Int J Clin Exp Med 2019;12(5):6109-6115
www.ijcem.com /ISSN:1940-5901/IJCEM0087774

Original Article

Study on regulation of articular chondrocyte proliferation and apoptosis in rabbit models with berchemia lineata massage cream by scrapping

Keqiang Hou[1], Guangyun He[2], Ming Fang[1], Enling Guan[3], Guanwei Fan[4], Chunxu Li[1]

Departments of [1]Rehabilitation, [2]Pharmacy, [3]Otolaryngology, Traditional Chinese Medical Hospital of Huangdao District, Qingdao, Shandong Province, China; [4]Graduate School, Tianjin University of Traditional Chinese Medicine, Tianjin City, China

Received November 2, 2018; Accepted December 12, 2018; Epub May 15, 2019; Published May 30, 2019

Abstract: Objective: To explore the influence of scrapping with berchemia lineata massage cream on the regulation of articular chondrocyte proliferation and apoptosis in rabbit models. Methods: A total of 80 healthy and clean Japanese white big-ear rabbits were selected to establish an arthritis model. They were divided into study group (n=40) and control group (n=40) in the principle of similar weight. The rabbits in the study group were scrapped with berchemia lineata massage cream for 4 weeks. TdT-mediated dUTP Nick-End Labeling (TUNEL) method was adopted to detect the apoptosis rate of chondrocytes, and Western blotting method was used to detect the expression levels of B-cell lymphoma-2 (Bcl-2) protein, Bcl-2 associated X (Bax) protein, Caspase-3 protein and proliferating cell nuclear antigen (PCNA) in the chondrocytes so as to analyze the effects of scrapping with berchemia lineata massage cream on the treatment of rabbit arthritis models. Results: The apoptosis rate of chondrocytes and the expression levels of Caspase-3 protein and Bax protein in the study group were significantly lower than those in the control group (all $P<0.001$). Moreover, the expression levels of PCNA protein and Bcl-2 protein in the study group were obviously higher than those in the control group ($P<0.001$). Conclusion: The scrapping with berchemia lineata massage cream can promote the proliferation of osteoarthritic chondrocytes and inhibit the apoptosis of chondrocytes. In addition, it can be used as a potential treatment for relieving the condition of arthritis.

Keywords: Scrapping with berchemia lineata massage cream, arthritis, chondrocytes, cell proliferation, apoptosis

Introduction

Osteoarthritis is a common arthropathy which is one of the major causes of loss of work and labor abilities in the population [1]. Osteoarthritis ranks fourth in disabling diseases and usually causes chronic musculoskeletal pain. Joint pain is the common clinical manifestation of osteoarthritis [2]. Osteoarthritis can occur at any age with the highest incidence among the elderly. Its incidence rate in the elderly aged above 70 years old is almost 100%, which is extremely harmful [3]. According to WHO statistics, there are currently 210 million patients with osteoarthritis in the world. Osteoarthritis occupies the 5th place among male diseases and the 7th among female diseases. The incidence rate has been high [4]. Osteoarthritis is likely to be a major factor endangering the health and labor ability of the elderly [5]. Articular cartilage degradation is the main characteristic of pathological changes in the development of patients with osteoarthritis. Chondrocytes play an important role in the protection and metabolism of cartilage [6]. The study of Poulet et al. showed that osteoarthritis in the body will gradually cause apoptosis of chondrocytes which is the main cause of arthropathy in the development of osteoarthritis [7]. Therefore, the exploration of a drug to inhibit the degradation of articular cartilage and the apoptosis of chondrocytes has been the focus of clinical research.

Berchemia lineata massage cream is composed of multiple drugs. It has the effects of stasis removing and bleeding stopping as well as cough preventing and pain relieving clinically [8]. However, there is little study on its effects on the treatment of osteoarthritis in patients

and its mechanism. Therefore, in this study, the effects of scrapping with berchemia lineata massage cream on the articular chondrocyte proliferation and apoptosis in rabbit arthritis models were observed, and the treatment effects and the mechanism of the cream were discussed, providing a reference for the future clinical application of scrapping with berchemia lineata massage cream.

Materials and methods

Experimental animals

Eighty healthy adult Japanese white big-ear rabbits aged 28-32 weeks and weighed 1.90± 0.11 kg which were purchased from Shanghai Fengxian Huihuang Farm (certificate of approval: SCXK (Hu) 2009-0022) and fed in a clean environment (temperature: 24.16±1.03°C; humidity: 54.06±4.76%). During the study, the rabbits could take food and drink water freely. This study strictly followed the hospital's ethical principles of welfare for animal experiments.

Instrument, reagents and drugs

Optical microscope purchased from Beijing Rico Zhongyi Technology Co., Ltd.; test kits for B-cell lymphoma-2 (Bcl-2) protein, Bcl-2 associated X (Bax) protein, Caspase-3 protein and proliferating cell nuclear antigen (PCNA) purchased from Abcam (Shanghai) Trading Co., Ltd.; the whole set of TdT-mediated dUTP Nick-End Labeling (TUNEL) kits purchased from Beyotime Institute of Biotechnology; berchemia lineata massage cream purchased from Anguo Qianxun Chinese Herbal Medicine Co., Ltd.

Experimental animal modeling and intervention

The 80 healthy and clean Japanese white big-ear rabbits were divided into study group (n= 40) and control group (n=40) in the principle of similar weight. Modeling was conducted for the two groups of rabbits. With reference to the modeling method reported in the literature of Rieger et al., the rabbit osteoarthritis model was established after the medial collateral ligament of the two back knees and the anterior and posterior cruciate ligament of the Japanese white big-ear rabbits were cut off, and the medial meniscus was excised [9].

Specific modeling method: The Japanese white big-ear rabbits lay on their back and were fixed on the operation table after they were anesthetized with 100 mg/kg chloral hydrate. Their knees bent to 90 degrees for disinfection. The medial knee joint patella was incised to make the anterior and posterior cruciate ligament exposed. Under direct vision, the medial collateral ligament was cut off, and the medial meniscus was excised. After surgery, the rabbits were immediately injected with penicillin. With reference to Animal Experimental Studies, the rabbits were injected with 200,000 U/d penicillin once every day for continuous 9 days to prevent infections [10]. After the surgery was completed, the Japanese white big-ear rabbits were driven to do activities for at least 15 min per day. During this time, they could take food and drink water freely. At the 4th week after surgery, X-ray examination of the knee joints was conducted for the white big-ear rabbits. The occurrence of subchondral bone sclerosis, bad joint alignment, joint space narrowing and subluxation of the joint indicated successful osteoarthritis modeling.

Treatment methods

Scrapping with berchemia lineata massage cream was used for the treatment of Japanese white big-ear rabbits in the study group, while those in the control group did not receive any treatment. The rabbits were fixed with their knees bent to 90 degrees so as to make the knee joints fully exposed. The berchemia lineata massage cream was applied on the internal and external knee eyes of the patella, and the beginning and ending points of the medial collateral ligament and the anterior and posterior cruciate ligament, and scrapping was conducted back and forth for 15 min. Later on, the aforementioned sites were massaged gently with the palm for 10 min so as to promote drug absorption. The treatment was conducted twice per day (one in the morning and the other in the evening) for continuous 4 weeks.

Specimen collection and index test

After the 4-week treatment was completed, the Japanese white big-ear rabbits were anesthetized with 3% pentobarbital sodium. The knee joint cavity was cut to take the cartilage tissue mass which was rinsed with frozen saline and

Table 1. General information of Japanese white big-ear rabbits in the study group and the control group

Group	Study group (n=38)	Control group (n=37)	t/χ^2	P
Gender			5.166	0.349
Male (%)	23 (60.53)	20 (54.05)		
Female (%)	15 (39.47)	17 (45.95)		
Age			2.153	0.186
≤30 weeks	20 (52.63)	19 (51.35)		
>30 weeks	18 (47.37)	18 (48.65)		
Weight			4.061	0.273
≤1.9 kg	24 (63.16)	22 (59.46)		
>1.9 kg	14 (36.84)	15 (40.54)		
Indoor temperature (°C)	24.12±0.63	23.89±0.51	1.735	0.087
Indoor humidity (%)	54.45±3.03	55.16±2.37	1.128	0.262

stored in a refrigerator (-80°C) for standby application.

Detection of apoptosis

Some cartilage specimens were immersed in the stationary liquid to make paraffin embedding tissue slices. After the slices were treated with dewaxing hydrated protease, the apoptosis of chondrocytes was detected with TUNEL method strictly in accordance with the instructions for the use of TUNEL kits. Microscopic examination was performed with an optical microscope. Five fields were randomly selected from each slice for microscopic examination. Brownish-yellow granules were regarded as positive in the microscopic examination of slices. The percentage of apoptotic cells per 100 cells was called as the apoptosis rate.

Detection of Bax, Bcl-2, Caspase-3 and PCNA in the cartilage tissues

Western blotting method was used to detect Bax, Bcl-2, Caspase-3 and PCNA in the cartilage tissues. The cartilage tissues were fully ground to make tissue homogenate. Proteins were extracted to carry out polyacrylamide gel electrophoresis experiment. The proteins were transferred to polyvinylidene fluoride membrane after electrophoresis. The membrane was reacted at 37°C after it was sealed. Then primary antibodies of Bax, Bcl-2, Caspase-3 and PCNA (1:1,000) were added and placed overnight at 4°C. Thereafter, the horse radish peroxidase (HRP)-labeled secondary antibodies (1:1,000) were added, and the membrane was reacted at 37°C for 1.5 h. Staining, developing and photographing were conducted. The photos were stored for analysis. The gel analysis software (Quantity One 4.6.2) was used to detect the expression levels of Bax, Bcl-2, Caspase-3 and PCNA.

Statistical methods

SPSS 18.0 (Shanghai Cabit Information Technology Co., Ltd.) was used for statistical analysis. The measurement data were expressed as mean ± standard deviation ($\bar{x}$ ± sd). t test was adopted for normally distributed data. Enumeration data were expressed with percentage. Chi-square test was adopted for the comparison between groups. P<0.05 suggested that there was statistical significance.

Results

General information of Japanese white big-ear rabbits in the study group and the control group

The osteoarthritis model was established for Japanese white big-ear rabbits in this study. A total of 38 rabbit arthritis models were established successfully in the study group. The success rate was 95.0% (38/40). A total of 37 rabbit arthritis models were established successfully in the control group. The success rate was 92.50% (37/40). The gender and the age of the Japanese white big-ear rabbits as well as the temperature and humidity of the room where the rabbits were fed had no influence on the study (all P>0.05). See **Table 1**.

Apoptosis rate of chondrocytes in the study group and the control group

The apoptosis rate of chondrocytes (37.62± 3.16%) in the study group was obviously lower than that (64.37±5.23%) in the control group (P<0.001). See **Table 2**.

Expression level of PCNA protein in the chondrocytes in the study group and the control group

The expression level of PCNA protein in the chondrocytes (0.535±0.231) in the study group

Table 2. Comparison of apoptosis rate of chondrocytes between the study group and the control group ($\bar{x}$ ± sd)

Group	Case	Apoptosis rate (%)
Study group	38	37.62±3.16
Control group	37	64.37±5.23
t		26.890
P		<0.001

Table 3. Comparison of the expression level of PCNA protein in the chondrocytes between the study group and the control group ($\bar{x}$ ± sd)

Group	Case	PCNA
Study group	38	0.535±0.031
Control group	37	0.259±0.019
t		46.340
P		<0.001

Note: PCNA, proliferating cell nuclear antigen.

was significantly higher than that (0.259± 0.019) in the control group (P<0.001). See **Table 3**.

Expression levels of Bax protein and Bcl-2 protein in the chondrocytes in the study group and the control group

The expression level of Bax protein in the chondrocytes in the study group was notably lower than that in the control group (0.631±0.023 vs. 0.973±0.026, P<0.001). The expression level of Bcl-2 protein in the study group was remarkably higher than that in the control group (0.596±0.150 vs. 0.443±0.011, P<0.001). The ratio of Bcl-2 protein to Bax protein in the chondrocytes in the study group was higher than that in the control group (0.701±0.028 vs. 0.462±0.014, P<0.001). See **Table 4**.

Expression level of Caspase-3 protein in the chondrocytes in the study group and the control group

The expression level of Caspase-3 protein in the chondrocytes (0.259±0.110) in the study group was lower than that (0.437±0.028) in the control group (P<0.001). See **Table 5**.

Discussion

Osteoarthritis is often manifested as articular cartilage damage, degeneration and osteoarthritis in the patients. In the beginning, the disease is manifested as degenerative changes of articular cartilage which gradually involve the periosteum, bone and joint capsule, and then cause joint swelling, pain and even deformities in the patients. It is a chronic arthritis with pathological changes [11, 12]. The incidence of osteoarthritis is related to the age and chronic injury. Currently, the medical problems for the occurrence of osteoarthritis take up about 10% in the global population. The incidence of osteoarthritis in the elderly population has been high. Its disability rate is up to 50%. Osteoarthritis has become a major factor for the loss of labor ability in the elderly [13, 14]. Cartilage damage is the main pathological feature of osteoarthritis, and chondrocyte apoptosis is an important factor for cartilage damage. Chondrocytes takes up 2% in the total volume of cartilage tissues. They have multiple functions including the secretion and synthesis of matrices as well as the control of the distribution of extracellular matrix [15].

Apoptosis is a normal physiological reaction process in the body. Excessive apoptosis indicates pathological changes. The excessive apoptosis of chondrocytes will result in pathological reduction of chondrocytes, leading to decreased secretion and synthesis of cartilage matrix and causing degenerative changes of articular cartilage [16, 17]. Bax, Bcl-2 and Caspase-3 are all apoptosis factors, and the Bcl-2 protein family has the functions of apoptosis resistance and apoptosis promotion. The ratio of apoptosis-inhibiting factor to apoptosis-promoting factor is closely related to cell viability [18, 19]. The Bcl-2 protein family, a major regulatory factor for apoptosis, plays a key role in maintaining cell differentiation and development as well as cell counts. Bax can promote apoptosis, while Bcl-2 can inhibit apoptosis. Therefore, the ratio of Bax to Bcl-2 determines the viability of chondrocytes [20, 21]. When the ratio of Bax to Bcl increases, cytochrome C can enter cells and mitochondria, which in turn can activate Caspase-3. Caspase-3 is the most important terminal cleavage enzyme in the process of apoptosis, which can activate endonuclease, inhibit repair of DNA damage and promote apoptosis [22]. The over-apoptosis of chondrocytes can simultaneously initiate the proliferation of chondrocytes in the body, thus maintaining a balanced state of the body [23].

Table 4. Comparison of the expression levels of Bax protein and Bcl-2 protein in the chondrocytes between the study group and the control group ($\bar{x}$ ± sd)

Group	Case	Bax	Bcl-2	Bcl-2/Bax
Study group	38	0.631±0.023	0.596±0.150	0.701±0.028
Control group	37	0.973±0.026	0.443±0.011	0.462±0.014
t		60.380	6.187	46.560
P		<0.001	<0.001	<0.001

Note: Bax, Bcl-2 associated X; Bcl-2, B-cell lymphoma-2.

Table 5. Comparison of the expression level of Caspase-3 protein in the chondrocytes between the study group and the control group ($\bar{x}$ ± sd)

Group	Case	Caspase-3
Study group	38	0.259±0.110
Control group	37	0.437±0.028
t		9.545
P		<0.001

The results of this study showed that after treatment, the apoptosis rate of articular chondrocytes in Japanese white big-ear rabbits was decreased obviously, the expression level of Bax protein in the chondrocytes was reduced significantly, the expression level of Bcl-2 protein in the chondrocytes was increased notably and that of Caspase-3 protein in the chondrocytes was reduced prominently, indicating that the scrapping with berchemia lineata massage cream could alleviate the condition of arthritis.

PCNA, a protein with a molecular weight of 36kd, is involved in the repair of DNA damage, plays a key role in the initiation of cell proliferation and can be used as an important index for evaluating the state of cell proliferation [24]. The results of this study showed that the expression level of PCNA protein in the chondrocytes of the Japanese white big-ear rabbits was remarkably increased after treatment, suggesting that the scrapping with berchemia lineata massage cream can promote the proliferation of chondrocytes.

The study conducted by Lin et al. revealed that the kneepad used for curing osteoarthritis can promote the proliferation of chondrocytes and inhibit the apoptosis of chondrocytes, which is similar to the conclusion of this study [25]. The difference is that the osteoarthritis is treated with the kneepad used for curing osteoarthritis. The mechanism still needs to be further validated.

In order to ensure the reliability of the results of this study, the purchased Japanese white big-ear rabbits were strictly screened, and the differences in the age, weight, and health condition of the rabbits were strictly controlled. In medical experiments, animal experiments are conducted to serve the people. As animals are different from humans, the results of animal experiments should be combined with the human body. The experiment that is effective in animals may not be effective in human body and vice versa. Therefore, it is hoped that the objects of next study are screened in clinical practice for treatment so as to further prove the results of this study.

In conclusion, the scrapping with berchemia lineata massage cream can promote the proliferation of osteoarthritic chondrocytes and inhibit the apoptosis of chondrocytes. In addition, it can be used as a potential treatment for relieving the condition of arthritis.

Acknowledgements

This work was supported by Technology Support Project in Huangdao District, Qingdao, Shandong Province (2014-1-100).

Disclosure of conflict of interest

None.

Address correspondence to: Guangyun He, Department of Pharmacy, Traditional Chinese Medical Hospital of Huangdao District, Qingdao, No.158 Hainan Island Road, Huangdao District, Qingdao 266500, Shandong Province, China. Tel: +86-0532-86858887, Fax: +86-0532-86858887; E-mail: heguangyun001234@163.com

References

[1] Kamik J, Reynolds J, Allison J and Camell C. Cryogenic enhancement of joint function, alleviation of joint stiffness and/or alleviation of pain associated with osteoarthritis: U.S. Patent 2017; 4.

[2] Stulberg SD. The natural history of untreated osteoarthritis of the hip. Springer New York 2017; 289-297.

[3] Blaney Davidson EN, van Caam AP, van der Kraan PM. Osteoarthritis year in review 2016: biology. Osteoarthritis Cartilage 2017; 25: 175-180.
[4] Kraus VB, Collins JE, Hargrove D, Losina E, Nevitt M, Katz JN, Wang SX, Sandell LJ, Hoffmann SC and Hunter DJ. Predictive validity of biochemical biomarkers in knee osteoarthritis: data from the FNIH OA biomarkers consortium. Ann Rheum Dis 2017; 76: 186-195.
[5] Ouyang X, Lu G and Zhang H. Clinical effect of joint replacement in the treatment of degenerative osteoarthritis of the knee joint in the elderly. Chinese Community Doctors 2017; 66-68.
[6] Jia PT, Zhang XL, Zuo HN, Lu X and Li L. Articular cartilage degradation is prevented by tanshinone IIA through inhibiting apoptosis and the expression of inflammatory cytokines. Mol Med Rep 2017; 16: 6285-6289.
[7] Poulet B. Models to define the stages of articular cartilage degradation in osteoarthritis development. Int J Exp Pathol 2017; 98: 120-126.
[8] Wu YQ, Deng JG, Zhong ZX and Yang X. Protective effect of the extracts of berchemia lineata (L.) DC. on Hepatic injury in mice. Lishizhen Medicine & Materia Medica Research 2009; 94-95.
[9] Rieger R, Boulocher C and Hoc T. How does hyaluronic acid-based viscosupplements modify the subchondral bone in a rabbit model of early OA. Osteoarthritis & Cartilage 2016; 24: S365.
[10] Arnold AR, Burnham CA, Ford BA, Lawhon SD, McAllister SK, Lonsway D, Albrecht V, Jerris RC, Rasheed JK, Limbago B, Burd EM and Westblade LF. Evaluation of an immunochromatographic assay for rapid detection of penicillin-binding protein 2a in human and animal staphylococcus intermedius Group, Staphylococcus lugdunensis, and Staphylococcus schleiferi Clinical Isolates. J Clin Microbiol 2016; 54: 745-748.
[11] Hochberg MC, Martel-Pelletier J, Monfort J, Moller I, Castillo JR, Arden N, Berenbaum F, Blanco FJ, Conaghan PG, Domenech G, Henrotin Y, Pap T, Richette P, Sawitzke A, du Souich P and Pelletier JP. Combined chondroitin sulfate and glucosamine for painful knee osteoarthritis: a multicentre, randomised, double-blind, non-inferiority trial versus celecoxib. Ann Rheum Dis 2016; 75: 37-44.
[12] Sundén A, Lidengren K, Roos EM, Lohmander LS, Ekvall Hansson E. Hip complaints differ across age and sex: a population-based reference data for the hip disability and osteoarthritis outcome score (HOOS). Health and quality of life outcomes 2018; 16: 200.
[13] Sions J, Coyle P, Velasco TO and Hicks G. Older adults with low back pain who meet a clinical cluster for hip osteoarthritis have ultrasound joint morphological findings consistent with hip osteoarthritis. Osteoarthritis & Cartilage 2016; 24: S248.
[14] Bischoff-Ferrari HA, Orav EJ, Egli A, Dawson-Hughes B, Fischer K, Staehelin HB, Rizzoli R, Hodler J, von Eckardstein A, Freystaetter G, Meyer U, Guggi T, Burckhardt P, Schietzel S, Chocano-Bedoya P, Theiler R, Willett WC, Felson D. Recovery after unilateral knee replacement due to severe osteoarthritis and progression in the contralateral knee: a randomised clinical trial comparing daily 2000 IU versus 800 IU vitamin D. RMD open 2018; 4: e000678.
[15] Zhang M, Mani SB, He Y, Hall AM, Xu L, Li Y, Zurakowski D, Jay GD and Warman ML. Induced superficial chondrocyte death reduces catabolic cartilage damage in murine posttraumatic osteoarthritis. J Clin Invest 2016; 126: 2893-2902.
[16] Jin L. Rhizoma drynariae on synovial cells apoptosis of rabbits with osteoarthritis. Chinese Archives of Traditional Chinese Medicine 2016; 144-147.
[17] Dai M, Sui B, Xue Y. Cartilage repair in degenerative osteoarthritis mediated by squid type II collagen via immunomodulating activation of M2 macrophages, inhibiting apoptosis and hypertrophy of chondrocytes. Biomaterials 2018; 180: 91-103.
[18] Lee JI, Balolong E, Jr., Han Y and Lee S. Stem cells for cartilage repair: what exactly were used for treatment, cultured adipose-derived stem cells or the unexpanded stromal vascular fraction? Osteoarthritis Cartilage 2016; 24: 1302-1303.
[19] Qi WU, Ganxiang HE, Yanfen HU. Study on time-effect and dose-effect of paeonol on the apoptosis of knee osteoarthritis articular chon-drocyte in rabbits and the mRNA expression of its related protein Bcl-2 and bax. China Pharmacy 2016; 27: 1337-1340.
[20] Song B, Song H, Wang W, Wang H, Peng H, Cui J, Wang R, Huang H, Wang W and Wang L. Beclin 1 overexpression inhibits chondrocyte apoptosis and downregulates extracellular matrix metabolism in osteoarthritis. Mol Med Rep 2017; 16: 3958-3964.
[21] Wang J, Wei T, Gao J. Effects of naringenin on inflammation in complete freund's adjuvant-induced arthritis by regulating Bax/Bcl-2 balance. Inflammation 2015; 38: 245-251.
[22] Yang S, Wang J, Chen F, Liu G, Weng Z and Chen J. Elevated Galectin-9 Suppresses Th1 effector function and induces apoptosis of activated CD4 (+) T cells in osteoarthritis. Inflammation 2017; 40: 1062-1071.
[23] Jorgensen C, Noël D, Toupet K. Mesenchymal stem cells derived exosomes and microparti-

cles protect cartilage and bone from degradation in osteoarthritis. Scientific Reports 2017; 7.

[24] Lee S, Kim CM, Lee JH, Lee K, Cho KS and Kim ES. Effect of hemp fiber on UVB-induced epidermal cell proliferation and PCNA expression. Genes & Genomics 2017; 39: 1-7.

[25] Lin MN, Guo JH and Liu JH. Influence of OA knee pad on cartilage cell proliferation and apoptosis of rabbit with knee osteoarthritis. Chinese Journal of Misdiagnostics 2010; 13-15.