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The Influence of Ursolic Acid on Bone Formation and Bone Mineral in Alcohol-induced Osteoporosis Rats*

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ABSTRACT Objective: To study influence of Ursolic Acid (UA) on bone formation and bone mineral in alcohol-induced osteoporosis in rats. **Methods:** Adult male wistar rats (n=10 for each group) were randomly divided into blank control group, UA control group, model group, UA low-, medium-, high group and were respectively given saline, 150 mg/kg UA, 50% alcohol, 50 mg/kg UA, 100 mg/kg UA, 150 mg/kg UA. UA control group was administered an equal volume of saline as the blank control group, and UA low-, medium-, high group were received an equal volume of alcohol as the alcohol model group. The rats were administered for 8 weeks. The content of serum phosphorus (P) was detected by phosphate method. The content of serum calcium (Ca) was detected by chromatometry method. Serum bone gla protein (BGP) and bone morphogenetic protein 2 (BMP-2) were detected by ELISA. **Results:** Compared with the blank control group, the contents of serum Ca, P, BGP and BMP-2 were reduced in the model group ($P<0.05$). Compared with the model group, the contents of serum Ca, P, BGP and BMP-2 were significantly increased in the medium-, and high-dose group ($P<0.05$). The result of HE staining revealed that bone trabecular was rules, density and uniform thickness in blank control group, and bone trabecular was irregular, sparse, and fracture in the model group. Compared with the model group, bone trabecular was intact, arranged regularly and continuous in the medium-, and high-dose group. **Conclusions:** Alcohol plays its role in AOP rats by reducing bone formation and promoting bone mineral loss. UA has antiosteoporotic effects on AOP rats by promoting bone formation and reversing bone mineral loss.

Key words: Alcohol-Induced Osteoporosis (AOP); Ursolic Acid (UA); Bone Formation; Bone Mineral

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Introduction

Osteoporosis (OP) is characterized by a decrease in bone mass as well as a deterioration of the bone architecture resulting in an increased risk of fracture. The main mechanism of osteoporosis is that bone absorption is more forceful than bone formation, which causes bone mineral loss and leads to the formation of osteoporosis. The present study found that the well-known risk factors of osteoporosis included women's gender, age, white, positive family history, insufficient calcium intake, use of glucocorticoid and sex hormone imbalance, smoking, and excessive drinking was one of the important factors [1]. Alcohol - Induced Osteoporosis (AOP) refers to the bone loss caused by long-term, large amounts of alcohol intake. Bone microstructure damage and osteopsathyrosis increases the risks of fracture of a whole body bone metabolism disorder which belongs to secondary osteoporosis and the low transformation type osteoporosis[2]. As the increasing of population of long-term heavy drinking and consumption of alcohol, it would bring serious influence to family and society. According to the WHO reported in 2011, each year the world because of alcohol dependence and abuse led to medical expenses increased by 4.5%, the mortality rate increased 3.8%[3]. AOP is common clinically, but for the problem has not been received enough attention. Therefore, the study on AOP becomes extremely important. Ursolic acid (UA) ,which is widely distributed in natural plants, has anti-inflammatory, anti-tumor, anti-oxidant, protecting liver, reducing blood lipid and other biological activities [4,5]. This study used animal experiment to investigate the effects of UA on bone formation and bone mineral in alcohol osteoporosis rats.

1 Materials and Methods

1.1 Materials

Ursolic acid (UA), its molecular formula is $C_{30}H_{48}O_3$, molecular weight 456.68, the chemical structure was shown in Figure 1 [6].

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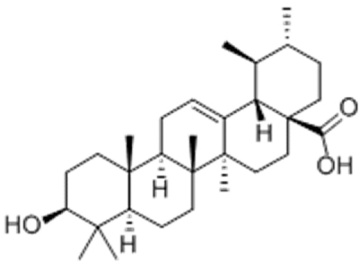

Fig. 1 Chemical structure of UA

1.2 Main chemicals and reagents

UA (98% purity, Changsha LuYuan Bio-Tech Co., Ltd.); Ethanol (Sinopharm Chemical Reagent Co., Ltd).

Calcium (Ca) Assay Kit and Phosphate (P) Assay Kit were provided by Nanjing Jiancheng Bioengineering Institute; Bone morphogenetic protein 2 (BMP-2) ELISA Kit and Bone gla protein (BGP) ELISA Kit were purchased from Shanghai DoBio Biotech Co.,Ltd.

1.3 Main experimental instruments

RM2135 paraffin section (Germany LEICA company); Semi-auto tissue machine (British SHANDON company); Olympus BX60 microscope (Japanese Olympus company); Constant temperature water bath (Tianjin Automatic Science Instrument Co., Ltd); 722s visible spectrophotometer (Shanghai precision scientific instrument Co., Ltd).

1.4 Experiment methods

60 male Wistar rats at 2 months were fed adaptively for 1 week, and then randomly divided into blank control group, UA control group, model group, UA low-, medium- , high group (n=10 for each group). All of them had common feed and water freely and were weighed every week. Model group was daily lavaged 50% alcohol 12 mL/kg·bw[7]. Blank control group was administrated with saline. UA control group was lavaged 150 mg/kg·bw UA and administered an equal volume of saline as the blank control group. UA low-, medium-, high group and were respectively given 50 mg/kg·bw UA, 100 mg/kg·bw UA, 150 mg/kg·bw UA and administered an equal volume of alcohol as the model group. All treatments lasted for 8 weeks. At the late of medication, all groups stop feeding about 12 hours. Abdominal aorta was an esthesiaed with 3% pentobarbital sodium. Serum was collected by abdominal aortic method to test Ca, P, BGP and BMP-2 content. Gathered femur of rats, fixed with dampen formaldehyde solution, paraffin embed, routine HE stained and morphology observed by microscope. The remainder was stored in - 80℃ refrigerator for follow-up study.

1.5 Statistical analysis

The results were statistically analyzed using SPSS 17.0 package. All the data were expressed as the Mean ± SD. Probability values lower 0.05 were considered statistically significant.

2 Results

2.1 Growth and development of rats

During the experiment, the animals from blank control group and UA control group showed normal behavioral activity, luster hair, no obvious abnormality in feces and weight increasing. Model group showed slow action, less activity, rough hair, appetite reduced, and part of the feces was soft. Compared with model group, the animals from UA intervention groups showed flexible activity, neat hair, no obvious abnormality in diet and feces of rats, and the effect of high and medium dose group was better than the low dose group. As shown in figure 2: the week weight in alcohol model group was significantly lower than the blank control group and higher in UA intervention groups than that in the alcohol model group.

2.2 Related index analysis of UA in serum of osteoporosis

2.2.1 Changes of serum BGP content The content of BGP

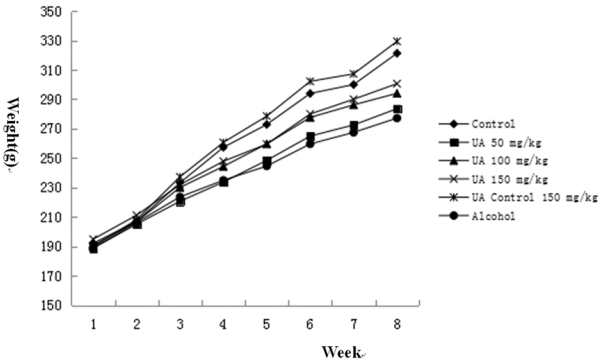

Fig. 2 Week weight curves of rats

was significantly reduced in model group compared to the blank control group ($P < 0.05$); There were no significant differences between UA control group and blank control group ($P > 0.05$); Compared with model group, the content of BGP was increased in UA medium-, high group, and the difference was statistically significant ($P < 0.05$); However, there was no significant difference between UA low group and model group ($P > 0.05$), as shown in table 1.

Table 1 Comparison of the femur BGP and BMP-2 of rats ($\bar{x}\pm s$, n=10)

Groups	BGP(ng/mL)	BMP-2(ng/mL)
Blank group	14.113± 0.162	19.970± 1.136
Model group	12.763± 0.430[a]	16.705± 0.515[a]
UA low group	13.042± 0.282[a]	17.444± 0.580[a]
UA medium group	13.543± 0.535[ab]	17.625± 1.363[a]
UA high group	13.624± 0.700[b]	18.256± 1.072[ab]
UA control group	14.393± 0.263[b]	19.679± 0.502

Note: Compared with normal control group, [a] $P<0.05$; Compared with model group, [b] $P<0.05$.

2.2.2 Changes of serum BMP-2 content There was no significant difference between UA control group and blank control group ($P > 0.05$); Compared with blank control group, the content of BMP - 2 was significantly reduced in model group, and the dif-

ference was statistically significant ($P < 0.05$); Compared with model group, the content of BMP - 2 was significantly increased in UA high group, and the difference was statistically significant ($P < 0.05$); Comparing the UA low-, medium group with the model group, the difference of serum BMP-2 was not statistically ($P > 0.05$), as shown in table 1.

2.2.3 Changes of serum Ca and P content The serum contents of Ca and P were significantly lower in model group than in blank control group ($P < 0.05$); Compared with model group, the contents of Ca and P were obviously improved in UA medium-, high group, and the difference was statistically significant ($P < 0.05$). But no significant difference was found between UA low group and model group ($P > 0.05$), as shown in table 2.

Table 2 Comparison of the femur Ca and P of rats ($\bar{x}\pm$ s, n=10)

Groups	Ca(mmol/L)	P(mmol/L)
Blank group	2.349± 0.116	2.505± 0.198
Model group	2.097± 0.136[a]	2.195± 0.113[a]
UA low group	2.162± 0.814[a]	2.199± 0.307[a]
UA medium group	2.279± 0.706[b]	2.556± 0.228[b]
UA high group	2.328± 0.112[b]	2.490± 0.263[b]
UA control group	2.348± 0.616	2.318± 0.227

Note:, [a] P<0.05 Compared with normal control group; [b] P<0.05 Compared with model group.

2.3 Femoral histomorphology observations

Bone trabecular was rules, density and uniform thickness in blank control group, and bone trabecular was irregular, sparse, and fracture in the model group. Compared with the model group, bone trabecular was intact, arranged regularly and continuous in the UA medium-, and high-dose group. But the improvement in UA low dose group was not obvious, as shown in figure 3.

3 Discussion

Long term excessive alcohol consumption can induce many diseases, such as alcoholic liver disease, alcoholic pancreatitis, alcoholic myocarditis and central nervous system lesions [8]. As for the influence of alcohol to skeleton, in 1965, Saville first found bone mass reduced significantly in corpse of excessive alcohol drinking [9]. Alcohol induced osteoporosis (AOP) was caused by long term excessive alcohol consumption, cooperated with other factors [10]. Many ways can induce the occurrence of AOP. Among them, the overexpression of a variety of inflammatory factors is one of the important ways. Perrien, et al. added ethyl (12 g / (kgod)) into liquid diet of SD rats, and 4w later immunohistochemical staining of femur marrow showed that expression of TNF-α and IL-1b increased significantly[11]. Dai, et al. administrated 5% ethyl to IL-6 knockout rats for 4 moth, and found that colony formation of osteoclast precursor cells in IL-6+/+ rats increased 40% compared with IL-6-/- rats, suggesting that IL-6 might play a role of intermediary in the process of osteoporosis induced by alcohol[12]. At present, in various index of assessing AOP, pathological observation of femoral tissue is the common in animal research, also is regarded as the golden standard. It was reported that bone trabecular was sparse significantly in rats administrated with white spirit (45°) for 8w[13]. Also the Zelanian rabbit was used as the subject, gavaged 45% ethyl per day according the dose of 10 ml/kg, the pathology observation result showed that bone trabecular became thin and sparse [14]. Our result also showed that bone trabecular was sparse, anomaly, even fracture in alcohol model group after 8w intervention of ethyl. In addition, drink heavily also was accompanied by nutrition imbalance, less exercise, weight loss et al, all of this improved osteoporosis [15]. In our study, the alcohol model group rats showed inappetence, excrement soft and significantly loss of weight compared with normal

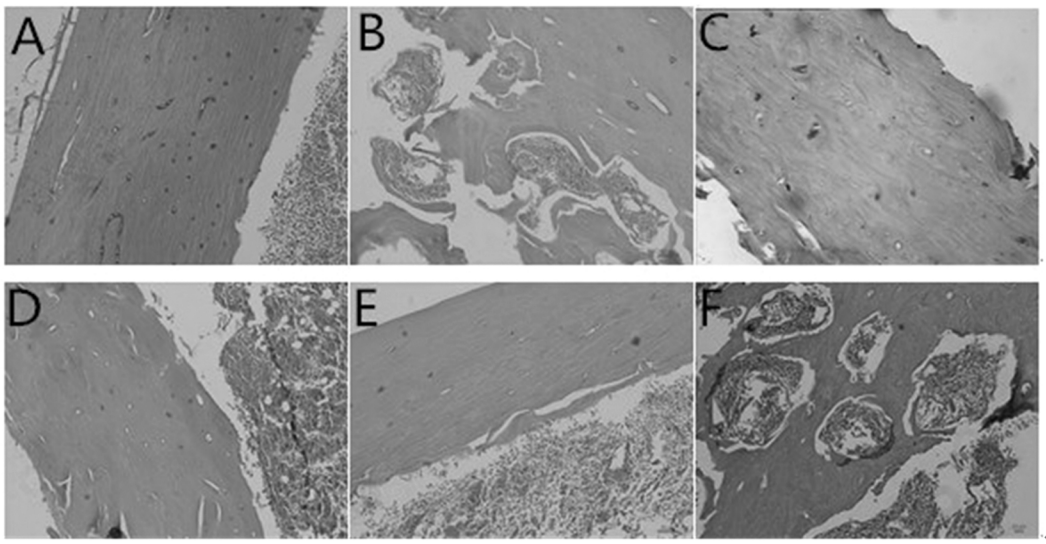

Fig. 3 Pathological changes of the femur of rats (HE, × 200)

A: Blank group; B: Low dose group; C: Medium dose group; D: High dose group; E: UA control group; F: Model group

control group.

UA is a pentacyclic triterpenoid. It exists in many different natural plants, such as pajasmine, glossy privet fruit, oldenlandia, hawthorn, see-buckthorn. UA has low toxicity (LD50>2,000 mg/kg·bw), various efficient biological activity. It has been reported that UA was safe for rabbits when suffered from acute and subacute toxicity test [6]. In our study, no significant difference were found between UA control group and blank control group, suggesting that UA had no toxic and side effect for normal rats. The most prominent activity of UA was anti-inflammatory. It was reported that under the role of LPS, THP-1 cell could promote the synthesis and release of IL-6. While after administration of UA, IL-6 was decreased significantly [16]. Saaby L, et al. found that UA can restrain the release of IL-6 [17]. Moreover, alcohol can affect type I insulin-like growth factor receptor (IGF-IR), which was the vital growth factor for the secretion of bone cell and regulated the activity of bone cell by autocrine and paracrine [18], interestingly, UA can strengthen the activity of IGF-IR [19]. Our results showed that after intervention of UA, pathology change of femur was significantly improved, especially in UA medium and high dose groups. Therefore, combined with our previous work, we surmise that UA had the role of protection skeleton, which might come true by the anti-inflammation of UA.

Bone of human body is in a dynamic balance, manifestation included the balance of bone formation and bone resorption. Osteoporosis could be induced when bone repsorption higher than bone formation. BGP is from osseous tissue, and is secreted in to skeleton by osteoblast, so the changed of serum BGP can specifically reflect the activity of osteoblast and can be used as the specific indicator to assess the formation of bone [20]. BMP-2 can promote the new bone growth by inducing bone marrow-derived mesenchymal stem cells (BMSCs) into cartilage cells and osteoblast. It was reported that BMP-2 had the capacity of promotion osteoblast differentiation and induction bone formation in vitro. BMP-2 was regarded as the only factor that had the highest activity and can induce bone formation solely in the family of transformation growth factor (TGF) [21]. In this study, we found that serum BGP and BMP-2 decreased significantly when exposed to ethyl, while BGP increased after administration of UA with medium and high dose and BMP-2 was increased in UA high group. These results suggest that UA facilitated bone formation in AOP. Calcium and phosphorus are the main minerals in bone and keep balance with the blood contents. But the balance would be disequilibrated when suffered from osteoporosis. Rese archers found that over and long term drinking could reduce rats serum calcium concentration, calcium-phosphorus product level and calcium salt deposition [7, 15]. Studies about human also found that people who drank alc ohol showed low bone mineral density [18]. In our study, the results showed that serum Ca and P significantly decreased in rats drank alcohol; this was in line with previous study. Treatment with UA at the dose of 100 mg/kg·bw and 150 mg/kg·bw can elevate serum Ca and P significantly, suggesting that UA had the function of inhibition the draining of bone mineral induced by AOP, and further promoted the deposition of calcium salt.

Finally, alcohol can induce rats' osteoporosis, showing destruction of bone trabecular construction, decrease of BGP and BMP-2 and loss of bone mineral. While UA administration can constrain the loss of bone mineral, accelerate the deposition of calcium salt and bone formation, showing protection role in AOP.

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熊果酸对酒精性骨质疏松大鼠骨形成、骨矿化的影响*

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摘要 目的：探讨熊果酸对酒精所致骨质疏松大鼠骨形成、骨矿化的影响。**方法：**雄性 Wistar 大鼠 60 只，按体重随机分为空白对照组、熊果酸对照组、模型组、熊果酸低、中、高剂量组，同时分别给予生理盐水、150 mg/kg 熊果酸、50%酒精，50 mg/kg 熊果酸，100 mg/kg 熊果酸，150 mg/kg 熊果酸灌胃。熊果酸对照组生理盐水剂量同空白组，熊果酸低、中、高剂量组酒精剂量同模型组。灌胃共持续 8 周。磷钼酸法检测血清磷(P)含量，比色法检测血清钙(Ca)含量，酶联免疫吸附（ELISA）法检测血清骨钙素(BGP)、骨形成蛋白 -2(BMP-2)浓度；HE 染色法观察股骨结构的病理学变化。**结果：**与空白对照组相比较，模型组血清 BGP、BMP-2 和 Ca、P 均明显降低，且有统计学差异($P < 0.05$)，但熊果酸对照与空白对照组各项指标结果相近。熊果酸中、高剂量组大鼠血清 BGP、Ca 和 P 水平均较模型组有显著升高，差异具有统计学意义 ($P < 0.05$)，但仅熊果酸高剂量组血清 BMP-2 显著升高($P < 0.05$)。股骨组织 HE 染色结果显示，空白对照组骨小梁致密、规则且较粗，粗细均匀；模型组骨小梁稀松、不规则、粗细不均匀，甚至可见骨小梁断裂；熊果酸中、高剂量组骨小梁致密、规则、较厚、粗细均匀，未见骨小梁断裂。**结论：**熊果酸能够促进酒精性骨质疏松大鼠的骨形成，抑制骨矿物质的流失，在改善酒精致骨质疏松方面有一定的保护作用。

关键词：酒精性骨质疏松；熊果酸；骨形成；骨矿物质

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