Implication of vinegar alone or in combination with Caltrate in a rat osteoporosis model induced by a low calcium diet and retinoic acid

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INTRODUCTION

Vinegar is not only a globally popular condiment and food ingredient but also a medicinal food having been used to fight diseases for thousands of years, which could be dated back to Hippocrates (460–377 BC) who used vinegar to manage wounds [1]. In China, vinegar is also regarded as a traditional Chinese medicine (TCM) with the tropism of taste of sour, warm, in the liver and stomach, and with various functions including soothing liver, dispersing blood stasis, relieving pain and reducing toxicity [2]. These medicinal effects of vinegar are further reflected in the processing of Chinese herbal medicine, vinegar processing [3]. In recent decades, there is increasing interest in the effect of vinegar on some metabolic disorders such as obesity, type 2 diabetes, liver diseases and tumor [1,4].

Osteoporosis is a global public health problem. It is characterized by reduced density and quality of bone, which mainly occurs progressively with ageing, in postmenopausal women or secondary to certain diseases and certain drug treatment [5,6]. To date, the role of vinegar in osteoporosis is not fully revealed. It has been reported that an appropriate amount of vinegar is beneficial to the absorption of calcium, thus reduces the bone turnover [7]. On the other hand, it is known that acetic acid, the main organic acid in vinegar, can lead to enamel demineralization in vitro [8,9], and there is even a reported case of osteoporosis caused by excessive intake of cider vinegar in Austria [10]. Therefore, some people may fear for that a long term consumption of vinegar will impair calcium metabolism leading to the calcium loss, and might worsen...
osteoporosis. Thus, the impact of vinegar on osteoporosis gains quite a few attentions.

Hengshun aromatic vinegar, one of the four famous vinegars in China, is produced by the fermentation of glutinous rice followed by a long ageing time. It has a history of 180 years and a large amount of consumers in China, and is also supplied to 160 countries as a basic flavoring material for Chinese cuisine [11,12]. To investigate the possibility of vinegar affecting the development of osteoporosis and the effectiveness of calcium supplements, we used this aromatic vinegar to explore the effect of vinegar in a rat osteoporosis model induced by a low-calcium (Ca) diet combined with retinoic acid. In addition, we would assess whether vinegar influenced the effect of Caltrate, a widely used calcium supplement.

MATERIALS AND METHODS

Materials

Hengshun aromatic vinegar (total acid: 4.5 g/100 mL) was provided by Jiangsu Hengshun Vinegar industry Co., Ltd (Zhenjiang, Jiangsu, China) and diluted with normal saline before use. Caltrate (Calcium Carbonate, Vitamin D, and Elements Tablets, hereafter referred as to Caltrate) was the product of Wyeth Pharmaceuticals (Suzhou, Jiangsu, China). The tablets were ground and suspended in 0.5% CMC-Na buffer (12 mg calcium carbonate/mL). All-trans-retinoic acid was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China) and suspended in 1% CMC-Na buffer. Kits for the measurements of inorganic phosphorus (P), inorganic calcium (Ca) and serum alkaline phosphatase (ALP), and Rat osteocalcin (Bone Gla Protein, BGP) ELISA kit were provided by Nanjing Jiancheng Biotechnology Co., Ltd (Nanjing, Jiangsu, China).

Animals and Experimental Design

SPF female and male SD rats (weighing 200-240 g; Animal quality certificate: SCXK (Su) 2018-0012) were purchased from the Experimental Animal Center of Jiangsu University (Zhenjiang, Jiangsu, China). Rats were housed in temperature-controlled rooms at 22 ± 2 °C with a 12 h of light/dark cycle alternatively and with free access to food and water. The animal care and all experimental procedures were approved by Animal Ethics Committee of China Pharmaceutical University. After acclimatization for 10 days, rats were divided into 7 groups (5 female and 5 male rats per group): normal rat control group, osteoporosis rat model control group and osteoporosis model groups administered with vinegar (total acid: 27.5, 55, 110 mg/kg), Caltrate (60 mg/kg) and combined treatment (60 mg/kg Caltrate plus 55 mg/kg vinegar), respectively. Except the normal group that received standard diet (1.1% w/w calcium, Qinglongshan Animal breeding ground, Nanjing, Jiangsu, China), other groups of rats were fed with a low-calcium (Low-Ca, 0.3% w/w Ca, Qinglongshan Animal breeding ground, Nanjing, China) diet for 6 weeks and received by gavage 70 mg/kg retinoic acid once daily during the last two weeks. Rats in treated groups were orally administered by gavage with Hengshun vinegar, Caltrate or combination of both once per day for the whole period. Instead, normal and model rats received the same volumes of 0.5% CMC-Na (5 mL/kg) for 6 weeks.

Assay of Serum Biochemical Markers

24 h after the final administration of vinegar or Caltrate, rats were anaesthetized for blood collection from the eye socket. The blood samples were centrifuged at 3000 rpm for 10 min and the serums were collected and stored at -20°C. Serum Ca, P, ALP and BGP were measured using commercial kits according to the protocols recommended by the manufacturers.

Measurement of Bone Mineral Density (BMD)

Rats were anesthetized with anesthetic ether and killed by cervical dislocation. Bilateral femurs and tibias and all the lumbar vertebrae were dissected, cleaned off the soft tissue, and stored at -20 °C before analysis, except that some tibias were fixed in 10% formalin as indicated below. The BMD of left femurs (F-BMD) and lumbar vertebrae (L1-L5) (L-BMD) were measured by dual energy X-ray absorptiometry using a Hologic Discovery system (Hologic Inc., Bedford, MA, USA).

Biomechanical Testing and Bone Ca and P Content Measurement

The biomechanical properties were detected via a three-point bending test as previously reported [13-14]. Briefly, the right femurs were thawed at room temperature and the femur length and midshaft diameter were recorded. After the femurs were placed on an electronic controlled universal material testing machine (Instron, Shanghai, China), a force was applied at 1 mm/min to the midshaft diaphysis until a fracture occurred. The force-displacement curves were recorded and processed using the corresponding software to calculate the ultimate load (N), stiffness (KN/mm), elastic modulus (Mpa), flexural strength (Mpa) and energy absorption (Nm).

For the assessment of the Ca and P content, left femurs were thawed at room temperature and dried to a constant weight at 110 °C to get the dry weights. After charring and ashing, the samples were dissolved in 6 mol/L HCl and pH was adjusted to neutral. The Ca and P contents were measured with commercial kits and expressed as the ratio of mineral content to dry weight (mg/g).

Bone Histological Analysis

The tibias were dissected from rats, fixed in 10% neutral buffered formalin for 5 days, and decalcified by 10% buffered EDTA for 4 weeks.
formic acid for 2 weeks. Samples were then dehydrated by passage through an ethanol series, embedded in paraffin and sectioned at a thickness of 3 μm. The sections were stained with 0.1% toluidine blue, cleared in xylene and observed under a microscope (100×). Three fields per section were selected for the measurement of trabecular number, trabecular thickness, trabecular bone area fraction and trabecular separation.

**Statistical Analysis**

All data were expressed as mean ± standard deviation (SD). Intergroup differences were analyzed by one-way analysis of variance (ANOVA) and Dunnett post hoc test with SPSS except for the analysis of body weight by two-way ANOVA and Tukey post hoc test. Statistical significance was set at $p < 0.05$.

**RESULTS**

**The Influence of Vinegar on Rat Body Weight**

Before treatment with low-Ca diet, all rats had a similar body weight. However, from the second to the sixth week, both female and male rats in model group showed an obvious loss in body weight compared with those of normal control (Table 1). Compared with normal group, an earlier weight loss occurred in male rats than in female ones receiving a lower dose of vinegar (27.5 and 55 mg/kg), which appeared from the second week after treatment of low-Ca diet. The decrease rate of body weight in male rats was also higher than that in female groups (Table 1). This difference in sex was also seen in the groups treated with Caltrate (Table 1). Rats received combined Caltrate and vinegar (55 mg/kg) also had a remarkable decrease in body mass in comparison to normal group (Table 1). There was no obvious difference in body mass between model rats and those received vinegar or Caltrate (Table 1). Most rats in model group had a reduction in the food intake and movements with the back arched and leg lame, whereas these changes were partially reversed by treatment with vinegar or Caltrate (data not shown).

**The Effect of Vinegar on Serum Biochemical Markers**

We found an elevated serum Ca in model rats in comparison to the normal control (Figure 1a), indicating the possible loss in bone minerals. This was effectively blunted by combined treatment with Caltrate and vinegar. Solo administration of vinegar or Caltrate also slightly decreased the serum Ca (Figure 1a). No significant difference in serum P was observed among all the groups (Figure 1b). In addition, model rats showed an obvious increase in serum BGP and ALP activity (Figure 1c,1d), demonstrating there was an enhanced activity of osteoblasts. These alterations, coupled with that of increased serum Ca, suggested that model rats had a higher bone turnover than normal ones. Vinegar, Caltrate or combined treatment reduced serum ALP markedly with less impact on BGP. These results suggested

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<th>Model ♀ Body weight (g)</th>
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* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ vs. Normal
vinegar could slightly suppress the high bone turnover caused by low-Ca diet plus retinoic acid.

**The Effect of Vinegar on Bone Mineral Density And Mineral Content**

We next measured the BMD of femurs and lumber vertebrae. As expected, both F-BMD and L-BMD of model rats were decreased significantly (Figure 2a,2b). Treatment with vinegar, Caltrate or both failed to reverse this alteration, indicating that neither vinegar nor Caltrate affected the BMD nor affected the effect of Caltrate in this model. However, the vinegar slightly alleviated the decrease in bone Ca content, and Caltrate showed a more obvious effect (Figure 2c). We did not observe the alterations in bone P content among all the groups. (Figure 2d).

**The Influence of Vinegar on Bone Biomechanical Properties**

We then assessed the biomechanical properties of the femurs via the three-point bending test. In line with the decrease in BMD, the bone quality indexes including the ultimate load, flexural strength, energy absorption and stiffness were all reduced in the model group (Figure 3a-3d). The elastic modulus in model group was also decreased, though the change did not reach a significant level (Figure 3e). Vinegar increased the flexural strength, stiffness and elastic modulus, nevertheless, only the high dose had a significant impact for the index of stiffness (Figure 3b-3e). Caltrate itself did not effectively improve the above indexes. However, it increased remarkably the ultimate load and stiffness when combined with 55 mg/kg vinegar (Figure 3a,3d). Together, these results indicated vinegar would not worsen the osteoporosis but might improve the bone biochemical properties to some extent.

**The Role of Vinegar in Bone Morphology**

We finally analyzed the bone morphology of tibias with toluidine blue staining. Consistent with the results of BMD and bone biochemical parameters, osteoporosis model rats exhibited a great decrease in trabecular number, trabecular thickness and trabecular bone area fraction, while had an increase in trabecular separation when compared with normal rat control (Figure 4a-4d), demonstrating the impairment of bone structure and the loss of bone mass. Treatment with vinegar partially reversed these alterations, and 27.5 mg/kg vinegar even elevated the trabecular number significantly in comparison with the model group. Treatment with Caltrate alone effectively restored the alterations in trabecular bone area and trabecular separation, and combination treatment with vinegar and Caltrate showed the most obvious effects as it markedly increased the trabecular number, trabecular bone area and trabecular separation (Figure 4a,4c,4d). These results demonstrated that an appropriate consumption of vinegar might have a mild and beneficial effect on the bone integrity, at least in this rat osteoporosis model induced with low-Ca and retinoic acid.

**DISCUSSION**

Osteoporosis is a chronic metabolic skeletal disorder and might be affected by dietary condiments. In the present study, we demonstrated the role of vinegar in a rat osteoporosis model induced by retinoic acid on a low-Ca diet. The vinegar alone did not worsen the progression of osteoporosis. In
Yu: Vinegar consumption, Caltrate, and osteoporosis

**Figure 2:** The effect of vinegar on the BMD and bone mineral contents. (a) Femur BMD. (b) Lumbar BMD. (c) Bone Ca content. (d) Bone P content. Values are expressed as mean±SD (n=10). ##p < 0.01, ###p < 0.001 vs normal; *p < 0.05 vs model.

**Figure 3:** The effect of vinegar on biomechanical parameters. (a) Ultimate load. (b) Flexural strength. (c) Energy absorption. (d) Stiffness. (e) Elastic modulus. Values are expressed as mean±SD (n=10). ##p < 0.01, ###p < 0.001 vs normal; *p < 0.05, **p < 0.01 vs model.
contrast, it showed a mild, positive effect on bone integrity,
which was more obvious in combined treatment with
Caltrate.

Calcium deficiency is a risk factor of osteoporosis and
tightly associated with the low BMD and decreased bone
quality [15-17]. For this, low-Ca diet is used to induce
animal osteoporosis, especially in combination with
ovariectomy [18-20]. All-trans-retinoic acid is a metabolite
of vitamin A. It can bind to nuclear retinoic acid receptors
(RARs), peroxisome proliferator-activated receptors (PPARs)
or retinoid-related orphan receptors (RORs) to activate or
initiate gene transcription. Its effect is also related to the
phosphorylation of cAMP response element-binding protein
(EREB) affecting the gene transcription [21]. Although
retinoic acid can regulate the formations and functions of
both osteoblasts and osteoclasts, treatment of animals with
a high dose of retinoic acid will lead to osteopenia and
fracture [21-25]. Induction of animal osteoporosis with
retinoic acid has the advantages of simple execution, short
duration and high rate of success. To investigate the effect
of vinegar on osteoporosis and further explore its impact
on the effect of calcium supplements, we reproduced a rat
osteoporosis model with a combination of a low-Ca diet and
70 mg/kg retinoic acid. The model had the features of high
bone turnover as it showed a significant elevation of serum
ALP and BGP, which was similar to that induced by retinoic
acid or low-Ca diet [19,24,26]. The balance between bone
formation and resorption determines the bone mass and
quality, which is depended on the functions of osteoblasts
and osteoclasts [5]. These functions are partially reflected by
the blood biochemical markers, such as serum ALP and BGP.
In bone tissue, ALP is secreted from osteoblasts and regarded
as a marker of osteoblast. It is crucial for bone mineralization.
BGP is an important non-collagen protein. Like ALP, BGP is
also a marker reflecting osteoblastic activity but is considered
indicative of bone turnover rather than bone formation. High
levels of ALP and BGP occur in osteoporosis of increased
bone turnover such as postmenopausal osteoporosis in
elderly women and in ovariectomized rats [27-30]. Vinegar at
doses of 27.5, 55 and 110 mg/kg, which is equivalent to about
6.5-26 mL/day consumption of vinegar for a 60 kg person,
markedly decreased serum ALP and slightly decreased BGP,
suggesting that it might suppress the high bone turnover
moderately. The vinegar moderately decreased serum Ca
level and increased the bone Ca content, although the effects
were not significant. These alterations in serum and bone Ca
level were also seen in the rats treated with Caltrate alone or
combination of vinegar and Caltrate. Thus, we speculated
this impact of vinegar might be in part due to the acetic and

Figure 4: The effect of vinegar on tibia histology. (a) Trabecular number. (b) Trabecular thickness. (c) Trabecular bone
area fraction. (d) Trabecular separation. (e) Toluidine blue staining. Values are expressed as mean±SD (n=10). #p < 0.05,
##p < 0.001 vs normal; *p < 0.05, **p < 0.01, ***p < 0.001 vs model.
lactic acid containing in vinegar, which have been reported to promote the intestinal absorption of Ca [7,31].

The vinegar failed to reverse the decrease in BMD in femur and lumbar vertebrae, nor did it worsen the loss of bone mass or influence the effect of Caltrate. Recent studies revealed that BMD alone cannot completely reflect the bone health, the latter is still affected by factors other than BMD, such as biomechanical factors and bone microstructures [5]. We next measured the biomechanical performance and histological indicators. Osteoporosis model rats had an overall decrease in the five indices including ultimate load, energy absorption, flexural strength, stiffness and elastic modulus. Vinegar itself did not impact the former two but reversed the reductions of the latter three markers, at least in part. Though these effects were not significant because of the individual difference, the vinegar reversed the decrease in flexural strength, stiffness and elastic modulus by 25%–59%, 35%–40% and 111%–124%, respectively. Although bone Ca content is important for bone strength [32], and the low-Ca diet was reported to impair the trabecular and cancellous bone structures [19], the above biomechanical improvements induced by vinegar were not simply attributed to the increase in bone Ca level, because the impact of Caltrate on flexural strength (increased by 27.6%) and elastic modulus (increased by 82.4%) were less than that of vinegar, particularly for the indicator of elastic modulus. Because there are various organic acids, amino acids, minerals and flavonoids containing in Hengshun vinegar [33,34], these compounds might modulate the glucose, fat and protein homeostasis during the dynamic process of bone remodeling, thus affected the bone biomechanics and microstructures [4,35-38]. Lee MY and co-workers have reported that Rubus coreanus vinegar was beneficial for the ovariectomized rats evidenced by the regulations in the contents of a series of metabolites in plasma [39]. While for the influence on stiffness, bone Ca seemed to play a key role. For this index, the impact of Caltrate was equivalent to that of vinegar, and combined treatment showed a most obvious effect.

Microstructure is another crucial factor impacting bone quality [5]. The vinegar moderately counteracted the impairments of trabecular bone induced by combination treatment of a low-Ca diet and retinoic acid, which should contribute to its impacts on biomechanical properties. Caltrate alone significantly ameliorated trabecular bone area and trabecular separation, and combined treatment showed a more obvious effect than Caltrate as it also increased trabecular number effectively. It is somewhat surprised that it is not completely consistent between the effects of Caltrate and co-treatment on biomechanical and trabecular indicators. One reason for this is that other factors, such as the cancellous bone microstructure, which we did not evaluated, influence the biomechanical performance. In addition, biomechanical parameters were assessed in femurs while morphological examinations were performed in tibias. The difference in detected position would also lead to the inconformity between the two indicators.

Together, Hengshun vinegar at doses of 27.5,55 and 110 mg/kg, which is equivalent to about 6.5-26 mL/day consumption of vinegar for a 60 kg person, will not affect the loss of BMD in the rat osteoporosis model induced by a combination of low-Ca diet and retinoic acid, nor did it decrease the effect of Caltrate. In contrast, it slightly ameliorated the bone biomechanical performance and microstructure, particularly in combined treatment with Caltrate. Here, we did not further explore the underlying mechanisms. However, according to the literature search, the mild beneficial effects of vinegar might be mainly due to acetic acid and polyphenols in the vinegar. Acetic acid can improve the Ca absorption, and both of acetic acid and polyphenols are reported to activate AMP-activate protein kinase (AMPK), mitigate inflammation and oxidative stress, and improve blood flow [4,7,37,38].

**CONFLICT OF INTEREST STATEMENT**

We declare that we have no conflict of interest.

**ACKNOWLEDGEMENTS**

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