

· 基础研究 ·

基于兔骨质疏松模型的骨质疏松症进展中骨髓代谢组学变化

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[摘要] 目的:探讨兔骨质疏松症(osteoporosis, OP)发生过程中骨髓代谢的变化特点。方法:通过兔双侧卵巢切除术(ovariectomy, OVX)制作兔OP模型模拟绝经期妇女的OP过程。双能X线吸收测量法测量骨密度(bone mineral density, BMD)的变化。通过活体磁共振氢质子波谱(proton magnetic resonance spectroscopy, ¹H-MRS)和离体液相色谱结合质谱(LC-MS/MS)分析等多种方法对骨髓进行代谢组学分析。OVX组和假手术组(Sham组)的取样分析节点均分别设定在术前、术后2个月和术后5个月。结果:随着时间的发展,术后5个月OVX组的BMD值较Sham组明显减低(2个月, $P < 0.05$; 5个月, $P < 0.01$)。¹H-MRS扫描分析表明,与Sham组相比,OVX组骨髓中的乳酸和脂类含量在术后2个月和5个月时均明显增加。OVX组骨髓中的胆碱、肌酸、谷氨酰胺、羟脯氨酸较Sham组减少。在术后2个月时,进行离体骨髓代谢组学分析,也得到了上述相似的骨髓代谢变化结果。此外,¹H-MRS扫描分析发现,OVX组的氨基酸、酰基肉碱、核苷酸和神经酰胺发生了显著变化。相关分析发现,BMD值与乳酸值之间呈明显负相关($r = -0.842, P < 0.01$),BMD与胆碱、肌酸、谷氨酰胺和羟脯氨酸呈显著正相关。结论:本研究发现随着OP进展,骨髓代谢会发生一系列复杂变化。利用¹H-MRS对活体进行骨代谢变化扫描分析可能是评价OP的一种潜在无创方法。

[关键词] 磁共振波谱;骨髓;骨质疏松症;代谢组学分析;时间动态变化

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Metabolic changes in bone marrow during the progression of osteoporosis using a rabbit osteoporotic model

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[Abstract] **Objective:** This study aims to investigate the metabolic changes in bone marrow of female rabbits during the development of osteoporosis (OP). **Methods:** The rabbit osteoporotic model was created by ovariectomy (OVX). The loss of bone mineral density (BMD) was evaluated by dual-energy X-ray absorptiometer (DXA). Metabolomic profiling of bone marrow was conducted through a multiplatform approach including *in vivo* proton magnetic resonance spectroscopy (¹H-MRS) and *in vitro* liquid chromatography coupled to mass spectrometry (LC-MS/MS)-based analysis. The analysis was conducted before the surgery (baseline), 2-month and 5-month after the surgery. **Results:** BMD in OVX group decreased gradually over time and was significantly lower than the Sham group at 5-month after OVX (2-month, $P < 0.05$; 5-month, $P < 0.01$). ¹H-MRS analysis showed lactate and lipid in bone marrow were significantly increased in OVX group. Compared to Sham group, choline, creatine, glutamine and hydroxyproline in bone marrow of OVX group decreased dramatically. Similarly, *in vitro* metabolomic analysis of bone marrow showed dramatic metabolic changes as early as 2-month during the development of osteoporosis. *In vivo* ¹H-MRS analysis showed that, compared to the Sham group, amino acids, acylcarnitines, nucleotides and ceramides were significantly altered. Correlation analysis identified a significant negative correlation between BMD and lactate ($r = -0.842, P < 0.01$). Significant positive correlations were found between BMD and

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choline, creatine, glutamine and hydroxyproline. **Conclusion:** Some complex and diverse changes in bone marrow metabolism was discovered in this study during the development of OP. *In vivo* analysis of bone metabolism using ^1H -MRS might be a potential non-invasive approach for the evaluation of OP.

[**Key words**] proton magnetic resonance spectroscopy; bone marrow; osteoporosis; metabolomics analysis; temporal changes

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随着世界上许多国家特别是亚洲国家逐步进入老龄化社会,骨质疏松症(osteoporosis, OP)已成为最为严重的公共健康问题之一^[1-2]。OP是一种骨代谢疾病,其特征是骨量减少,骨微结构改变,从而导致骨强度降低,骨折风险增加^[3-4]。随着老年人年龄的不断增加,死亡率和一些疾病的发病率都与OP有着越来越密切的关系。对于年长妇女特别是绝经后的妇女,OP也被认为是威胁此类人群健康的重要因素。OP已经严重影响了老年人的生活质量,增加了家庭和社会的医疗负担,因此临床上对于OP的预防、预测和治疗已经越来越重要。

目前已经有多种OP动物模型的建立方法,其中,卵巢切除(ovariectomy, OVX)诱导的兔OP模型是模仿女性绝经后OP的最佳动物模型之一。大鼠因为不能达到真正的骨骼成熟度并且其骨骼缺乏哈弗斯系统,限制了大鼠OP模型的建立^[5]。相比之下,新西兰兔模型有以下优点,包括发育期短、可取材的解剖结构较大、解剖结构尺寸足够支持完成用于评估诱发性OP的手术操作^[6]。此外,兔的OVX模型可以在不使用糖皮质激素等辅助剂的情况下,支持后续对OP进行化疗和外科干预的研究。

骨密度(bone mineral density, BMD)是目前国际学术界公认的诊断OP的金标准^[7],它可以反映骨骼强度的参数,它受骨的几何结构、微结构、骨量和骨基质质量的影响^[8]。BMD可以直接用双能X线吸收法(dual-energy X-ray absorptiometer, DXA)测量,DXA是目前诊断OP的标准方法。根据WHO给出的指导参数,腰椎(L₁~L₄)、股骨颈或髌骨任何部位BMD值的阈值即T值, $-2.5 < T \text{ 值} < -1.0$ 为骨量减少, $T \text{ 值} < -2.5 \text{ SD}$ 则可诊断为OP^[7,9]。此外,高分辨率外周骨定量CT(HR-pQCT)可获得BMD值甚至是骨的微观结构和骨几何结构^[10]。BMD值低的患者往往骨折风险较大,但是,临床上BMD值往往只能反映骨强度的60%~70%^[11]。一些回顾性研究显示,BMD值在识别骨折风险高的个体时并不是最优指标^[12-14]。Maghraoui等^[15]研究发现,16%骨量减少的妇女和8.5%BMD正常的妇女没有较大的骨折风险,但是她

们被检查出有未被发现的脊柱骨折。此外,DXA和HR-pQCT都是放射性检查,不宜被频繁使用。

无论是BMD的测量还是骨微观结构的扫描探查,临床上很多患者在做上述检查时都已经处于OP较长时间,并且因OP引起的并发症(如骨折等)而就诊。Christenson等^[2]研究发现,骨形成和再吸收的生物标志物可能是评估骨代谢的一种有效方法,可以以此来预防OP的进展。最近的相关研究报道^[16-17],骨髓变化可影响骨的代谢,并与OP有关,骨髓间充质干细胞的分化在此机制中起着重要作用。由于OP是一种与骨代谢相关的疾病,了解OP发育过程中骨髓代谢的变化,对OP的早期诊断和治疗至关重要。目前,一些研究主要集中在利用各种功能性核磁共振技术对骨髓成分的变化进行研究^[18],试图找到一种OP生物标志物,以尽早预测OP的发生。

磁共振氢质子波谱(proton magnetic resonance spectroscopy, ^1H -MRS)是一种分子水平成像技术,可以无创地获取活体局部组织中的多种化学物质并进行定量分析。在近期一些研究中^[18-20], ^1H -MRS被用来测量骨髓中的脂肪,但他们的关注点只放在了骨髓脂肪含量或骨髓脂肪与骨体积之间的关系,并没有关注骨髓中其他代谢产物的变化,尤其是缺少对于OP代谢组学分析的研究。

本研究旨在研究OP发展过程中骨代谢变化的特点,为OP的预防、早期诊断和治疗提供帮助。

1 材料和方法

1.1 材料

60只4个月大的新西兰兔(2.50 ± 0.42)kg,均购于广东省医学实验动物中心[实验动物合格证编号:SCXK(粤)2016-0035],在手术前给予2周的适应性喂养。所有兔子均在标准环境下饲养(12 h:12 h 日夜循环,不限制水和食物,温度22~24℃,湿度40%~60%)。本研究中的所有动物实验方案均经江苏医药职业学院伦理委员会的批准(YCZYX-2015-1327),并按照美国国立卫生研究院(NIH)实

验动物使用指南执行。

1.2 方法

1.2.1 OP动物模型与时间序列的建立

新西兰兔被随机分为两组:卵巢切除组(OVX组)和假手术组(Sham组),每组30只。OVX组在无菌条件下进行双侧卵巢切除术,以产生类似于绝经后的雌激素缺乏症^[21]。Sham组也在同样条件下进行手术,但是并不切除双侧卵巢。动物处死后解剖发现OVX组未发现卵巢组织,并且子宫角明显萎缩,证实了卵巢切除术的成功。

分别在术前、术后2个月和术后5个月时对两组进行¹H-MRS扫描、骨密度测量和脂类分析。每个时间节点每组取8只兔子进行实验。

1.2.2 ¹H-MRS扫描分析

用1.5%的异氟醚麻醉动物,并允许其自主呼吸,¹H-MRS扫描期间,兔的体温保持在36.5~37.5℃。固定兔腹部,使其后肢与躯干展开,扫描左侧股骨,扫描时间点分别在术前(此数据作为基线)、术后2个月和5个月。每一时间点扫描8只兔子,按照前期研究中的描述进行¹H-MRS扫描^[22]。使用德国Bruker公司动物专用的9.4T/20磁共振仪(Avance 400 MHz)对兔子进行活体¹H-MRS扫描,采用点分辨自旋回波波谱(point-resolved echo spin spectroscopy, PRESS)序列单体素扫描,取样框定位于股骨近端。扫描参数:体素大小为6 mm×6 mm×14 mm,TR/TE=4 000 ms/15 ms,NA=512,频谱宽度为8 012 Hz。频谱处理使用MRS数据分析包和LC-Model(代谢物溶液模型光谱的线性组合)软件。骨髓脂肪分数(fat fraction, FF)作为脂肪信号相对于总信号(脂肪和水)的百分比,按以下公式计算:
$$FF = [I_{\text{脂肪}} / (I_{\text{脂肪}} + I_{\text{水}})] \times 100\%$$

1.2.3 BMD分析

对被检测兔子实施全身麻醉。分别在术前(此数据作为基线)、术后2个月和5个月,用双能X线吸收仪(DXA)(GE Healthcare公司, LUNARIDXA™, 美国)测量兔子左侧股骨BMD值,每组8只。使用小型实验动物的数据处理软件enCORE software platform(GE Healthcare公司, 美国)进行数据处理^[23]。

1.2.4 骨髓代谢组学分析

每组在每个实验节点(术前、术后2个月、术后5个月)处死8只兔子以获得代谢组学分析的骨髓样本。从左股骨中提取骨髓,并将骨髓细胞颗粒化。代谢组学分析按照先前研究的方法进行,但进行了微调^[24-25]。将400 μL的提取缓冲液(MeOH-Acn-

H₂O, 4:4:2, V/V/V)添加到含有约1×10⁶个骨髓细胞的离心管中(标准偏差≤10%)。将混合物放在冰上静置10 min以沉淀蛋白质和DNA等大分子。之后,在4℃ 16 000 g条件下将混合物离心10 min。上清液用于进一步分析。

采用Agilent 1290高效液相色谱法结合Sciex-qtrap 5500三重四极质谱仪(LC-MS/MS)进行代谢分析。注入10 μL提取物,并在LunaNH₂高效液相色谱柱(250.0 mm×2.0 mm, 5 μm, Phenomenex公司, 美国)上分离代谢物。代谢组学分析采用多反应监测模式,在正负两种模式下,快速切换极性(50 ms)。停留时间设定为3 ms。目标设定共有420种代谢产物,覆盖了所有主要代谢途径^[26]。

使用多量子3.0(Sciex公司, USA)手动检查和整合色谱峰。数据在统计分析前被转换成以2为底的对数。如前所述,使用代谢分析3.0进行多变量分析(PLS-DA、PCA)、代谢通路分析和热图聚类(www.metaboanalyst.ca)^[24]。

1.3 统计学方法

使用GRAPHPAD Prism8.0进行数据分析。采用Shapiro-Wilk分析数据正态分布情况。符合正态分布,数据用均数±标准差($\bar{x} \pm s$)表示。数据为偏态分布,则用中位数与四分位间距表示。多组(3组或以上)分析,采用单因素方差分析,或者重复测量单因素方差分析,Greenhouse-Geisser校正双侧Student's *t*检验用于比较两组之间的显著性水平。线性回归分析或者Pearson相关性分析观察两组之间的相关性。以 $P < 0.05$ 为差异有统计学意义。

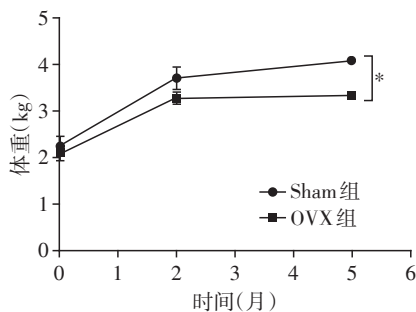
2 结果

2.1 基本特点

在实验最初备用的60只兔子中,有58只在整个研究过程中存活下来,没有观察到手术并发症或肉眼可见的感染迹象。Sham组和OVX组体重均有增加,Sham组体重增长较OVX组快。5个月时,Sham组体重明显高于OVX组[(4.10 ± 0.06) kg vs. (3.80 ± 0.05) kg, $P < 0.05$, 图1]。

2.2 BMD值的变化

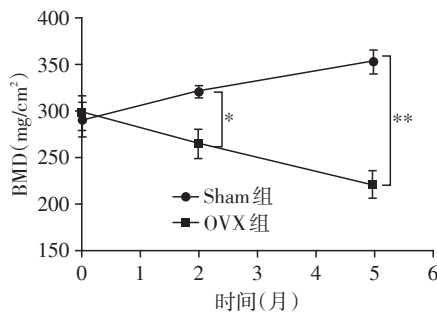
Sham组的BMD随着时间逐渐升高。相反,OVX组的BMD随着时间而逐渐降低。与Sham组相比,术后2个月时OVX组的BMD值显著下降($P < 0.05$),在术后5个月时两组BMD值的差异进一步增大[(319.0 ± 20.1) mg/cm² vs. (76.3 ± 11.3) mg/cm², $P < 0.01$, 图2]。



两组比较, * $P < 0.01$, $n = 8$ 。

图1 OP进展过程中Sham组和OVX组的体重变化

Figure 1 Changes of body weight during the development of OP in Sham group and OVX group



两组比较, * $P < 0.05$, ** $P < 0.01$, $n = 8$ 。

图2 Sham组和OVX组兔左侧股骨BMD值的变化

Figure 2 Changes of bone BMD in rabbit left femur of Sham group and OVX group

2.3 ^1H -MRS 数据分析

为了研究OP进展过程中骨髓代谢的变化,采用 ^1H -MRS技术对兔骨髓中必需的代谢产物进行定量分析。图3显示的是OVX组在术前(基线)、术后2个月和术后5个月时获得的具有代表性的 ^1H -MRS波谱。

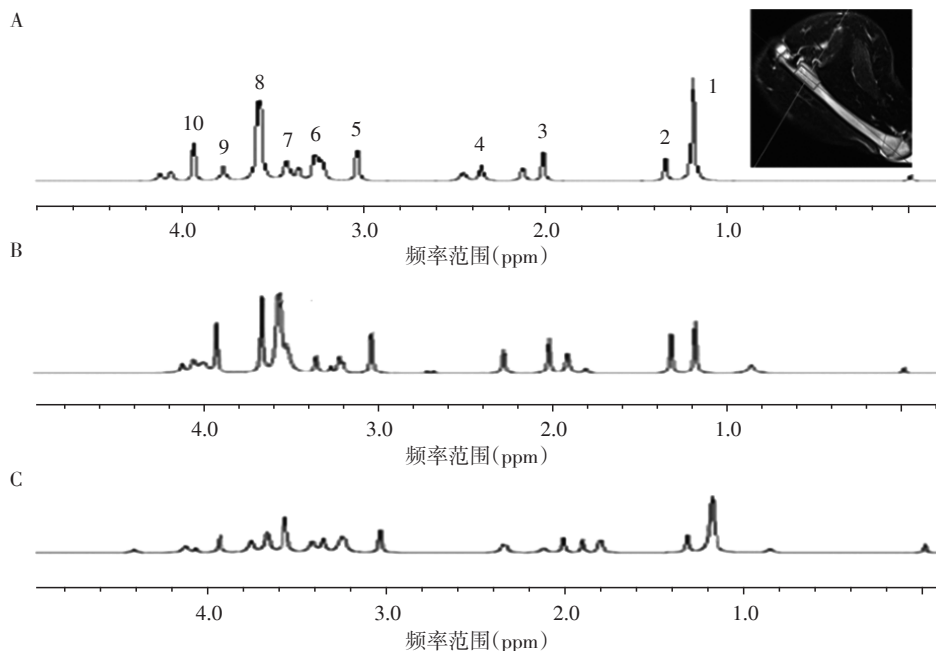
通过 ^1H -MRS扫描,定量分析了骨髓中的9种代谢产物,包括氨基酸、脂类和有机酸。结果显示OVX组兔子的骨髓中,胆碱(choline, Cho)、肌酸(creatine, Cr)、谷氨酰胺(glutamine, Glx)和羟脯氨酸(hydroxyproline)显著降低(图4)。

Sham组兔子骨髓中的Cho含量在5个月内略有增加。而在术后第5个月时,OVX组的Cho含量显

著降低,仅有术前基线值的40%。类似地,Sham组骨髓中的羟脯氨酸水平随着时间的推移显著升高,而OVX组的羟脯氨酸水平与基线水平比较,显著降低($P < 0.01$)。在基线水平上,OVX组和Sham组的乳酸(lactate, Lac)和脂肪含量差异无统计学意义。然而,随着OP的进展,OVX组兔子骨髓中Lac和总脂类积累,在术后5个月时,OVX组的Lac和脂肪含量明显高于基线水平。

2.4 随OP进展骨髓代谢变化的分析

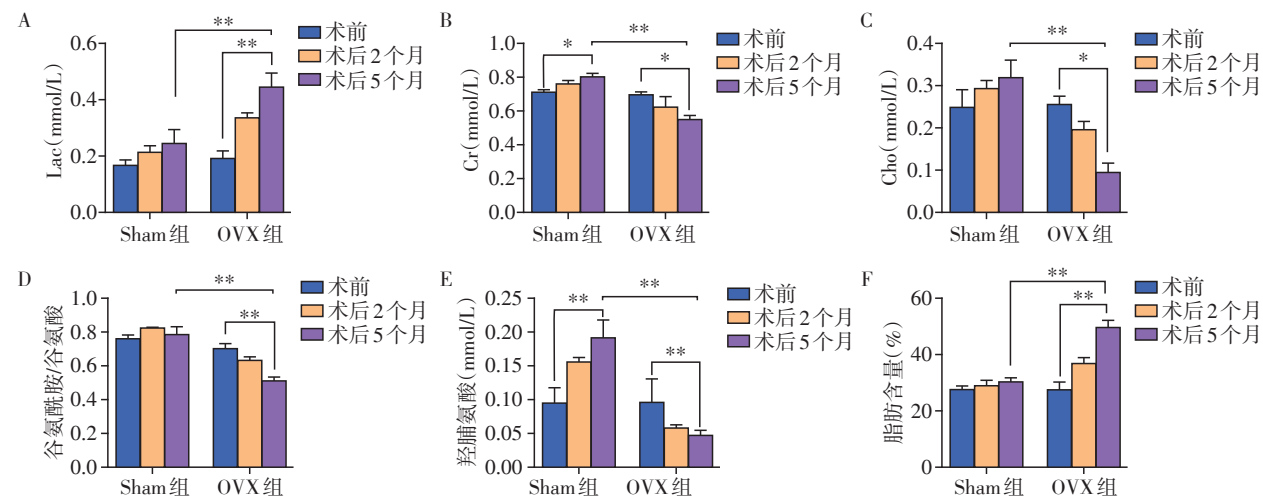
随着OVX组兔子OP进展,对其骨髓代谢变化进行了较广范围的研究。图5是1组30种代谢产物的热图,它们的浓度在OP进展过程中发生了显著



A: 术前(基线); B: OVX术后2个月; C: OVX术后5个月。1: 脂类; 2: 乳酸; 3: 羟脯氨酸; 4: γ -氨基丁酸; 5: 总肌酸; 6: 胆碱; 7: 牛磺酸; 8: 肌醇; 9: 谷氨酰胺; 10: 总肌酸。右上角显示的是在T2WI上选取股骨近端的体素进行 ^1H -MRS扫描。

图3 OVX组兔子左侧股骨的 ^1H -MRS波谱图

Figure 3 *In vivo* ^1H -MRS spectra of rabbit left femur in OVX group



A: 两组 Lac 变化; B: 两组 Cr 变化; C: 两组 Cho 水平变化; D: 两组谷氨酰胺/谷氨酸比值变化; E: 两组羟脯氨酸变化; F: 两组脂肪含量比较。每组不同时间点的差异, 采用重复测量方差分析, 和 Greenhouse-Geisser 校正。Shan 组与 OVX 组相同的时间点的分析, 采用双侧 *t* 检验, 两组比较, **P* < 0.05, ***P* < 0.01, *n* = 8。

图4 两组兔子骨髓中各主要代谢物随OP进展的¹H-MRS数据变化

Figure 4 Changes of metabolites in rabbit bone marrow of two groups during the development of OP by ¹H-MRS

变化。对 OVX 组进行的全球代谢数据 PLS-DA 分析, 显示了 3 个时间点之间的显著代谢差异(图 6)。在 VIP(variance in projection)分析后进行 PLS-DA 分析, 图 7 所示的是前 30 种变化最明显的代谢物。

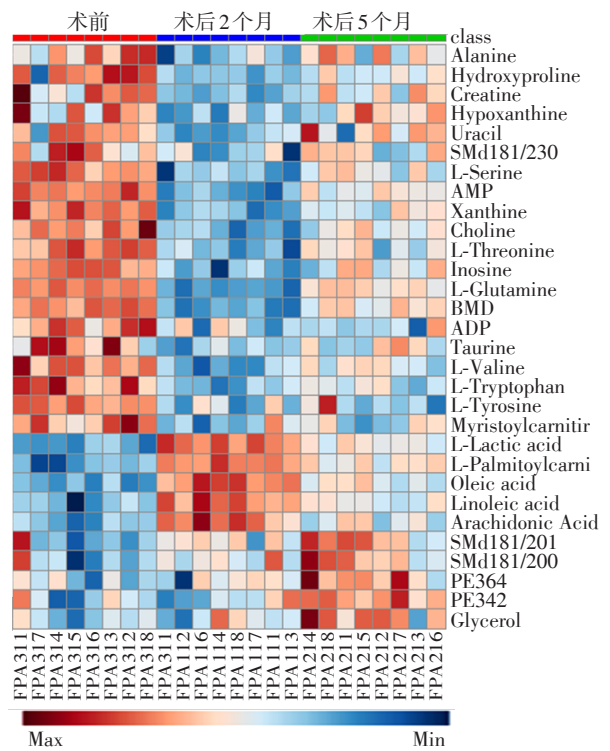


图5 OVX组随OP进展浓度变化最明显的30种物质的热图
Figure 5 Heat map of a subset 30 metabolites significantly changed in OVX group during the development of OP

本研究结果显示, 在 OP 进展过程中, 骨髓中的氨基酸、酰基肉碱、核苷酸和神经酰胺发生了较大变化(图 7)。特别是 OVX 组的骨髓代谢表现出脂肪酸氧化抑制、游离脂肪酸积聚和酰基肉碱减少的特征(图 8)。此外, 还发现 OVX 组骨髓的氨基酸和嘌呤代谢显著降低(图 8)。

2.5 利用所选生物标志物进行¹H-MRS预测

OVX 组 Lac 水平与 BMD 值呈明显负相关(*r* = -

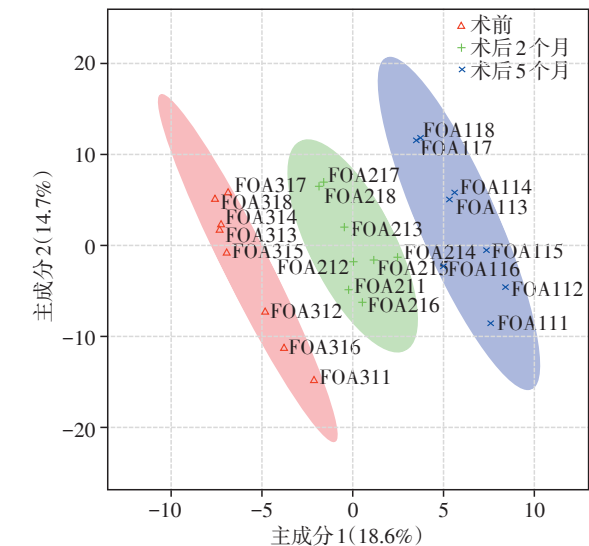


图6 PLS-DA显示了OVX组骨髓代谢产物在术前、术后2个月和术后5个月的显著差异
Figure 6 PLS-DA showed the dramatic metabolic differences in OVX group between baseline, 2 month and 5-month samples

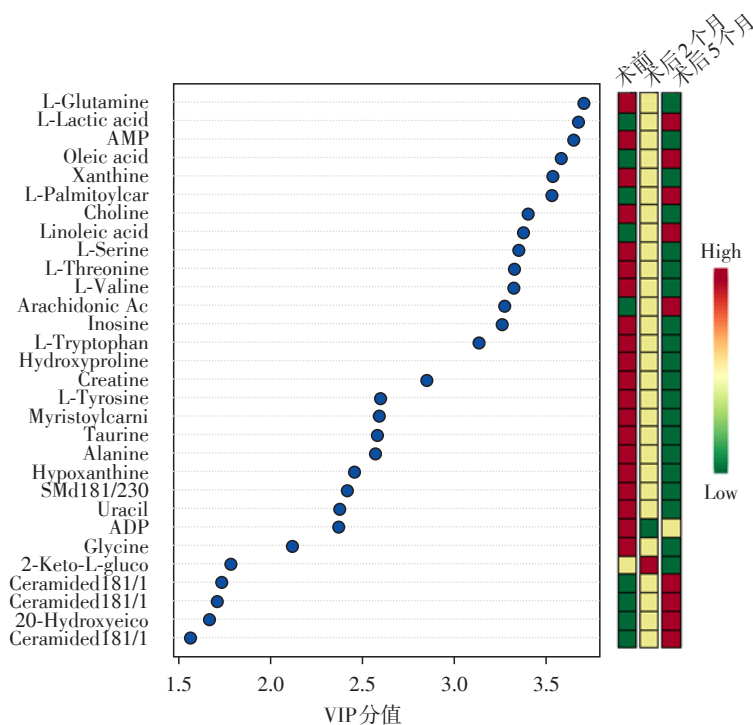


图7 VIP分析显示两组间30种代谢变化最明显的物质

Figure 7 VIP analysis showed the top 30 most discriminating metabolites between two groups

0.842, $P < 0.01$, 图9)。相反, BMD值和谷氨酰胺、一磷酸腺苷(adenosine monophosphate, AMP)和苏氨酸(L-threonine)之间呈明显正相关(图10)。

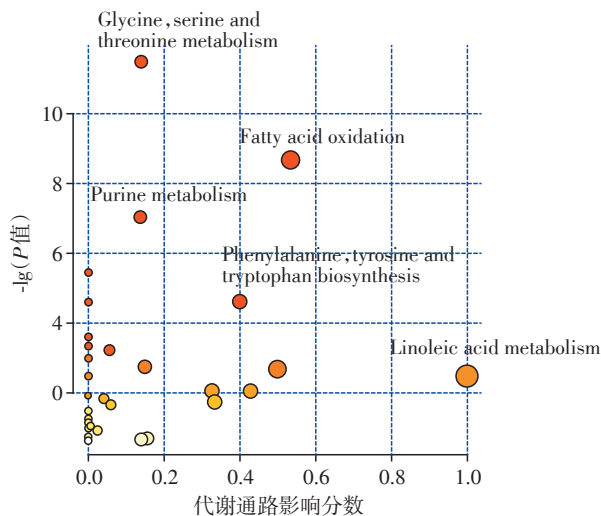


图8 代谢通路分析显示OP进展过程中骨髓组织的代谢途径发生了显著变化

Figure 8 Pathway analysis showed significantly altered metabolic pathways in bone marrow tissue during the development of OP

3 讨论

OP是一种与年龄相关的骨骼代谢疾病,其特点

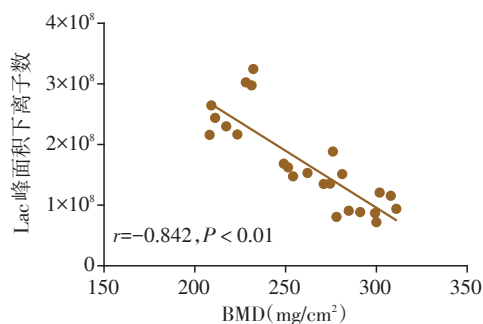


图9 OVX组骨髓中Lac水平和BMD值呈负相关

Figure 9 Level of lactic acid in bone marrow correlated negatively with BMD value in OVX group

是骨密度低、骨微结构改变,OP对绝经后妇女影响较大^[3,27]。OP是由于体内代谢环境失衡而引起的,是在骨形成与吸收过程中发生的变化^[28]。先前研究指出^[20,29-31],OP患者的骨髓总脂质与BMD值呈负相关,本研究结果与这些报道一致。有研究表明,磷脂抑制剂能够通过破坏脂质筏的形成,从而阻止RANKL引起的Akt激活,进而抑制破骨细胞的形成,减少骨质疏松^[32]。

除此之外,本研究还发现随着OP的进展,不同亚类的脂质中,神经酰胺显著增加,而鞘磷脂降低。鞘磷脂与许多细胞生物活动有关,包括增殖、分化、凋亡。此外,鞘磷脂在调节细胞生长和生存方面发挥着重要作用^[33-35]。神经酰胺是鞘脂合成和

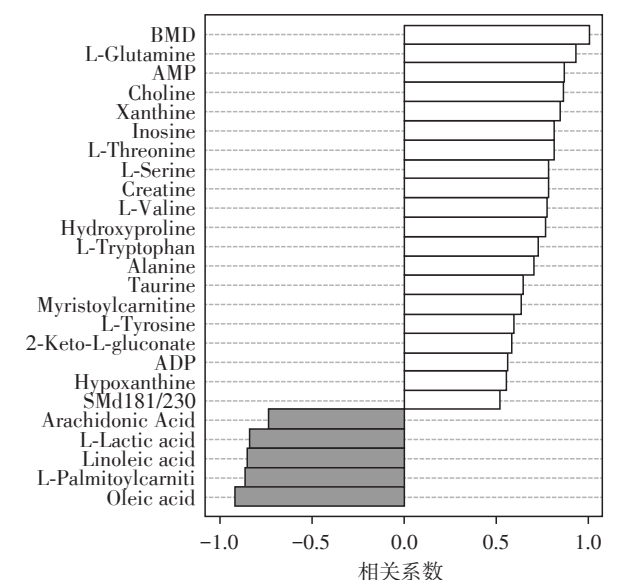


图10 Pearson回归分析揭示与BMD值相关的25个代谢物
Figure 10 Pearson's correlation analysis revealed the top 25 metabolites in bone marrow correlated with BMD

降解代谢的关键中间产物^[36-37]。神经酰胺广泛分布于人体不同组织中,但在不同组织中,神经酰胺的种类和生物调节作用不同。¹⁸C神经酰胺和¹⁸C-SM在脑和骨骼肌中含量较高^[36],能诱导细胞凋亡^[38]。神经酰胺已被证实与癌症有密切关系,但很少有研究关注它们在OP进展中的作用。研究表明神经酰胺能够通过调控NFAT2转录因子来促进破骨细胞的活性,进而造成骨质疏松^[39]。

¹H-MRS和LC-MS/MS的代谢组学分析显示,随着OP的进展,Lac在骨髓代谢过程中不断积聚。早期研究发现,上述代谢产物在骨的形成和吸收过程中起着重要作用,其数量变化在一定程度上反映了骨代谢过程的变化。Lac是成骨细胞葡萄糖代谢的主要最终产物^[40]。Lac是骨骼肌中的氧化底物,是肌肉中糖异生的前体,通过糖酵解产生^[41]。并且与败血症和其他感染性疾病相关的中性粒细胞损伤的机制中,Lac也起着重要作用^[42]。很少有人报道乳酸盐或Lac在骨和骨髓代谢中的机制。此外,本研究发现骨髓中的Lac水平与BMD值呈负相关。最近研究发现,与正常组对比,乳酸脱氢酶的含量在OP患者血清中显著增高^[43]。

相反,OVX组中的Cr、Cho、Glx和羟脯氨酸较Sham组明显下降。这一结果与先前关于OP代谢机制的研究一致^[2,18,44-45]。Glx不仅有助于产生能量,而且还有助于促进成骨细胞氧化还原稳态的保持^[46-48]。Cr可能在成骨过程中增加成骨细胞的

代谢活性,促进其分化和无机化^[49]。尿羟脯氨酸含量的测定可被作为评估骨吸收^[2]或代谢活性的标准测量^[44]。

综上所述,本研究表明,骨代谢产物Glx、Lac可作为MRS评价骨代谢活性的生物标志物,这是一种先进的、可以用于活体、无创、无辐射的医学成像技术。最重要的是,在OP进展过程中,代谢产物的变化比BMD值的变化发生早得多。从这一点来看,MRS检测关键生物标志物可能是一种比BMD更好的预测和预防OP的新方法。

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