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Original Article

Myrtol ameliorates cartilage lesions in an osteoarthritis rat model

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Abstract: Purpose: The aim of this study is to evaluate the effects of myrtol standardized on cartilage lesions in osteoarthritis (OA) rats. Methods: Fifty-six healthy Sprague-Dawley rats were randomly divided into sham group (13 rats) and OA model group (43 rats) with interior meniscus excision. Then serum estradiol (E_2) and glycosaminoglycan (GAG) content in cartilage tissue were measured by radioimmunoassay and toluidine blue staining, respectively. After that, the model rats were randomly divided into low dose myrtol (LDM) group, middle dose myrtol (MDM) group and high dose myrtol (HDM) group (10 rats in each group) with treatment of 450 mg/kg, 300 mg/kg and 150 mg/kg myrtol, respectively. Then, Mankin scores were used to evaluate lesion extent of knee joint cartilage. Expression of tumor necrosis factor α (TNF-α), transforming growth factor β1 (TGF-β1), interleukin (IL)-6, Bax and Bcl-2 were investigated using PCR gel electrophoresis method. Results: Mankin cores were lower in sham group and myrtol group than in model group. There were statistically significant differences ($P < 0.01$) between sham group and model group in expression of TNF-α, TGF-β1, IL-6, Bax and Bcl-2 in the cartilage tissue. Myrtol significantly reduced the expression of TNF-α, IL-6 and Bax, and increased the expression of TGF-β1 and Bcl-2 in myrtol group, comparing with those in model group ($P < 0.01$). Conclusions: Myrtol could down-regulate the expression of TNF-α, IL-6 and Bax, and up-regulate the expression of TGF-β1 and Bcl-2. Myrtol standardized is a promising drug to ameliorate knee cartilage lesions in the OA rat model.

Keywords: Osteoarthritis, myrtol standardized, estradiol (E_2), glycosaminoglycan (GAG), cytokines

Introduction

Osteoarthritis (OA) is a severe chronic progressive disease and prevalent in women, especially in menopause women [1], which could lead to long-term disability [2]. The knee is the most susceptible joint and the major symptoms are pain and restricted joint function. The cost for OA therapy increase with population age [3]. Thus, it is necessary to study the OA therapy method.

Administration of drugs is an effective method to ameliorate these symptoms. The OA therapy drugs included acetaminophen, anti-inflammatory drugs and capsacin [4, 5]. Nonsteroidal anti-inflammatory drugs (NSAIDs) is frequently chosen as fist-line therapy for OA [6]. Herbal medicine is an promising source for new drug discovery [7]. The main components of myrtol which was extracted from eucalyptus leaves of *Myrtuscommunis* were cineol, limonene and α-pinene [8] and it has been applied in the treatment of respiratory system disease, such as acute bronchitis, chronic bronchitis and chronic obstructive pulmonary disease [9]. In addition, myrtol also has broad spectrum antibacterial activities, anti-inflammatory and anti-allergy activities [9-11].

The mechanism of pathogenesis of OA is not clear and thought to be related with trauma, cartilage metabolic abnormalities, immune abnormalities and increase of age [12, 13]. Numbers of cytokines were related with OA, such as interleukin (IL), tumor necrosis factor alpha (TNF-α), prostaglandin and nitric oxide [14]. OA pathogenesis is related with progres-

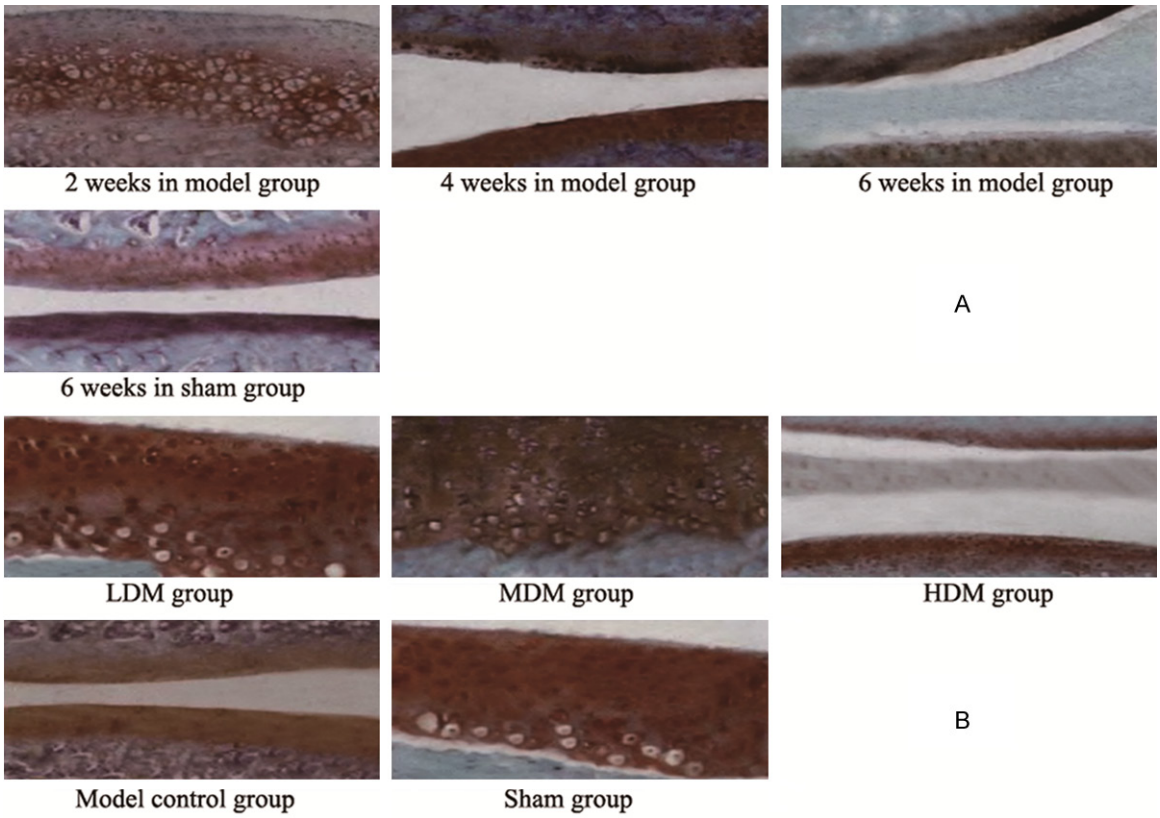

Figure 1. Hematoxylin and eosin staining of cartilage tissue (×20). A: Morphology of cartilage tissues in different groups after surgery; B: Morphology changes of cartilage tissue after myrtol administration. LDM, MDM and HDM group represents low dose myrtol group, middle dose myrtol group and high dose myrtol group, respectively.

sive loss of cartilage cells which included cell senility and apoptosis. In addition, several reports have reported an increased amount of apoptosis cell death in OA cartilage [15-17].

In our study, effects of myrtol standardized on OA rat model were investigated. The expression levels of TNF-α, TGF-β1, IL-6, Bax and Bcl-2 were measured in OA model rats, normal rats and OA model rats with myrtol standardized treatment. The results would be helpful for understanding of cytokines and apoptosis function in pathogenesis of OA and provide new drug selections for OA therapy.

Material and methods

Animals

The average body weight of 56 rats was 160 ± 15 g and the average age was 8 weeks. Rats were divided into 10 cages and kept under controlled environmental conditions (21 ± 1°C, 60% relative humidity, 12 h alternate light-dark cycles). The noise was less than 50 decibel. Rats were fed with standard diet and had access to Milli-Q water ad libitum. All studies have been approved by China Ethics Committee and performed in accordance with the ethical standards.

Performance of interior meniscus excision and sham surgery

Rats were randomly divided into two groups: 13 rats in sham group and 43 rats in model group. Rats were anaesthetized with intraperitoneal injection of sodium pentobarbital (3%). Rats in model group were subjected to interior meniscus resection. The operations were performed under aseptic conditions. Interior knee was made a 2 cm longitudinal incision and exposed. Then cruciate and interior collateral ligament were cut off. The interior meniscus was resected and articular cartilage surface was left. Then joint capsule and skin were sewed by 4-0 silk thread. The rats were not immobilized post-

Table 1. Comparison of estradiol (E_2) and sugar glycosaminoglycans (GAG) content in serum between sham group and OA (osteoarthritis) model group

Group	n	E_2	GAG
Sham	3	201.05 ± 39.52	0.632 ± 0.060
OA model	3	90.23 ± 35.39*	0.248 ± 0.053*

*P < 0.01, comparing with sham group.

operatively. Rats in sham group were subjected to incision without interior meniscus excision. After surgery, rats received intramuscular injection with amikacin for 4 days to prevent infection and were forced to walk 30 min twice per day. The morphologies of knees were observed at 2 weeks, 4 weeks and 6 weeks postoperatioin, respectively.

Blood sampling and radioimmunoassay of estradiol (E_2)

After 4 weeks of surgery, 3 rats were selected from sham group and model group respectively and blood were collected from abdominal aortic. Serum was centrifuged at 3000 r/min for 10 min. Then ^{135}I antibody, target antibody, target standard substance and immune separating agents were prepared. After adding responding agents, tubes were incubated at 37°C for 1 h. After incubation, 500 μL immune separating agents were added into tubes and incubated for 15 min. After centrifugation at 3000 r/min for 10 min, supernatants were discarded. Finally, precipitation count (CPM) was measured by γ radioimmunoassay counter (SN-695, Shanghai hesuorihuan photoelectric company).

Myrtol administration

After surgery, rats of OA model group were fed for 4 weeks and then were given Myrtol standardized which was purchased from Pohl-Boskamp GmbH & Co. Forty rats were randomly divided into OA model group as control, high dose myrtol (HDM) group, middle dose myrtol (MDM) group and low dose myrtol (LDM) group and the doses of myrtol were 0 mg/kg, 450 mg/kg, 300 mg/kg and 150 mg/kg, respectively. Drug administration was performed once for myrtol group every day. Sham group and model group were given equal volume of saline. On the 12th week, rats were executed euthanasia death. Lateral femoral condyle cartilage and subchondral bone were cut by sharp blade and then 10% paraformaldehyde was used for tissues fixation. After that, tissues were paraffin-embedded and stored at 4°C.

Toluidine blue staining for glycosaminoglycan (GAG) analysis and haematoxylin and eosin staining for Mankin scores

Paraffin blocks were cut into 4 μm sections by paraffin slicing machine (RM2235, Leica, German) and then were stained with 0.5% toluidine blue for 20 min. After that, slides were washed twice by distilled water for each 1 min. Finally, cells were observed under light microscopy (BH3-MJL, Olympus, Japan).

Paraffin blocks were cut into 4 μm sections and stained by Maryer hematoxylin for 2 min and eosin for 10 s. Then slides were washed twice by distilled water for each 2 min and observed by microscope. Mankin method was used to evaluate cartilage lesion [18].

Gel electrophoresis analysis

Total RNA in the cartilage tissue was extracted by TRIzol and was transcribed to cDNA according to manufacture's instruction (rimeScript RT reagent Kit Perfect Real Time kit). The primers of TNF-α, TGF-β1, IL-6, Bax and Bcl-2 were synthesized by Takara (China). The PCR products were analyzed by gel imaging analysis software.

Statistical analysis

Data were subjected to SPSS 16.0 software for analysis and represent as means ± standard deviation. ANOVA was performed for significant analysis, followed by independent-Sample T Test. Differences were considered to be significant when P < 0.05.

Results

Model establishment

After postoperative 1 week for rats in model group, both knees demonstrated spindle swelling and the strength of both hind legs were reduced, showing the adynamia phenomenon. After postoperative 2 weeks, both knees joints were red and swollen. Condyle cartilage was lackluster and with mild hyperemia phenomenon. Cartilage was mildly damaged and cell cluster was obvious with disordered arrangement. After postoperative 4 weeks, the knee

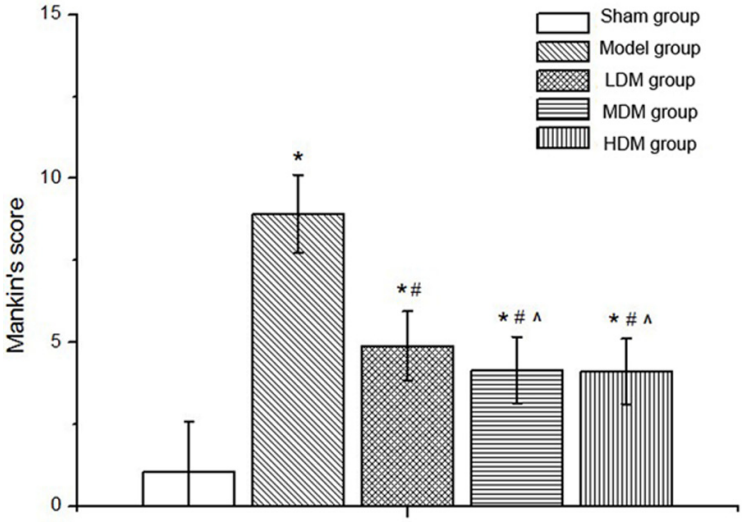

Figure 2. Mankin scores of cartilage lesions in the rats (haematoxylin and eosin staining). LDM, MDM and HDM group represent low dose myrtol group, middle dose myrtol group and high dose myrtol group, respectively. *P < 0.01, comparing with sham group; #P < 0.01, comparing with model group; ^P < 0.05, comparing with LDM.

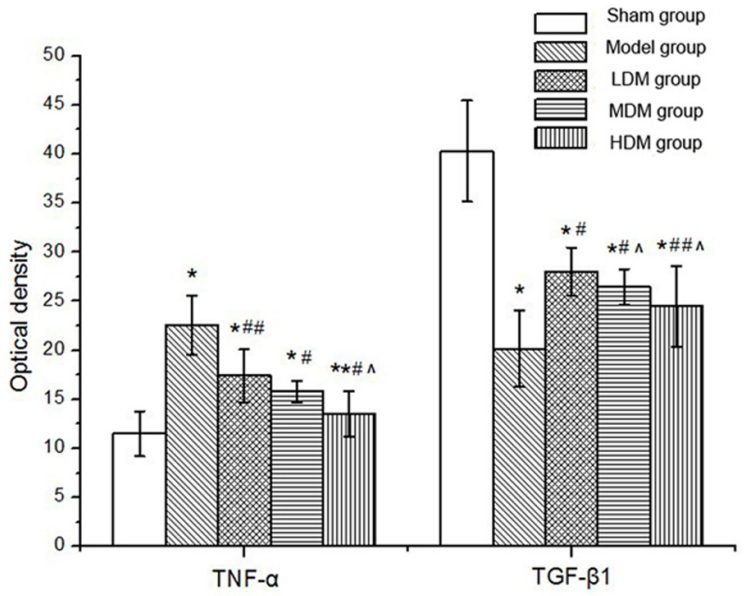

Figure 3. Effect of myrtol on expression of TNF-α and TNF-β1 in cartilage tissue of rats with knee osteoarthritis. LDM, MDM and HDM group represent low dose myrtol group, middle dose myrtol group and high dose myrtol group, respectively. *P < 0.01, **P < 0.05, comparing with sham group; #P < 0.01, ##P < 0.05, comparing with model group; ^P < 0.05, comparing with LDM group.

joint were deformity swelling. The surface of cartilage was dim, rough, thin and soft. Broken nucleus and karyolysis appeared in the cartilage cells. After postoperative 6 weeks, joint cartilage was with severe congestion and cracking. Cartilage defect appeared in the medial femoral condyle. In sham group, both knees were without swelling and the surface of cartilage was smooth, transparency and without congestion and cartilage defect (**Figure 1**). Thus, the OA model was successfully established.

E_2 and GAG content

The OD means of images by radioimmunoassay method and toluidine blue staining were used to evaluate the content of E_2 and GAG, respectively. **Table 1** showed the content of serum E_2 and GAG in sham group and model group. E_2 content was 90.23 ± 35.39 in model group and 201.05 ± 39.52 in sham group (**Table 1**). GAG content was 0.248 ± 0.053 in model group and 0.632 ± 0.060 in sham group. The results showed that the content of E_2 and GAG were both higher in sham group than that in model group (P < 0.01).

Mankin scores

Figure 1B showed the histology of cartilage changes under microscope after Myrtol. Mankin scores were used to evaluate lesion extent of cartilage. **Figure 2** showed the Mankin scores of cartilage after 12 weeks. The highest score of cartilage was obtained by model group, which indicated that the lesion extent of model group was the highest. In contrast, sham group got the lowest score (P < 0.01). The score of cartilage in LDM group was higher than that in MDM and HDM groups (P < 0.05).

Expression levels of TNF-α and TGF-β1

Figure 3 showed expression levels of TNF-α and TGF-β1 in cartilage tissue of rats. TNF-α showed the highest expression level in model

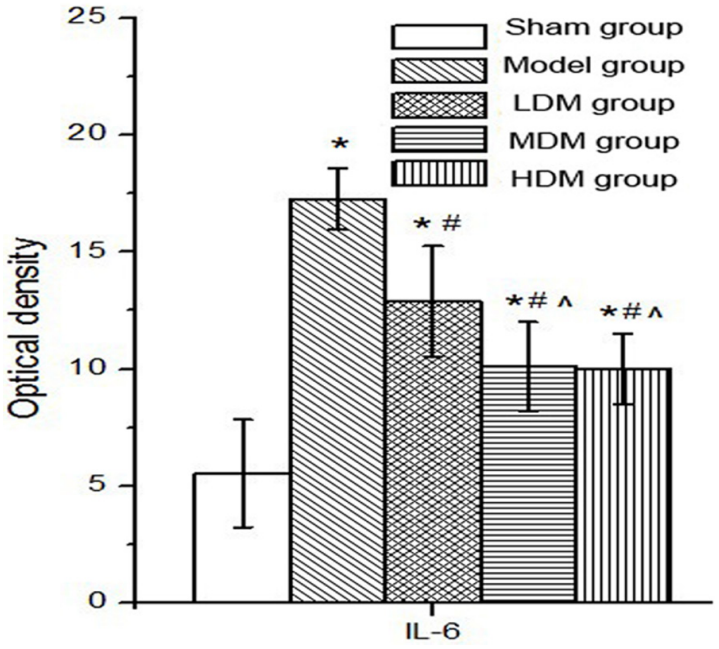

Figure 4. Effect of myrtol on expression of IL-6 in cartilage tissue of rats with knee osteoarthritis. LDM, MDM and HDM group represent low dose myrtol group, middle dose myrtol group and high dose myrtol group, respectively. *P < 0.01, **P < 0.05, comparing with sham group; #P < 0.01, ##P < 0.05, comparing with model group; ^P < 0.05, comparing with LDM group.

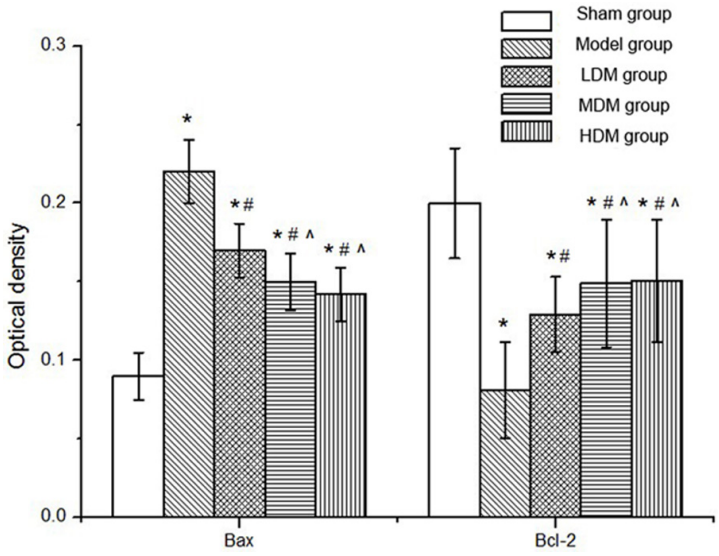

Figure 5. Effect of myrtol on expression of Bax and Bcl-2 in cartilage tissue of rats with knee osteoarthritis. LDM, MDM and HDM group represent low dose myrtol group, middle dose myrtol group and high dose myrtol group, respectively. *P < 0.01, **P < 0.05, comparing with sham group; #P < 0.01, ##P < 0.05, comparing with model group; ^P < 0.05, comparing with LDM group.

group and lowest in sham group (P < 0.05). The expression level of TNF-α was lower in myrtol group than that in model group (P < 0.05). In addition, expression level of TNF-α was higher in LDM group than that in HDM group (P < 0.05).

Expression pattern of TGF-β1 was different from TNF-α. TGF-β1 showed the highest expression level in sham group and lowest in model group (P < 0.05). The expression level of TGF-β1 was higher in myrtol group than that in model group (P < 0.05). In addition, expression level of TGF-β1 was higher in LDM group than that in HDM group (P < 0.05).

Expression level of IL-6

Figure 4 showed the expression levels of IL-6 in cartilage tissue of rats. Expression pattern of IL-6 showed the lowest expression level in sham group and highest in model group (P < 0.01). Expression level was lower in myrtol group than that in model group (P < 0.01). There was no difference between HDM group and MDM group and expression level of IL-6 were higher in LDM than that in HDM group (P < 0.05).

Expression levels of Bcl-2 and Bax

Figure 5 showed expression levels of Bax and Bcl-2 in cartilage tissue of rats. Bax showed the highest expression level in model group and lowest in sham group (P < 0.01). The expression level of Bax was lower in myrtol group than that in model group (P < 0.01) and higher in LDM group than that in HDM and MDM group (P < 0.05).

In contrast, Bcl-2 showed the lowest expression level in model group and the highest in sham group (P < 0.01). The expression level of Bcl-2 was higher in myrtol group than that in model group (P < 0.01) and lower in LDM group than that in HDM and MDM group (P < 0.05).

Discussion

In the study, we demonstrated that myrtol ameliorated OA. The OA model was verified by the

morphology of cartilage. Myrtol down-regulated expression of TNF-α, IL-6, Bax and up-regulated expression of TGF-β1, Bcl-2.

Decline of E_2 was an important reason that induced knee OA in women, especially in postmenopausal women and supplementary of estradiol could reduce morbidity of knee OA [19]. The decrease of GAG could be observed in arthroedema animals with OA [20]. Thus, measurements of E_2 and GAG could reflect occurrence of OA. In our study, E_2 and GAG concentrations were employed to evaluate if the model was successful. The content of E_2 and GAG were both lower in model group than those in sham group. Thus, the OA model was successfully established.

Mankin scores were used for evaluation of lesion extent of cartilage and higher scores indicated higher lesion extent [21]. In our study, Mankin scores of myrtol group were higher than those in model group ($P < 0.1$) which indicated regression of cartilage in myrtol group was much lighter than that in model group. The results showed that myrtol could repair cartilage lesion and protect cartilage.

TNF-α is a cytokine which involves in inflammation. There are several evidences showing that TNF-α was related with pathogenesis of human OA. For example, studies in murine arthritis animal model have demonstrated that TNF-α was sufficient to drive inflammation [22]. TNF-α mediated the loss of GAG [23]. In myrtol, 1,8-cineole is one of the main monoterpenes and could reduce cellular TNF-α release [24]. Smith et al. found that when the cartilage cells were transfected with TGF-β1, which promoted repair of damaged cartilage and inhibition of cartilage degeneration [25]. In our study, myrtol down-regulated expression of TNF-α but up-regulated expression of TGF-β1 in OA rats. The results demonstrated that myrtol ameliorated OA mediated expression of cytokines TNF-α and TGF-β1.

IL-6 played critical role in the regression OA and was an important factor to destroy joint and the reason of inflammatory occurrence [26]. In OA, cartilage released amounts of IL-6 in vivo conditions [27]. In our study, myrtol could down-regulate IL-6 expression in cartilage. The function of myrtol on OA may be involved in expression of IL-6.

Apoptosis plays an important role in pathogenesis of OA. Blancop et al. have demonstrated that apoptotic cells account for 11%-22% in OA cartilage and 2%-4% in normal cartilage [28] and Kim et al. have reported that apoptotic cells accounts for 51% in OA cartilage [29]. Although the data were so different, the results showed that apoptosis occurred in OA. Bcl-2 family consisted of two antipodal protein classes: one is cell apoptosis inhibition proteins including Bcl-2, Bcl-xL and Bcl-w and another class is cell apoptosis promoting proteins including Bax, Bid and Bak [30]. The proportion of these two classes decided whether cells would induce apoptosis when stimulated. Oltval has reported that Bax and Bcl-2 played important roles in the apoptosis process [31]. In our study, myrtol down-regulated Bax expression in OA rat cartilage cells and up-regulated expression of Bcl-2, which meant myrtol could inhibit the apoptosis induced by OA.

In summary, myrtol ameliorated cartilage lesion in OA rats. Myrtol down-regulated expression of TNF-α and IL-6 which may inhibit cartilage matrix degradation, bone resorption, inflammation of cartilage cells and up-regulated expression of TGF-β1 which may promote the synthesis of cartilage proteoglycan and collagen by cartilage cells, restoration of the damaged cartilage and inhibit cartilage lesions. Myrtol also could down-regulate expression of Bax and up-regulate Bcl-2 which may inhibit chondrocytes apoptosis in OA rats. However, correlations between TNF-α, TGF-β1, IL-6, Bax, Bcl-2 and clinical experiments were needed for further investigation. These results indicated that myrtol ameliorated lesion in OA rats mediated cytokines and apoptosis and is a promising drug for clinical therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

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