

· 药物研究 ·

牛膝活性成分β-蜕皮甾酮通过Akt信号干预地塞米松诱导的骨细胞凋亡

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摘要: **目的** 探讨牛膝活性成分β-蜕皮甾酮对地塞米松诱导 MLO-Y4 骨样细胞凋亡的抑制作用及其可能的作用机制。**方法** 10 μmol/L 地塞米松(dexamethasone, Dex)作用于 MLO-Y4 骨样细胞,以 PI3K/Akt 抑制剂 LY294002 为对照,β-蜕皮甾酮(β-Ecdysone, β-Ecd)干预,分为 6 组:①正常组;②10 μmol/L Dex 模型组;③ 10 μmol/L Dex+10 μmol/L β-Ecd 组;④ 10 μmol/L Dex+10 μmol/L β-Ecd+50 μmol/L LY294002 组;⑤10 μmol/L Dex+0.1 μmol/L β-Ecd 组;⑥ 10 μmol/L Dex+0.1 μmol/L β-Ecd+50 μmol/L LY294002 组。作用 48 h 后,Annexin V-FITC/PI 法检测细胞早期凋亡率,Western-Blot 检测 Akt1、Phospho-Akt (Thr308)、Connexin43 (CX43)、Caspase9、Bad、Phospho-Bad 蛋白表达。**结果** 与正常组比较,Dex 组细胞凋亡率升高,Caspase9、Bad 蛋白表达升高,Akt1、CX43 蛋白表达降低;与造模组比较,β-Ecd 减弱 Caspase9 蛋白表达,使 Akt1、CX43 蛋白表达增强;LY294002 使 Akt1、CX43 蛋白表达明显减弱,而 β-Ecd 并不能使 LY294002 降低的 Akt1 蛋白表达升高。**结论** 0.1 μmol/L、10 μmol/L β-蜕皮甾酮可能通过 Akt 信号途径干预地塞米松诱导的骨细胞凋亡。

关键词: β-蜕皮甾酮;地塞米松;骨细胞;凋亡;Akt

β-Ecdysone protects osteocytes from glucocorticoid-induced apoptosis via Akt signal pathway in vitro

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Abstract: Objective To investigate the effects of β-ecdysone on glucocorticoid-induced apoptosis by MLO-Y4 osteocyte-like cells and the possible mechanism. **Methods** MLO-Y4 cells were treated with 10 μM dexamethasone (Dex) and divided into 6 groups: (1) Control group; (2) 10 μmol/L Dex group; (3) 10 μmol/L Dex + 10 μmol/L β-Ecd group; (4) 10 μmol/L Dex + 10 μmol/L β-Ecd + 50 μmol/L LY294002 group; (5) 10 μmol/L Dex + 0.1 μmol/L β-Ecd group; and (6) 10 μmol/L Dex + 0.1 μmol/L β-Ecd + 50 μmol/L LY294002 group. After 48 hours, the cell apoptosis was measured by Annexin V-FITC / PI assay. The protein expressions of Caspase9, Bad, Phospho-Bad, Akt1, Phospho-Akt (Thr308) and Connexin43 (CX43) were examined with Western blotting. **Results** Comparing to those in control group, apoptosis rate, protein expressions of Caspase9 and Bad increased, and Akt1 and CX43 decreased in Dex group. Comparing to those in model groups, β-ecdysone inhibited Caspase9 expression, and increased the expressions of Akt1 and CX43. LY294002 significantly reduced the protein expressions of Akt1 and CX43. β-Ecdysone could not rescue the decreased Akt1 expression by LY294002. **Conclusion** Ecdysone of 0.1 μmol/L and 10 μmol/L may prevent the osteocyte apoptosis induced by glucocorticoid through Akt signal pathway.

Key words: β-ecdysone; dexamethasone; osteocyte; apoptosis; Akt

临床上糖皮质激素 (glucocorticoid, GC) 被广泛

用于抗炎、抗过敏、抗中毒、抗休克的治疗,但易导致骨质疏松症的发生^[1-3]。GC 诱导的骨质疏松症 (glucocorticoid induced osteoporosis, GIOP) 是继发性骨质疏松症的最常见病因。临床流行病学研究显示,在美国约 50% 风湿性关节炎患者长期使用 GC,

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治疗数周后,骨量开始流失,最初数月内的骨量丢失迅速,达5%~15%。长期接受GC治疗(半年以上)的患者骨质疏松发生率高达30%~50%^[3]。

β -蜕皮甾酮(β -Ecdysone, β -Ecd)为牛膝根内主要活性成分之一。本研究发现, β -Ecd有助于改善去势小鼠的脊椎骨小梁体积与骨强度,一定剂量的 β -Ecd可以提高成骨细胞的活性而抑制破骨细胞的活性^[4]。骨细胞在骨组织中占90%~95%,形成遍布骨基质的三维骨细胞网络(osteocyte network)。其被认为是机械应力感受细胞及活跃的旁分泌细胞。但近年研究表明,骨细胞可能是骨重塑的调节中心,起着包括骨重塑的启动、骨吸收的抑制以及骨形成调节等的重要作用^[5]。本实验研究 β -Ecd对Dex诱导的骨细胞凋亡的干预作用,并检测Akt1、P-Akt(Thr308)、CX43、Caspase9、Bad、P-Bad蛋白的表达,以探讨 β -Ecd作用于骨细胞可能的分子途径。为 β -Ecd干预GIOP的作用机制提供实验依据。

1 材料和方法

1.1 药物和试剂

β -蜕皮甾酮(批号:10020231,规格:99%,分子量:480.6,上海同田生物技术有限公司),地塞米松(批号:D9184,分子量:434.5,Sigma公司), α -MEM培养基(Hyclone公司),胎牛血清(Hyclone公司),青链霉素(Hyclone公司),PI3K抑制剂LY294002(LY,编号:9901S,Cell Signaling Technology公司),Annexin V-FITC/PI(编号:V13241,Thermo fisher公司),BCA蛋白浓度检测试剂盒(编号:P0010,碧云天公司),Akt1、P-Akt-T308、CX43、Caspase9、Bad、P-Bad、GAPDH单克隆兔抗小鼠抗体(Cell Signaling Technology公司),羊抗兔IgG-HRP二抗(Santa Cruz公司),ECL显色试剂盒(批号:P0 018 A,上海碧云天生物技术有限公司)。

1.2 仪器

CO₂培养箱(型号:FORMA STERI-CYCLE i160,Thermo Scientific公司),生物安全柜(型号:MSC 1.8,Thermo Scientific公司),电泳仪与凝胶垂直电泳系统(型号:Mini Protean,美国BIO-RAD公司),酶标仪(型号:Synergy2,Bio Tek公司),流式细胞仪(型号:C6,BD公司)。

1.3 实验方法

1.3.1 MLO-Y4骨样细胞给药与分组:MLO-Y4骨样细胞以 α -MEM培养基(2.5%胎牛血清+2.5%小牛血清+1%青链霉素)于37℃、5%CO₂培养箱

中培养。当细胞密度达到80%~90%时,分别以Dex、LY(PI3K抑制剂)、不同浓度的 β -Ecd进行干预,将细胞分为6组:①正常组;②10 μ mol/L Dex模型组;③10 μ mol/L Dex+10 μ mol/L β -Ecd组;④10 μ mol/L Dex+10 μ mol/L β -Ecd+50 μ mol/L LY294002组;⑤10 μ mol/L Dex+0.1 μ mol/L β -Ecd组;⑥10 μ mol/L Dex+0.1 μ mol/L β -Ecd+50 μ mol/L LY294002组。作用时间为48h。

1.3.2 Annexin V-FITC/PI法检测凋亡:MLO-Y4骨样细胞用0.25%胰酶消化,洗涤,1300g,4℃离心5min,收集。加入5 μ L FITC标记的Annexin-V室温避光15min,再加入PI,避光反应5min后,加入5 μ L缓冲液(binding buffer),进行流式细胞凋亡检测(N=3)。

1.3.3 Western-Blot检测Dex、 β -Ecd作用下Akt、P-Akt(Thr308)、CX43、Caspase9、Bad、P-Bad、GAPDH蛋白表达:提取各组细胞总蛋白,以BCA法进行蛋白定量测定,变性蛋白,SDS-PAGE电泳,转膜,封闭,分别加入稀释后的一抗(Akt1、P-Akt(Thr308)、CX43、Caspase9、Bad、P-Bad稀释比例为1:200,GAPDH稀释比例为1:400),置于4℃冰箱过夜,PBST洗涤8次,每次5min;将PVDF膜放入含辣根过氧化物酶标记的稀释二抗(HRP Goat anti-Rabbit IgG,对磷酸化一抗稀释比例为1:3000,对其余一抗稀释比例为1:15000),室温孵育1h,PBST洗涤8次,每次5min。ECL试剂显色,凝胶图像分析仪曝光、拍照。

1.4 统计学处理

实验结果以 $\bar{x}\pm s$ 表示,采用统计学软件SPSS 21.0进行数据分析,行单因素方差分析, $P<0.05$ 认为差异有统计学意义。

2 结果

2.1 流式细胞仪Annexin V-FITC/PI法检测各组细胞凋亡

流式细胞仪检测细胞凋亡结果(图1)显示:与正常组比较,造模组(10 μ mol/L Dex)凋亡率升高;与造模组比较,用药组(10 μ mol/L β -Ecd,0.1 μ mol/L β -Ecd)降低了凋亡率;而加入抑制剂LY(第4、6组)凋亡率明显升高。同时发现,第4组(10 μ mol/L β -Ecd)凋亡率比第6组(0.1 μ mol/L β -Ecd)低。

2.2 Western-Blot检测各组细胞相关基因蛋白表达与正常组比较,Dex组Akt1、CX43蛋白表达降

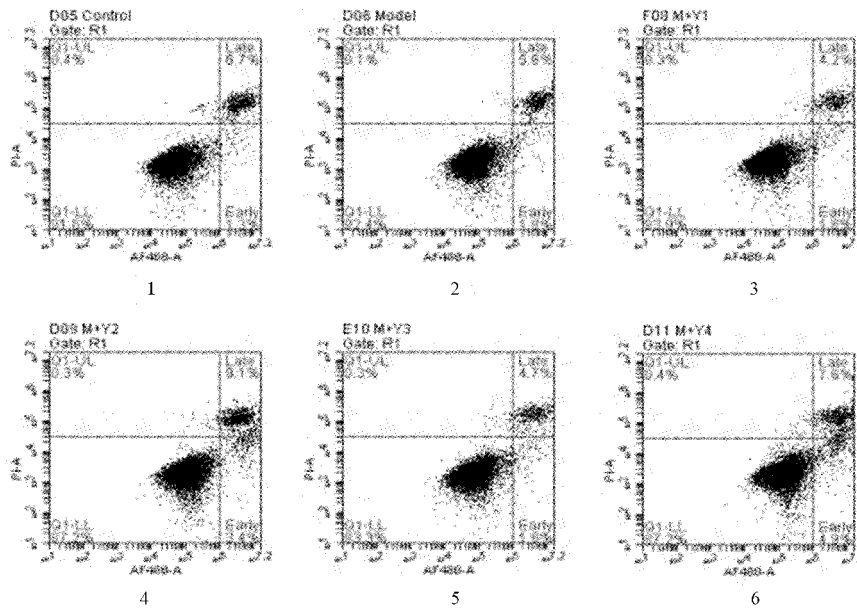


图1 流式细胞仪检测凋亡结果

Fig.1 Apoptosis results detected with flow cytometry

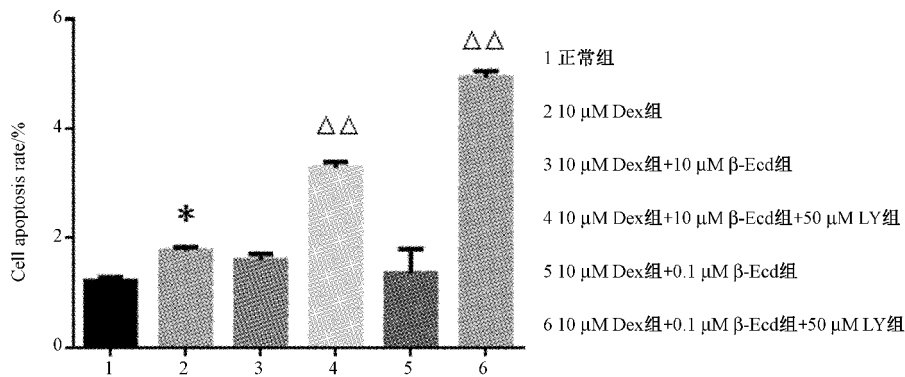


图2 MLO-Y4 细胞凋亡率

与正常组比较, * $P < 0.05$; 与造模组比较, $\Delta\Delta P < 0.01$ 。

Fig.2 Apoptosis rate of MLO-Y4 cells

低;与造模组 (10 $\mu\text{mol/L}$ Dex) 比较, $\beta\text{-Ecd}$ (10 $\mu\text{mol/L}$, 0.1 $\mu\text{mol/L}$) 使 Akt1、CX43 蛋白表达增强; 与给药干预组比较, 加入 LY 后, Akt1、P-Akt (Thr308)、CX43 蛋白表达均降低。

表1 各组细胞相关蛋白表达结果 ($\bar{x} \pm s$, $n=3$)

Table 1 The results of cell-associated protein expressions in each group ($\bar{x} \pm s$, $n=3$)

| 组别 | 蛋白相对表达率 (%) | | | | | |
|----|---------------------|----------------------|-----------------------|---------------------|---------------------|-----------------------|
| | Akt1 | P-Akt-Thr308 | CX43 | Caspase9 | Bad | P-Bad |
| 1 | 103.88±1.79 | 263.43±3.28 | 183.25±22.36 | 65.50±3.39 | 32.41±0.26 | 168.08±51.21 |
| 2 | 75.04±11.66 * | 125.55±22.12 * | 119.09±19.03 * | 102.77±18.25 * | 58.81±0.10 * | 69.46±14.63 * |
| 3 | 85.44±0.71 Δ | 137.49±14.69 | 147.01±16.50 | 44.11±4.06 Δ | 60.18±4.95 | 51.77±14.88 |
| 4 | 53.54±5.21 Δ | 76.67±15.25 Δ | 22.35±6.01 Δ | 26.77±7.92 Δ | 45.80±6.23 Δ | 151.85±22.42 Δ |
| 5 | 86.92±0.90 Δ | 73.16±7.39 Δ | 164.73±22.99 Δ | 57.58±4.83 Δ | 46.55±7.16 Δ | 51.59±3.02 |
| 6 | 43.32±1.88 Δ | 44.15±6.44 Δ | 25.88±1.22 Δ | 28.6±4.52 Δ | 35.44±4.27 Δ | 282.67±12.47 Δ |

注:与正常组比较 * $P < 0.05$;与造模组比较 $\Delta P < 0.05$ 。

Caspase9、Bad 是促进凋亡的因子, Bad 磷酸化 (P-Bad) 则抑制 Bad 活性。与正常组比较, Dex 组 Caspase9、Bad 表达增强、P-Bad 表达减弱, 提示诱发凋亡; 与造模组 (10 $\mu\text{mol/L}$ Dex) 比较, $\beta\text{-Ecd}$ (10 $\mu\text{mol/L}$, 0.1 $\mu\text{mol/L}$) 减弱 Caspase9 的表达; 加入抑制剂 LY 组 (第 4、6 组), Caspase9、Bad 表达明显减弱、P-Bad 表达明显升高, 且第 4 组、第 6 组表达因 $\beta\text{-Ecd}$ 浓度不同而有差异。

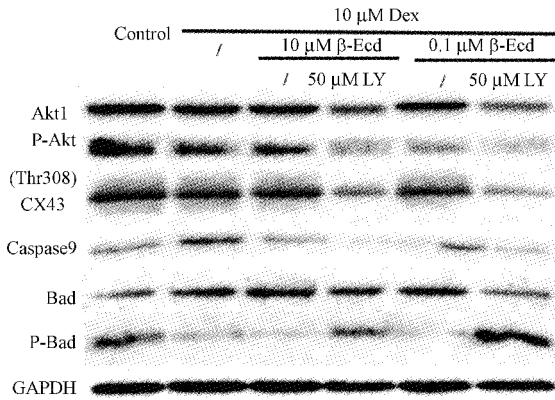


图 3 Western-Blot 检测结果
Fig.3 Results of Western-Blot

3 讨论

GC 广泛应用于临床, 但同时也伴随不良反应, 如导致骨质疏松的发生。目前认为, 骨形成的抑制和骨细胞的凋亡在糖皮质激素诱导的骨质疏松症的发病机制中起到一定且关键的作用, 而在 GC 使用期间骨吸收的变化是可变的^[6]。过量的 GC 抑制 Akt 蛋白的活性, 从而抑制了骨形成^[7]; 高剂量或者长时间使用 GC 则影响骨的水化、骨内血管及骨的强度而导致骨细胞的凋亡^[8], 约 25% 的患者由于凋亡的细胞的聚集而发生骨坏死, 从而增加股骨头塌陷的风险^[9]。本研究结果发现, 高浓度 (10 $\mu\text{mol/L}$) Dex 使 MLO-Y4 骨细胞凋亡率升高。

有学者发现, 衰老会导致骨细胞凋亡增加^[10]。牛膝活性成分 $\beta\text{-蜕皮甾酮}$ 具有明显的延长果蝇寿命、降低 MDA (malonaldehyde, 丙二醛)、升高 SOD (superoxide dismutase, 超氧化物歧化酶) 活性等抗衰老作用^[11]。

CX43 (骨连接蛋白) 是骨细胞中表达最丰富的半通道蛋白, 由两个并列连接子或半通道形成的间隙连接调节骨细胞、成骨细胞和破骨细胞之间的细胞间通讯, 因此在骨形成和骨重塑中发挥关键作用^[12]。有实验发现, Dex 可诱导 CX43 降解, 而 Akt

磷酸化则抑制 CX43 降解, CX43 降解可能导致骨质流失^[13]。PI3K/Akt 信号通路在调节细胞增殖、生长和凋亡中起重要作用, 本研究结果表明, 10 $\mu\text{mol/L}$ 地塞米松使 MLO-Y4 骨细胞 Akt1、CX43 蛋白表达降低, 而 $\beta\text{-Ecd}$ 使 Akt1、CX43 蛋白表达升高。LY294002 是 PI3K/Akt 信号通路特异性抑制剂, 体外和体内实验表明 LY 通过抑制 Akt 磷酸化而抑制 Akt 蛋白活性并增加凋亡率^[14]。研究发现, $\beta\text{-Ecd}$ 并不能使 LY 降低的 Akt1 蛋白表达升高。由此反证 $\beta\text{-Ecd}$ 在骨细胞中可能通过 Akt 途径对 Dex 诱导的凋亡起干预作用。

据报道线粒体是调节细胞凋亡的关键。激活 Bad 使线粒体通透性提高, 细胞色素 C 从膜间隙释放到细胞质中, 最终激活 Caspase 酶介导的凋亡程序。而 P-Bad 与分子伴侣蛋白 14-3-3 结合, 能抑制 Bad 的表达^[15-16]。研究发现, 10 $\mu\text{mol/L}$ Dex 抑制骨细胞 Bad 蛋白的磷酸化, 促进 Bad、caspase9 蛋白的表达, 而一定浓度的 $\beta\text{-Ecd}$ 干预则使 Caspase9 蛋白的表达降低。表明 $\beta\text{-Ecd}$ 可能通过线粒体凋亡途径抑制 Dex 诱导的骨细胞凋亡。

综上所述, 牛膝活性成分 $\beta\text{-蜕皮甾酮}$ 通过激活骨细胞中的 PI3K/Akt 信号通路, 从而干预 Dex 诱导的骨细胞凋亡, 该过程可能依赖于线粒体凋亡途径。

【参 考 文 献】

- [1] Trijau S, De LG, Pradel V, et al. Osteoporosis prevention among chronic glucocorticoid users: results from a public health insurance database[J]. RMD Open, 2016, 2(2): e000249.
- [2] Komori T. Glucocorticoid signaling and bone biology[J]. Horm Metab Res, 2016, 48(11): 755-763.
- [3] Buckley L, Guyatt G, Fink HA, et al. 2017 American College of Rheumatology Guideline for the prevention and treatment of glucocorticoid-induced osteoporosis [J]. Arthritis Rheumatol, 2017, 69(8): 1095.
- [4] Dai W, Zhang H, Zhong ZA, et al. $\beta\text{-Ecdysone}$ augments peak bone mass in mice of both sexes[J]. Clin Orthop Relat Res, 2015, 473(8): 2495-2504.
- [5] Duan P, Bonewald LF. The role of the wnt/ $\beta\text{-catenin}$ signaling pathway in formation and maintenance of bone and teeth[J]. Int J Biochem Cell Biol, 2016, 77: 23-29.
- [6] Lems W F, Saag K. Bisphosphonates and glucocorticoid-induced osteoporosis: cons[J]. Endocrine, 2015, 49(3): 628-634.
- [7] 丁香莹, 梁敏. 凋亡在糖皮质激素性骨质疏松症中的研究进展[J]. 中国骨质疏松杂志, 2017, 23(12): 1660-1663.
- [8] 任辉, 魏秋实, 江晓兵, 等. 糖皮质激素性骨质疏松的研究新进展[J]. 中国骨质疏松杂志, 2014, 20(9): 1138-1142.
- [9] Sato AY, Tu X, Mcandrews KA, et al. Prevention of

- glucocorticoid induced-apoptosis of osteoblasts and osteocytes by protecting against endoplasmic reticulum (ER) stress in vitro and in vivo in female mice[J]. *Bone*, 2015,73(6):60-68.
- [10] Davis HM, Pacheco-Costa R, Atkinson EG, et al. Disruption of the Cx43/miR21 pathway leads to osteocyte apoptosis and increased osteoclastogenesis with aging[J]. *Aging Cell*, 2017,16(3):551-563.
- [11] Kapur P, Wuttke W, Jarry H, et al. Beneficial effects of β -Ecdysone on the joint, epiphyseal cartilage tissue and trabecular bone in ovariectomized rats[J]. *Phytomedicine*, 2010,17(5):350-355.
- [12] Kar R, Riquelme MA, Werner S, et al. Connexin 43 channels protect osteocytes against oxidative stress-induced cell death[J]. *J Bone Miner Res*, 2013,28(7):1611-1621.
- [13] Gao J, Cheng TS, An Q, et al. Glucocorticoid impairs cell-cell communication by autophagy-mediated degradation of connexin 43 in osteocytes[J]. *Oncotarget*, 2016,7(19):26966-26978.
- [14] Xia LJ, Wu YL, Zhang FC. Combination of cecropin XJ and LY294002 induces synergistic cytotoxicity, and apoptosis in human gastric cancer cells via inhibition of the PI3K/Akt signaling pathway [J]. *Oncology Letters*, 2017, 14(6):7522-7528.
- [15] Xu T, Pang Q, Wang Y, et al. Betulinic acid induces apoptosis by regulating PI3K/Akt signaling and mitochondrial pathways in human cervical cancer cells[J]. *Int J Molecul Med*, 2017,40(6):1669-1678.
- [16] Zhou Q, Ye M, Lu Y, et al. Curcumin improves the tumoricidal effect of mitomycin C by suppressing ABCG2 expression in stem cell-like breast cancer cells [J]. *PLoS One*, 2015, 10(8):e0136694.
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【参 考 文 献】

- [1] Drake MT, Clarke BL, Lewiecki EM. The Pathophysiology and Treatment of Osteoporosis [J]. *Clin Therapeut*, 2015,37(8):1837-1850.
- [2] Yoon HK, Lee YK, Ha YC. Characteristics of patients diagnosed with osteoporosis in South Korea; results from the national claim registry [J]. *J Bone Metab*, 2017,24(1):59-63.
- [3] Muka T, Oliver-Williams C, Kunutsor S, et al. Association of age at onset of menopause and time since onset of menopause with cardiovascular outcomes, intermediate vascular traits, and all-cause mortality: a systematic review and meta-analysis [J]. *JAMA Cardiol*, 2016,1(7):767.
- [4] Hodis HN, Mack WJ, Henderson VW, et al. Vascular effects of early versus late postmenopausal treatment with estradiol [J]. *N Engl J Med*, 2016,374(13):1221-1231.
- [5] Karwowski W, Naumnik B, Szczepański M, et al. The mechanism of vascular calcification- a systematic review [J]. *J Exp Clin Res*, 2012,18(1):RA1.
- [6] Lampropoulos CE, Kalamara P, Konsta M, et al. Osteoporosis and vascular calcification in postmenopausal women; a cross-sectional study [J]. *Climacteric*, 2016,19(3):303-307.
- [7] Sumino H, Ichikawa S, Kasama S, et al. Relationship between carotid atherosclerosis and lumbar spine bone mineral density in postmenopausal women [J]. *Hypertens Res*, 2008,31(6):1191-1197.
- [8] Farhat GN, Cauley JA. The link between osteoporosis and cardiovascular disease [J]. *Clin Cases Miner Bone Metab*, 2008,5(1):19.
- [9] Yamada S, Inaba M, Goto H, et al. Significance of intima-media thickness in femoral artery in the determination of calcaneus osteo-sono index but not of lumbar spine bone mass in healthy Japanese people [J]. *Osteoporos Int*, 2005,16(1):64.
- [10] Fodor D, Bondor C, Albu A, et al. Relation between intima-media thickness and bone mineral density in postmenopausal women: a cross-sectional study [J]. *Sao Paulo Medical J*, 2011,129(3):139-145.
- [11] Khromov BM. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk; a consensus statement from the American Society of Echocardiography Intima-Media Thickness Task Force [J]. *J Am Soc Echocardiogr*, 2008,21(2):93-111.
- [12] Chambless LE, Folsom AR, Clegg LX, et al. Carotid Wall Thickness is Predictive of Incident Clinical Stroke: The Atherosclerosis Risk in Communities (ARIC) Study [J]. *Am J Epidemiol*, 2000,151(5):478-487.
- [13] Sumino H, Ichikawa S, Shu K, et al. Relationship between carotid atherosclerosis and lumbar spine bone mineral density in postmenopausal women [J]. *Hypertens Res*, 2008,31(6):1191-1197.
- [14] Shin J, Park JH, Song YM, et al. Association between lumbar bone mineral density and carotid intima-media thickness in Korean adults; a cross-sectional study of healthy twin study [J]. *J Korean Med Sci*, 2017,32(1):70-76.
- [15] Anagnostis P, Karagiannis A, Kakafika AI, et al. Atherosclerosis and osteoporosis: age-dependent degenerative processes or related entities [J]. *Osteoporos Int*, 2009,20(2):197-207.
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