Acetylcarnitine, P5. UDP-N-acetylglucosamine, P6. L-Isoleucine, P7. 3-Hydroxydecanoic acid, P8. LysoPE(18:3), P9. LysoPC(18:3), P10. LysoPE(16:1), P11. LysoPC(16:1), P12. LysoPC(16:0), P13. Linoleyl carnitine, P14. LysoPE(18:1), P15. LysoPC(18:1), P16. L-Palmitoylcarnitine, P17. Elaidic-carnitine. The color scale represents Pearson correlation coefficients, brown and blue represent positive or negative correlations, respectively. The deep color represents large correlation coefficients and strong correlation

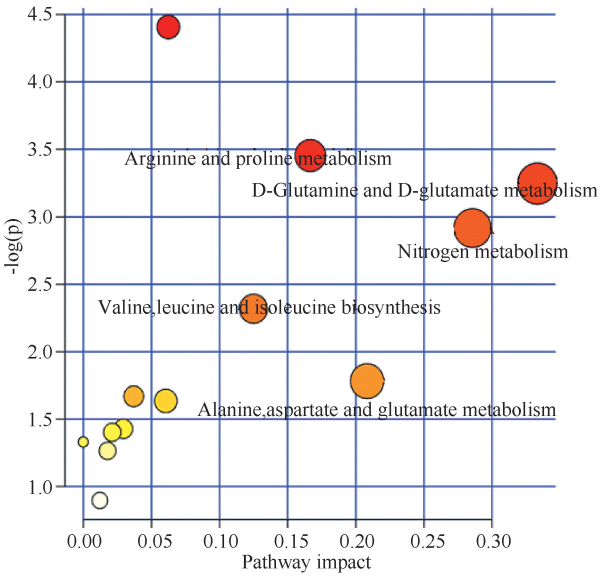

Fig. 8 MetPA analysis of the metabolic pathway MetPA analysis was performed on all potential biomarkers by MetaboAnalyst 3.0 (http://www.metaboanalyst.ca). The abscissa represents the significance value of the metabolic pathway calculated by the topological analysis and the ordinate represents the significance level of the metabolic pathway enrichment analysis. The large Pathway impact and -Log(p) values, large circles, and deep colors indicate stronger correlations between metabolites and metabolic pathways

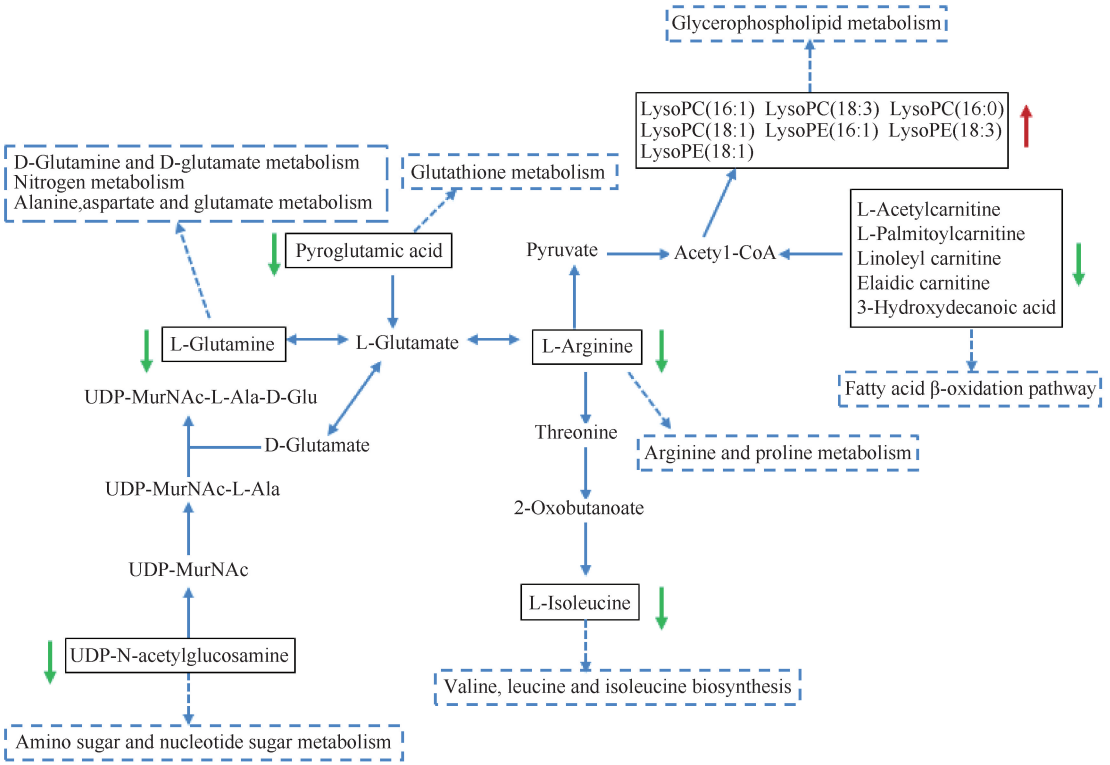

Fig. 9 Network of the disturbed metabolites and metabolic pathways based on the KEGG pathway database The metabolites in the black box are potential biomarkers, "↑" or "↓" indicates a significant increase or decrease with age. After wogonoside treatment, all labeled metabolites were returned

酸、亮氨酸和异亮氨酸合成)以及能量代谢(氮代谢)。氨基酸代谢伴随着能量代谢,能量代谢异常是机体衰老时的一个重要表现,包括线粒体 ATP 生成减少,自由基生成增多并损害细胞,神经内分泌调节紊乱等[46]。线粒体不仅是细胞进行氧化的主要场所,同时也是能量代谢最主要的场所。研究发现,线粒体功能障碍导致的神经损伤是衰老以及神经退行性疾病发生的原因之一[47-50]。另外,线粒体 DNA 的突变,会影响线粒体内氧化磷酸化获得能量的过程,随着年龄的增加,线粒体 DNA 突变明显增加[51]。因此,汉黄芩苷可能通过调控代谢途径保护线粒体功能,进而调节衰老过程中的能量代谢平衡,从而达到延缓衰老的作用。

综上所述,机体衰老过程伴随着氧化应激损伤、线粒体功能障碍和能量代谢紊乱。汉黄芩苷能够通过上调抗氧化基因表达和调控不同的代谢途径来调节衰老过程中的氧化应激和代谢紊乱,从而达到延缓衰老的效果。本研究从基因表达水平和代谢组学角度,对汉黄芩苷干预果蝇衰老的作用机制进行了初探,为后期深入探讨汉黄芩苷延缓衰老的作用机制提供了科学基础。

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