

· 基础研究 ·

柚皮苷对体外培养骨髓间充质干细胞 Runx-2 和 Osterix 表达及骨质疏松模型大鼠骨强度的影响

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摘要 **目的:**探讨柚皮苷对体外培养骨髓间充质干细胞 Runx-2 和 Osterix 表达及骨质疏松模型大鼠骨强度的作用。**方法:**采用髓腔冲洗离心法从雌性 Lewis 大鼠股骨获取骨髓间充质干细胞, 将传代培养后的细胞分为 5 组。A 组不进行干预, B 组加入二甲基亚砷, C、D、E 组分别加入浓度为 $1 \mu\text{g} \cdot \text{mL}^{-1}$ 、 $10 \mu\text{g} \cdot \text{mL}^{-1}$ 、 $100 \mu\text{g} \cdot \text{mL}^{-1}$ 的柚皮苷。培养 6 h 后收集细胞采用 RT-PCR 法测定各组细胞 Runx-2 和 Osterix 的 mRNA 表达水平。将 24 只雌性 SD 大鼠的卵巢切除, 3 个月后将大鼠随机分为 4 组, 每组 6 只。I 组、II 组、III 组分别以 $60 \text{ mg} \cdot \text{kg}^{-1}$ 、 $300 \text{ mg} \cdot \text{kg}^{-1}$ 和 $1500 \text{ mg} \cdot \text{kg}^{-1}$ 柚皮苷灌胃, IV 组给予等体积的 PBS 灌胃, 每天灌胃 1 次, 持续 2 个月。药物干预结束后, 测定大鼠股骨强度和胫骨骨小梁面积。**结果:**①Runx-2 mRNA 和 Osterix mRNA 表达水平。B、C、D、E 组大鼠骨髓间充质干细胞 Runx-2 mRNA 表达水平比较, 差异有统计学意义 [(1.10 ± 0.02) , (2.05 ± 0.31) , (2.76 ± 0.14) , (1.82 ± 0.10) , $F=44.021$, $P=0.000$]。进一步两两比较, C、D、E 组 Runx-2 mRNA 表达水平高于 B 组 ($P=0.000$, $P=0.000$, $P=0.000$); D 组高于 C 组和 E 组 ($P=0.001$, $P=0.000$); C 组和 E 组比较, 差异无统计学意义 ($P=0.153$)。B、C、D、E 组 Osterix mRNA 表达水平比较, 差异有统计学意义 [(1.02 ± 0.10) , (1.11 ± 1.35) , (3.24 ± 0.30) , (2.55 ± 0.35) , $F=7.037$, $P=0.012$]。进一步两两比较, D 组和 E 组 Osterix mRNA 表达水平高于 B 组 ($P=0.031$, $P=0.005$); C 组和 B 组比较, 差异无统计学意义 ($P=0.886$); C 组低于 D 组和 E 组 ($P=0.006$, $P=0.039$); D 组和 E 组比较, 差异无统计学意义 ($P=0.267$)。②股骨生物力学强度。4 组骨质疏松模型大鼠股骨强度比较, 差异有统计学意义 [$(773.36 \pm 9.21) \text{ N} \cdot \text{mm}^{-1}$, $(805.66 \pm 16.00) \text{ N} \cdot \text{mm}^{-1}$, $(766.70 \pm 38.96) \text{ N} \cdot \text{mm}^{-1}$, $(707.46 \pm 15.88) \text{ N} \cdot \text{mm}^{-1}$, $F=37.776$, $P=0.000$]。进一步两两比较, I、II、III 组股骨强度均高于 IV 组 ($P=0.000$, $P=0.000$, $P=0.000$); I 组和 III 组比较, 差异无统计学意义 ($P=0.509$); II 组高于 I 组和 III 组 ($P=0.004$, $P=0.001$)。③胫骨骨小梁面积。4 组骨质疏松模型大鼠胫骨骨小梁面积比较, 差异有统计学意义 [(22.26 ± 2.32) , (25.10 ± 2.18) , (23.66 ± 3.26) , (15.63 ± 2.00) , $F=14.168$, $P=0.000$]。进一步两两比较, I、II、III 组胫骨骨小梁面积均大于 IV 组 ($P=0.001$, $P=0.000$, $P=0.000$); I 组与 II 组和 III 组比较, 差异均无统计学意义 ($P=0.090$, $P=0.387$); II 组和 III 组比较, 差异无统计学意义 ($P=0.374$)。**结论:**柚皮苷可促进体外培养的骨髓间充质干细胞中 Runx-2 和 Osterix 的表达, 增强骨质疏松模型大鼠的骨强度, 增大骨小梁面积。

关键词 骨质疏松 柚皮苷 骨碎补 骨髓间充质干细胞 Runt 相关转录因子 2 Osterix 生物力学 动物实验

Effect of naringin on expression of Runx-2 and Osterix in bone marrow stem cell cultured in vitro and on bone strength for osteoporosis rat model Xu Zhanwang*, Li Nianhu. *The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250014, Shangdong, China

ABSTRACT **Objective:** To explore the effect of naringin on the expression of Runx-2 and Osterix in bone marrow stem cell (BMSC) cultured in vitro and on the bone strength for osteoporosis rat model. **Methods:** The BMSC were obtained from femur of femina Lewis rats by medullary cavity flushing and centrifugation, and the cells were divided into 5 groups after serial subcultivation. The cells in group A were not intervened and the cells in group B were placed in the culture fluids supplemented with dimethyl sulfoxide, while cells in other groups were placed in the culture fluids respectively supplemented with naringin with final concentration of $1 \mu\text{g}/\text{mL}$ (group C), $10 \mu\text{g}/\text{mL}$ (group D) and $100 \mu\text{g}/\text{mL}$ (group E). The expression level of Runx-2 mRNA and Osterix mRNA were detected through RT-PCR after 6-hour culture. Twenty-four female SD rats were administrated with ovariectomy to creat models of osteoporosis and were randomly divided into 4 groups 3 months later, 6 cases in each group. The rats in group I, II and III were intragastric administrated with naringin with dose of $60 \text{ mg}/\text{kg}$ (group I), $300 \text{ mg}/\text{kg}$ (group II) and $1500 \text{ mg}/\text{kg}$ (group III), while the rats in group IV were intragastric administrated with PBS,

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once a day for consecutive 2 months. After the drug intervention, the femur strength and the tibia trabecular bone area of the rats were measured. **Results:** There were statistical differences in Runx-2 mRNA expression level of BMSC among group B, C, D and E ((1.10 ± 0.02) , (2.05 ± 0.31) , (2.76 ± 0.14) , (1.82 ± 0.10) , $F = 44.021$, $P = 0.000$). Further pairwise comparison showed that the Runx-2 mRNA expression level of group C, D and E were higher than that of group B ($P = 0.000$, $P = 0.000$, $P = 0.000$), and group D surpassed group C and E ($P = 0.001$, $P = 0.000$), and there was no statistical difference between group C and group E ($P = 0.153$). There were statistical differences in Osterix mRNA expression level of BMSC among group B, C, D and E ((1.02 ± 0.10) , (1.11 ± 1.35) , (3.24 ± 0.30) , (2.55 ± 0.35) , $F = 7.037$, $P = 0.012$). Further pairwise comparison showed that the Osterix mRNA expression level of group D and group E were higher than that of group B ($P = 0.031$, $P = 0.005$), and there was no statistical difference between group C and group B ($P = 0.886$), and the Osterix mRNA expression level of group C were lower than that of group D and group E ($P = 0.006$, $P = 0.039$), and there was no statistical difference between group D and group E ($P = 0.267$). There were statistical differences in the femur strength among groups I, II, III and IV ((773.36 ± 9.21) , (805.66 ± 16.00) , (766.70 ± 38.96) , (707.46 ± 15.88) N/mm, $F = 37.776$, $P = 0.000$). Further pairwise comparison showed that the femur strength of group I, II and III were higher than that of group IV ($P = 0.000$, $P = 0.000$, $P = 0.000$), and there was no statistical difference between group I and group III ($P = 0.509$), and the femur strength of group II were higher than that of group I and group III ($P = 0.004$, $P = 0.001$). There were statistical differences in the tibial bone trabecula area among group I, II, III and IV ((22.26 ± 2.32) , (25.10 ± 2.18) , (23.66 ± 3.26) , (15.63 ± 2.00) , $F = 14.168$, $P = 0.000$). Further pairwise comparison showed that the tibial bone trabecula area of group I, II and III were larger than that of group IV ($P = 0.001$, $P = 0.000$, $P = 0.000$), and there were no statistical differences among group I, II and III ($P = 0.090$, $P = 0.387$), and there was no statistical difference between group II and group III ($P = 0.374$). **Conclusion:** Naringin can promote the expression of Runx-2 and Osterix in BMSC cultured in vitro, and it can increase bone strength and bone trabecula area of osteoporosis rat.

Key words Osteoporosis; Naringin; Drynaria fortunei; Bone marrow stem cell; Runt-related transcription factor 2; Osterix; Biomechanics; Animal experimentation

50 岁以上女性中, 20% 存在骨质疏松^[1], 患者骨强度减低, 骨折风险增大^[2]。Runt 相关转录因子 2 (Runt-related transcription factor 2, Runx-2) 是骨髓间充质干细胞 (bone marrow stem cell, BMSC) 向成骨细胞分化的特异性转录调节因子^[3], 而 Osterix 是成骨细胞分化和骨形成所必须的转录因子^[4-5]。柚皮苷是中药骨碎补的主要活性成分^[6], 可以促进 BMSC 的增殖与分化, 并改善去卵巢大鼠的骨质疏松^[7]。为了探讨 Runx-2 和 Osterix 在柚皮苷防治骨质疏松中的作用, 我们进行了相应的实验研究, 现报告如下。

1 材料与仪器

1.1 实验动物 雌性 Lewis 大鼠 5 只, 体质量 150 ~ 180 g, 购自美国 Harlan 实验室, 实验动物合格证号: HLU20111023; 3 月龄雌性 SD 大鼠 24 只, 平均体质量 220 g, 购自山东中医药大学实验动物中心, 实验动物合格证号: SCXK(鲁)20050015。

1.2 实验试剂 柚皮苷 (Sigma-Aldrich 公司), 采用二甲基亚砜重溶, 原液浓度 $100 \mu\text{g} \cdot \text{mL}^{-1}$; DMEM 培养液 (Sigma-Aldrich 公司); 10% 胎牛血清、2 mM 谷氨酰胺、100 单位 $\cdot \text{mL}^{-1}$ 青霉素、100 $\mu\text{g} \cdot \text{mL}^{-1}$ 链霉素 (Invitrogen 公司)。

1.3 实验仪器 Bose ElectroForce 3220 载荷框架 (Bose 公司); Nikon Eclipse E800 倒置相差显微镜 (Nikon 公司); StepOnePlus 实时 PCR 测量仪 (Life Technologies 公司)。

2 方法

2.1 检测柚皮苷对 BMSC 中 Runx-2 和 Osterix 基因表达的影响

2.1.1 BMSC 获取及培养 取雌性 Lewis 大鼠双侧股骨, 采用髓腔冲洗离心法获取 BMSC^[8]。将冲洗得到的细胞在 37 °C、5% CO₂ 和饱和湿度条件下培养, 培养液为 DMEM 培养液 + 10% 胎牛血清 + 2 mM 谷氨酰胺 + 100 单位 $\cdot \text{mL}^{-1}$ 青霉素 + 100 $\mu\text{g} \cdot \text{mL}^{-1}$ 链霉素。3 d 后以 PBS 冲洗, 已贴壁细胞继续培养直至达到 90% 融合。然后将所得细胞进一步扩增, 采用 5 代以前的细胞进行实验。

2.1.2 BMSC 分组及药物干预 按 1×10^5 个 $\cdot \text{mL}^{-1}$ 将细胞接种于 12 孔培养板中, A 组不进行干预, B 组加入二甲基亚砜, C 组、D 组、E 组分别加入浓度为 1 $\mu\text{g} \cdot \text{mL}^{-1}$ 、10 $\mu\text{g} \cdot \text{mL}^{-1}$ 、100 $\mu\text{g} \cdot \text{mL}^{-1}$ 的柚皮苷。柚皮苷浓度的选择依据我们以前的实验结果确定^[7]。

2.1.3 Runx-2 和 Osterix 基因表达水平检测 培养

6 h 后收集细胞采用 RT-PCR 法测定各组细胞 Runx-2 mRNA 和 Osterix mRNA 表达水平。所用引物序列见表 1。

表 1 实时荧光定量 PCR 所用引物序列

基因名称	引物序列
Runx-2	Forward 5'-AAGCCACAGTGGTAGGCAGT-3'
	Reverse 5'-TTGTTTGTGAGGCGAATGAA-3'
Osterix	Forward 5'-AAGGCAGTTGGCAATAGTGG-3'
	Reverse 5'-TGAATGGGCTTCTTCTCAG-3'
管家基因 18S	Left CGGCTACCACATCCAAGGAA
	Right GCTGGAATTACCGCGGCT

2.2 检测柚皮苷对骨质疏松大鼠骨强度的影响

2.2.1 骨质疏松大鼠模型建立 将 24 只 3 月龄雌性 SD 大鼠的卵巢切除,术后饲养 3 个月,建立骨质疏松大鼠模型。卵巢切除术后所有大鼠均存活,未发生感染及其他并发症。

2.2.2 分组及药物干预 卵巢切除术 3 个月后,将动物随机分为 4 组,每组 6 只。I 组、II 组、III 组分别以 $60 \text{ mg} \cdot \text{kg}^{-1}$ 、 $300 \text{ mg} \cdot \text{kg}^{-1}$ 和 $1500 \text{ mg} \cdot \text{kg}^{-1}$ 柚皮苷灌胃,IV 组给予等体积的 PBS 灌胃,每天灌胃 1 次,持续 2 个月。

2.2.3 骨生物力学强度测定 药物干预结束后,切除所有大鼠的左侧股骨,去除软组织,用生理盐水纱布包裹。应用 Bose ElectroForce 3220 载荷框架采用三点弯曲法测定股骨生物力学强度,加载速度 $0.1 \text{ mm} \cdot \text{min}^{-1}$ (图 1)。



图 1 骨质疏松模型大鼠左股骨三点弯曲实验
(股骨前面朝上,股骨头朝内)

2.2.4 骨小梁面积测定 药物干预结束后,切除所有大鼠的左侧胫骨,去除软组织,用甲醛固定后进行常规病理切片,HE 染色。在显微镜下观察拍照,并采用 Image-Pro Plus 6.0 图像处理软件测量骨小梁面积。

2.3 数据统计分析 采用 SPSS17.0 软件对所得数

据进行统计分析,各组大鼠 BMSC 中 Runx-2 mRNA 和 Osterix mRNA 表达水平及骨质疏松模型大鼠股骨骨强度和胫骨骨小梁面积的组间总体比较采用单因素方差分析,组间两两比较采用 LSD-*t* 检验,检验水准 $\alpha = 0.05$ 。

3 结 果

3.1 BMSC 培养结果 原代细胞初接种时呈圆形 (图 2),24 h 后细胞开始贴壁,3 d 后细胞基本完全贴壁并呈梭形。换液冲洗后细胞呈集落样生长,形态主要为多角形或不规则形,少数为梭形。

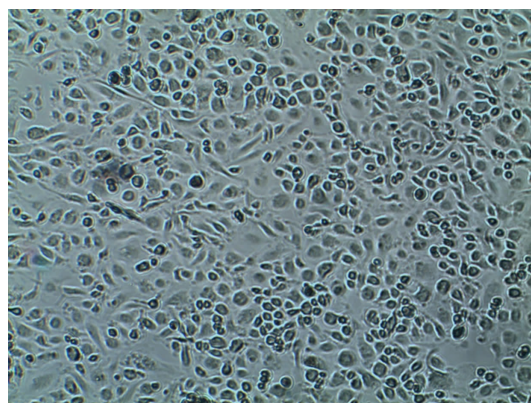


图 2 用髓腔冲洗离心法获得的大鼠 BMSC ($\times 100$)

3.2 Runx-2 mRNA 和 Osterix mRNA 表达水平 B、C、D、E 组大鼠 BMSC 干预培养 6 h 后 Runx-2 mRNA 表达水平比较,差异有统计学意义。进一步两两比较,C、D、E 组 Runx-2 mRNA 表达水平高于 B 组 ($P = 0.000$, $P = 0.000$, $P = 0.000$);D 组高于 C 组和 E 组 ($P = 0.001$, $P = 0.000$);C 组和 E 组比较,差异无统计学意义 ($P = 0.153$)。B、C、D、E 组 Osterix mRNA 表达水平比较,差异有统计学意义。进一步两两比较,D 组和 E 组 Osterix mRNA 表达水平高于 B 组 ($P = 0.031$, $P = 0.005$);C 组和 B 组比较,差异无统计学意义 ($P = 0.886$);C 组低于 D 组和 E 组 ($P = 0.006$, $P = 0.039$);D 组和 E 组比较,差异无统计学意义 ($P = 0.267$)。见表 2。

表 2 4 组大鼠 BMSC 的 Runx-2 mRNA 和 Osterix mRNA 表达水平

组别	Runx-2 mRNA	Osterix mRNA
B 组	1.10 ± 0.02	1.02 ± 0.10
C 组	2.05 ± 0.31	1.11 ± 1.35
D 组	2.76 ± 0.14	3.24 ± 0.30
E 组	1.82 ± 0.10	2.55 ± 0.35
F 值	44.021	7.037
P 值	0.000	0.012

3.3 股骨生物力学强度 4 组骨质疏松模型大鼠股骨强度比较,差异有统计学意义。进一步两两比较, I、II、III 组股骨强度均高于 IV 组 ($P=0.000, P=0.000, P=0.000$); I 组和 III 组比较,差异无统计学意义 ($P=0.509$); II 组高于 I 组和 III 组 ($P=0.004, P=0.001$)。见表 3。

3.4 胫骨骨小梁面积 4 组骨质疏松模型大鼠胫骨骨小梁面积比较,差异有统计学意义。进一步两两比较, I、II、III 组胫骨骨小梁面积均大于 IV 组 ($P=0.001, P=0.000, P=0.000$); I 组与 II 组和 III 组比较,差异均无统计学意义 ($P=0.090, P=0.387$); II 组和 III 组比较,差异无统计学意义 ($P=0.374$)。见表 3。

表 3 4 组骨质疏松模型大鼠股骨强度和胫骨骨小梁面积

组别	股骨强度 ($N \cdot mm^{-1}$)	胫骨骨小梁面积
I 组	773.36 ± 9.21	22.26 ± 2.32
II 组	805.66 ± 16.00	25.10 ± 2.18
III 组	766.70 ± 38.96	23.66 ± 3.26
IV 组	707.46 ± 15.88	15.63 ± 2.00
F 值	37.776	14.168
P 值	0.000	0.000

4 讨 论

柚皮苷是中药骨碎补的主要活性成分,近年来的研究表明其具有骨保护的药理作用。Wu 等^[9]的研究表明,柚皮苷具有预防卵巢切除小鼠骨量丢失的潜能和作用,并认为其作用机制是柚皮苷在成骨细胞中通过磷脂酰肌醇 3 激酶/蛋白激酶 B 通路,转录因子 c-Fos/c-Jun 和活化蛋白-1 旁路诱导骨形态发生蛋白 2 (bone morphogenetic protein-2, BMP-2) 表达。另外,柚皮苷还可通过抑制核因子 κB 受体活化因子配体诱导的核因子 κB 和细胞外信号调节蛋白激酶的活性来阻止破骨细胞形成,从而减少骨吸收^[10]。

在 BMSC 向成骨细胞分化过程中有 2 条重要的信号传导通路,即 Wnt/ β 连锁蛋白信号通路和转化生长因子 β /BMP-2 信号通路,但最终的靶基因都是 Runx-2^[11]。目前多数学者认为,Runx-2 在成骨细胞分化的起始阶段可触发骨基质蛋白的形成,为后续的分化成熟提供大量的未成熟成骨细胞。而成骨细胞的最终分化成熟则需要 Osterix 的参与^[12]。

从本研究的结果可以看出,柚皮苷可有效促进大鼠 BMSC 中 Runx-2 mRNA 和 Osterix mRNA 表达,其中以 $10 \mu g \cdot mL^{-1}$ 柚皮苷的作用最为显著。据此可以推测,柚皮苷可通过上调 Runx-2 和 Osterix 的表达

来促进 BMSC 分化。另外,本研究结果也显示,柚皮苷可有效增加骨质疏松模型大鼠的骨强度,增大骨小梁面积,以 $300 mg \cdot kg^{-1}$ 的柚皮苷效果最佳。

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