The Protective Effect of Dietary Administration of Puerarin on Canine Osteoarthritis Model With Anterior Cruciate Ligament Transected

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Abstract

Background

Lameness caused by osteoarthritis (OA) is one of the main causes of disability in elderly dogs. Non-steroidal anti-inflammatory drugs (NSAIDs) are important tools in the treatment of canine OA. In recent years, due to the many side effects of NSAIDs, patients cannot tolerate or do not want to take the risk of NSAIDs. People are becoming more and more interested in new treatments for canine OA, and so-called nutritional supplements have emerged. Puerarin has a wide range of pharmacological activities and is often used as a clinical prescription drug and dietary supplement in China. However, the effect of puerarin on canine OA has not been evaluated. Therefore, the purpose of this study is to evaluate the anti-inflammatory and anti-cartilage degradation effects of puerarin in a canine OA model induced by anterior cruciate ligament transection (ACLT), and to detect the serum inflammatory factor interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α) levels and cartilage degradation biomarker C-terminal telopeptides of collagen type II (CTX-II), cartilage oligomeric matrix protein (COMP) and chondroitin sulfate 846 epitope (CS 846) levels in serum and synovial fluid at different periods of puerarin administration.

Results

Eight weeks after the administration, the veterinarian performed clinical and imaging evaluations to comprehensively evaluate the protective effect of puerarin on canine OA. Daily oral administration of 20 mg/kg puerarin can significantly inhibit the expression of IL-1 β , IL-6 and TNF- α in serum within 8 weeks ($P < 0.05$), and its anti-inflammatory effect is similar to oral celecoxib (negative control group). Puerarin has a certain protective effect on articular cartilage and can reduce the level of biomarkers CTX-II, COMP and CS 846 in serum and synovial fluid in the early stage of OA ($P < 0.05$). In addition, the clinical scores and radiographs scores were significantly reduced after 8 weeks of puerarin treatment ($P < 0.05$).

Conclusions

Canine OA cartilage may be mediated through anti-inflammatory, anti-metabolism and anabolic effects, and strongly down-regulate the inflammatory factors IL-1 β , IL-6 and TNF- α and cartilage degradation biomarkers CTX-II, COMP and CS 846 are related, providing a good alternative therapy for OA.

Background

paragraph 1

Osteoarthritis (OA), also known as degenerative joint disease, is an inflammatory disease in which articular cartilage undergoes degenerative degradation [1]. OA is a common joint disease in veterinary clinics. It often occurs in horses, cattle and dogs, seriously affecting animal health and quality of life, and causing huge economic losses [2]. OA is very common in overweight and large breed dogs and lameness

caused by OA is also the main reason for the early retirement of police dogs [3]. There are 20% of dogs over 1 year old with varying degrees of OA [4, 5]. The clinical symptoms are chronic pain, joint swelling, claudication, dysfunction and reduced quality of life, which ultimately leads to loss of joint function and mobility.

paragraph 2

Clinically, OA has no specific drugs, and is usually limited to palliative measures to relieve pain and relieve symptoms. In small animals and horses, non-steroidal anti-inflammatory drugs (NSAIDs) are important tools for the treatment of OA [4, 6, 7]. Sulfated glycosaminoglycan or hyaluronic acid injections are often used in horses [7]. NSAIDs exert anti-inflammatory effects by self-inhibiting cyclooxygenase (COX-1 and COX-2), which is produced by the breakdown of arachidonic acid due to cell wall damage [8]. However, the use of NSAIDs usually causes side effects in the gastrointestinal tract, and long-term use is likely to cause organ damage and drug dependence [9]. In addition to reducing pain, preventing cartilage matrix degradation is the key to treating OA. This requires long-term use of safe alternative therapies. In recent years, studies have found that dietary supplements and herbs have played some positive and beneficial effects through anti-catabolic and anti-inflammatory effects [10, 11]. They have attracted much attention because of their high safety, no adverse reactions, convenient management and prevention of the occurrence and development of diseases [2].

paragraph 3

Puerarin (C₂₁H₂₀O₉) is the major bioactive ingredient isolated from the root of *Pueraria lobata* (Willd.) Ohwi [12]. Puerarin has a wide range of pharmacological properties and is used to treat cardiovascular and cerebrovascular diseases, Alzheimer's disease, osteonecrosis, Parkinson's disease, diabetes and diabetic complications, endometriosis and cancer [13]. In addition, puerarin has a wide range of applications in clinical prescriptions and dietary supplements [14]. Puerarin in collagen matrix has the effect of increasing new bone formation locally and can be used for bone grafting or for bone induction [15]. Previous studies have shown that in rat and mouse OA models, puerarin can effectively inhibit inflammation and improve OA [16, 17]. However, the effect of puerarin on canine OA has not been evaluated.

paragraph 4

Inflammation plays a vital role in the pathogenesis of OA [18]. Interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor α (TNF- α) play a significant role in regulating the metabolism of chondrocytes and synovial cells. Articular cartilage is mainly composed of extracellular matrix and chondrocytes [19]. A large amount of proteoglycan and collagen form the network scaffold outside the cartilage cells, which play a role in supporting and shaping the cartilage cells. In the early stage of OA, the destruction of articular cartilage leads to increased decomposition and accumulation of metabolites in the joint synovial fluid (SF). These products are released into the blood circulation and finally released outside the body through filtration and excretion, or decomposed in the body [20, 21]. Biomarkers play an important

role in the early diagnosis and condition evaluation of OA [22]. In the pathological state of canine OA, the biomarkers of C-terminal telopeptides of collagen type II (CTX-II), cartilage oligomeric matrix protein (COMP) and chondroitin sulfate 846 epitope (CS 846) in blood and joint fluid exchange were significantly increased [23-25].

paragraph 5

We hypothesize that Puerarin can protect canine OA and is an effective herbal supplement for canine OA. In the canine OA model with anterior cruciate ligament transection, we studied clinical symptoms and knee joint imaging changes after puerarin administration, as well as changes in serum inflammatory factors and serum/synovial cartilage biomarkers. Explore the anti-inflammatory and anti-matrix degradation effects of puerarin on canine OA.

Results

Clinical Trial

As shown in Fig. 1, with the aggravation of canine OA, the lameness (Fig. 1A), joint mobility (Fig. 1B), pain on palpation (Fig. 1C), weight-bearing (Fig. 1D) and overall score of clinical condition (Fig. 1E) in the OA group showed an upward trend, while feeding puerarin or celecoxib all scores showed a downward trend. Compared with the OA group, pain on palpation score and overall score of clinical condition were significantly lower in the puerarin group and celecoxib group at 6 weeks (T3) and 8 weeks (T4) after modeling ($P < 0.05$). Lameness, joint mobility and weight-bearing score decreased significantly at T4 ($P < 0.05$). In addition, there was no significant difference in lameness and weight-bearing score between the puerarin group and the celecoxib group at T4 ($P > 0.05$), and there was no significant difference in joint mobility, pain on palpation and overall score of clinical condition at T3 and T4 ($P > 0.05$).

Radiographic progression

Fig. 2 is a representative image of X-ray of the right knee joint of each group of dogs after the ACLT induced OA model 8 weeks (T4). In the OA group, the surface of the joints was hardened, and the internal space of the joints became narrow, resulting in joint instability. The width of the tibial plateau and the lower end of the femur is narrowed (Fig. 2A arrow), the patellar space is significantly narrowed, and osteophytes are formed on the edges of the tibial plateau and the femur and are accompanied by osteoporosis (Fig. 2E arrow). In the control group, the knee joint space was larger and there was no osteophyte formation (Fig. 2D rectangle frame). Lateral position radiographs showed that the patellar space was large (Fig. 2H arrow), the tibial plateau surface was smooth, the imaging density of surrounding soft tissues was normal, and the subchondral bone density was evenly distributed. In addition, compared with the control group, the radiographs score of the OA group was significantly higher ($P < 0.001$) (Fig. 2I). At the 6th week (T4) of puerarin administration, the joint space is slightly narrowed, with slight osteophyte formation, the tibial plateau and lower end of the femur have normal widths, but the patella space is slightly narrow (Fig. 4F). The surface of the tibial plateau is rough, but the damage is

not serious (Fig. 2B). After administration of celecoxib, the joint space became smaller than the control group (Fig. 2C), osteophytes formed on the right knee joint, the soft tissue of the knee joint showed high-density shadows, and the patella space was significantly narrowed (Fig. 2G). Compared with the OA group, the radiographs score of the puerarin group and celecoxib group were significantly lower ($P < 0.05$), but it was still statistically significant with the control group ($P < 0.05$) (Fig. 2I).

Effect of puerarin on inflammatory cytokines

Fig. 3 shows the trend of changes in the levels of IL-1 β (Fig. 3A), IL-6 (Fig. 3B) and TNF- α (Fig. 3C) in canine serum after 6 weeks of administration. Compared with the control group, the serum IL-1 β , IL-6 and TNF- α levels in the OA group increased significantly from T0 to T4 ($P < 0.001$). From the second week of administration (T2) to the end of administration (T4), the serum levels of IL-1 β , IL-6 and TNF- α in the puerarin group and celecoxib group were significantly lower than those in the OA group ($P < 0.05$). In addition, there was no significant difference in serum IL-6 concentration between the puerarin group and the celecoxib group at T4 compared with the control group ($P > 0.05$). There was no significant difference in serum levels of IL-1 β , IL-6 and TNF- α between the puerarin group and the celecoxib group ($P > 0.05$). The results showed that the puerarin group and the celecoxib group could significantly reduce the serum levels of IL-1 β , IL-6 and TNF- α in the canine OA model, with similar anti-inflammatory effects.

Effect of puerarin on the biomarkers of cartilage degradation in serum

Fig. 4 shows the trend of changes in the levels of cartilage degradation biomarkers CTX-II (Fig. 4A), COMP (Fig. 4B) and CS 846 (Fig. 4C) in canine serum within 8 weeks (T4) after administration. Compared with the control group, the serum CTX-II, COMP and CS 846 levels of the OA group increased significantly at the time points of T1, T2, T3 and T4 ($P < 0.001$). Compared with the OA group, the serum CS 846 of the puerarin group was significantly reduced at T2 and T3 ($P < 0.001$), the serum CTX-II, COMP and CS 846 of the puerarin group were significantly reduced at T4 ($P < 0.001$), and the celecoxib group at T4 Serum CTX-II was significantly reduced ($P < 0.01$). In the puerarin group, serum CS 846 at T3 and serum CTX-II, COMP and CS 846 levels at T4 were significantly lower than those in the celecoxib group ($P < 0.05$). In addition, within 8 weeks (T4) of the canine ACLT model, we observed that the serum COMP and CS 846 of the OA group increased first and then decreased. However, the concentration of CTX-II showed an upward trend and no peak was detected.

Effect of puerarin on the biomarkers of cartilage degradation in SF

Fig.5 shows the trend of changes in the levels of cartilage degradation biomarkers CTX-II (Fig. 5A), COMP (Fig. 5B) and CS 846 (Fig. 5C) in canine SF within 8 weeks (T4) after administration. Compared with the control group, the levels of CTX-II, COMP and CS 846 in the SF of the OA group increased significantly at time points T1, T2, T3 and T4 ($P < 0.001$), and CTX-II biomarkers showed an upward trend. However, the COMP and CS 846 biomarkers increased and then decreased. Compared with the OA group, CS 846 in the SF of the puerarin group was significantly reduced at T2 and T3 ($P < 0.001$), CTX-II, COMP and CS 846 in the SF of the puerarin group were significantly reduced at T4 ($P < 0.001$), and at T4 Synovial fluid CTX-II

in the celecoxib group was significantly reduced ($P < 0.01$). In addition, the levels of CTX-II, COMP and CS 846 in SF at T2 and T3 in the puerarin group and SF at T4 were significantly lower than those in the celecoxib group ($P < 0.05$).

Discussion

paragraph 1

This study shows that puerarin can improve the severity of OA in the canine model of ACLT, including reducing clinical symptoms, inflammation and erosion of articular cartilage, and can effectively alleviate the condition. The effect of drug treatment is related to the decrease in the level of key cartilage metabolism biomarkers in the structural changes of OA. ACLT is a model of OA traditionally used in large animals (dog, goat/sheep, and horse). It was later incorporated into small mammals, mainly rabbits, rats, and mice [26, 27]. It is considered today the most widely used model of OA [28]. The ACLT model causes changes in the physiology of cartilage cells, cartilage destruction and osteophyte formation, and ultimately leads to joint instability. It is the preferred model for pharmacological research [29]. In addition, since the thickness of the cartilage in dogs is less than half the size of humans, the canine OA model has more advantages in studying cartilage degeneration and cartilage damage [26]. In our study, the clinical symptoms increased significantly after eight weeks of surgery, X-rays showed surface sclerosis, narrowing of the medial joint space, narrowing of the width of the tibial plateau and lower end of the femur, and obvious narrowing of the patella space. Osteophytes formed on the edges of the tibial plateau and femur and accompanied by osteoporosis. Inflammatory factors IL-1 β , IL-6 and TNF- α in canine serum were significantly increased. Cartilage degradation biomarkers CTX-II, COMP and CS 846 were significantly higher in serum and synovial fluid, showing significant OA feature. The above shows that the canine OA model was successfully established. The experiments were conducted under therapeutic conditions to reproduce conditions that are as close as possible to the natural disease.

paragraph 2

Lameness is one of the most common causes of canine OA. It is caused by degeneration of articular cartilage, pain, inflammation, and joint mobility disorders [30]. The drug treatment of OA is limited to the use of NSAIDs. The clinical efficacy of NSAIDs is mainly related to the inhibition of cyclooxygenase 2 (COX-2), and most of them are toxic, especially adverse reactions to the gastrointestinal tract [31]. We use the typical COX-2 inhibitor celecoxib as a positive control. Celecoxib has relatively small side effects and is widely used in the treatment of OA [32, 33]. In recent years, more and more researches on herbal extracts and dietary supplements have opened up new horizons for the treatment of OA [34-36]. Curcumin down-regulates TNF- α by inhibiting the proliferation of macrophages and participates in the activation of fibrinolysis, so it provides a good complementary therapy for canine OA treatment [11]. Avocado/soybean unsaponifiables inhibit nitric oxide synthase and matrix metalloproteinase-13 to exert a protective effect on experimental canine OA [37]. The modern stabilized and freeze-dried green-lipped mussel is more effective than the carprofen in treating chronic pain due to moderate to severe Canine OA and that it has

no side-effects [38]. Studies have shown that puerarin has a positive effect on rat and mouse OA models. It can reduce mechanical hyperalgesia and cartilage damage in OA rats [16], increase the proliferation of cartilage cells in mice and reduce the recruitment of inflammatory monocytes [17]. We evaluated the effects of puerarin in a canine OA model for the first time.

paragraph 3

Inflammation is a variable feature of OA and is associated with joint symptoms and disease progression [39, 40]. We found that the IL-1 β , IL-6 and TNF- α in the serum of the canine OA model increased significantly in the second week, indicating that the inflammatory response was involved in the early stage of OA, even before the appearance of joint structure and clinical symptoms. Canine serum IL-1 β , IL-6 and TNF- α have been continuously increasing at the 8th week of the induced OA model, and no peak value was detected, indicating that the pathological process of OA continued to aggravate, the inflammation reaction was severe, and the damage of articular cartilage increased. After the puerarin group was administered for 2 weeks, the serum levels of IL-1 β , IL-6 and TNF- α were significantly reduced, and puerarin played a significant anti-inflammatory effect in a short period of time in OA. At this time, puerarin was administered. The clinical symptom scores of the posterior dogs did not change (lameness, joint mobility, pain on palpation, weight-bearing and overall score of clinical condition), which further verified that severe inflammatory reactions had occurred before clinical symptoms appeared. After 6 weeks of puerarin administration, compared with OA, serum IL-1 β , IL-6 and TNF- α were significantly reduced, and the clinical symptoms were improved. X-rays showed that the structure and morphology of the dog's knee joint improved. Interestingly, there is no significant difference between the IL-6 concentration in serum and the control group. We speculate that puerarin is more sensitive to IL-6, the inflammatory factor, and its mechanism of action needs further verification. The test results show that puerarin exerts an anti-inflammatory effect in the early stage of canine OA and is similar to the anti-inflammatory effect of the positive control celecoxib group.

paragraph 4

The degradation of type II collagen and proteoglycan in the extracellular matrix of the cartilage will result in the weakening of the tensile strength of the cartilage [41]. CTX-II is one of the degradation products of type-II collagen, mainly derived from its carboxy terminal peptide [24, 42]. COMP is a tissue-specific protein that binds to type II collagen network and plays a role in collagen bundles and collagen network structure [23, 43]. CS 846 epitope is a chondroitin sulfate synthetic biomarker and inseparable from the degree of joint injury in patients with OA [24, 44]. Our study found that the concentration of CTX-II, COMP and CS 846 in the SF is higher than that in the serum. This is because when the articular cartilage is destroyed, the biomarkers CTX-II, CS 846 and COMP are first released into the SF, and then enter the blood circulation. Within 8 weeks after the establishment of the OA model, the levels of COMP and CS 846 in the serum and SF of the OA group increased first and then decreased, while CTX-II continued to increase. This may be because the degradation of proteoglycan in canine OA is earlier than the degradation of collagen, which is similar to the pathological process of human OA. After 6 weeks of administration of

puerarin or celecoxib, CTX-II, COMP, and CS 846 in serum and SF were significantly reduced. However, after 6 weeks of puerarin treatment, the levels of CTX-II, COMP and CS 846 in blood and SF were significantly reduced compared with the celecoxib group. It shows that puerarin can effectively inhibit the degradation of articular cartilage, and the effect is significantly better than celecoxib after taking it for 8 weeks, and it has a protective effect on articular cartilage.

paragraph 5

This research is largely limited by the research design. One of the limitations is that the duration of this study is 8 weeks. Future long-term studies will provide more information about the potential impact of puerarin on the long-term development of OA. In addition, although the ACLT model mimics the pathological changes of spontaneous OA well, it is still different from dogs with spontaneous OA. In the next step, we will evaluate the effect of puerarin on spontaneous canine OA on a large scale. The mechanism of action of puerarin to improve canine OA, especially their overall effect on cartilage metabolism, requires further research to better understand the ways in which puerarin can alter the disease.

Conclusions

Current research shows that the protective effect of puerarin on canine OA cartilage may be mediated through anti-inflammatory, anti-metabolism and anabolic effects, and strongly down-regulate the inflammatory factors IL-1 β , IL-6 and TNF- α and cartilage degradation biomarkers CTX-II, COMP and CS 846 are related, providing a good alternative therapy for OA.

Methods

paragraph 1

Experimental design

16 male German shepherd dogs (6-8 years old, 20-30 kg), all dogs are provided by the Harbin Police Dog Training Base (Harbin, China), each dog is individually housed in a high-strength iron tube kennel (125 cm [length] $\times$ 95 cm [width] $\times$ 110 cm [height]), the temperature is $23 \pm 1^\circ\text{C}$. All kennels are equipped with an automatic watering system. Before being included in the study, the veterinarian performed clinical scores, assessed knee flexion and circumference, lameness scores, and knee X-rays on all dogs to ensure that all measured variables were within the reference range.

Dogs were excluded from the study if they had received any anti-inflammatory or opiate drugs during the four weeks preceding the study.

Dogs were allowed a 2-week acclimatisation period with once-daily hand-walking. The dogs were ranged by weight and randomly divided into control group (sham operation, $n = 4$) and model group (ACLT

surgery, n = 12) to which the animal care personnel were blinded. The model group is divided into 3 subgroups (n = 4 in each group): OA group: untreated; celecoxib group: 200 mg/day celecoxib [32] (Pfizer Pharmaceutical, New York, USA) treated canine OA (positive control); Puerarin group: puerarin (Chengdu Mansite Biological Technology, Chengdu, China) 20 mg/kg/day to treat canine OA; The dosage of puerarin was determined according to previous studies [16, 17] and according to FDA guidelines [45]. The Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University (#NEAU-2017-06-0384-12) approved the experimental design and animal surgery procedures in this study. Make every effort to minimize animal suffering and reduce the number of animals used.

paragraph 2

OA induction and treatment

After induction with propofol (Fresenius Kabi, Uppsala, Sweden) (5.5 mg/kg intravenously), the dogs in the model group were anesthetized by inhalation of 3% isoflurane (Hebei Yibin Pharmaceutical, Hebei, China) in oxygen/nitrous oxide. Dogs in the model group had their right hind limbs shaved and disinfected, and the surgical site was isolated with gauze or wound towel. Make a 3-4 cm incision before the medial collateral ligament of the dog's right knee to open the joint capsule. After the patella is displaced, expose the tibial plateau and femoral condyle, trim nearby muscles (do not damage any ligaments and meniscus). Use a No. 12 scalpel blade to cut off the anterior cruciate ligament (ACL), flush the joint cavity with saline, and put the patella back in place, taking care to prevent bleeding and soft tissue damage. The veterinarian performed a drawer test on the dog to ensure that the ACL was completely cut off. Polyglycolide absorbable suture (Jinhuan Medical Products, Shanghai, China) to close the wound (Fig. 6). No surgery on the left hind limb. During the postoperative period, if needed, dogs were treated with fentanyl patch 75 µg (Janssen Pharmaceutica, Xi'an, China), amoxicyclin (400 mg/kg), repeated as necessary. The control group used the same method for sham operation, but did not undergo any ligament or meniscus resection. After the operation, under the supervision of an animal caregiver, all dogs are actively running for 2 h/day, five times a week.

In the second week after ACLT-induced OA model, dogs in the puerarin group were fed 20 mg/kg puerarin every day, and dogs in the celecoxib group were fed 200 mg celecoxib every day by oral administration daily between 07:30 and 08:30 h, at least 2 h before feeding, starting from the second week (T1) after surgery and continuing for the eighth week (T4). Canine serum and SF samples were collected at 2 weeks (T1), 4 weeks (T2), 6 weeks (T3), and 8 weeks (T4) after induction of the OA model (Fig. 7). For arthrocentesis, the dog is sedated with medetomidine hydrochloride (0.02 mg/kg intravenously). Under aseptic conditions, arthrocentesis is performed to aspirate SF (beside the ligament patellae). The aspirated SF was centrifuged at 2500 g at 25°C for 10 min, and the supernatant was collected and stored at -80°C. All dogs were not euthanized because we conducted a long-term follow-up test based on this research to evaluate the long-term effect of puerarin on canine OA.

paragraph 3

Clinical Trial

At T1, T2, T3 and T4, the veterinarian, blinded to treatment, used an ordinal scoring system to assess the severity of clinical symptoms [46]. The degree of lameness was graded on a scale of 1-5 as follows: The degree of lameness was graded on a scale of 1-5 as follows: 1: (Walks normally); 2: (Slightly lame when walking); 3: (Moderately lame when walking); 4: (Severely lame when walking); and 5: (Severely lame when walking). The degree of joint mobility was graded on a scale of 1-5 as follows: 1: (Full range of motion); 2: (Mild limitation (10-20%) in range of motion; no crepitus); 3: (Mild limitation (10-20%) in range of motion; with crepitus); 4: (Moderate limitation (20-50%) in range of motion; $\pm$ crepitus); and 5: (Severe limitation (>50%) in range of motion; $\pm$ crepitus). The degree of pain on palpation was graded on a scale of 1-5 as follows: 1: (None); 2: (Mild signs; dog turns head in recognition); 3: (Moderate signs; dog pulls limb away); 4: (Severe signs; dog vocalises or becomes aggressive); and 5: (Dog will not allow palpation). The degree of weight-bearing was graded on a scale of 1-5 as follows: 1: (Equal on all limbs standing and walking); 2: (Normal standing; favours affected limb when walking); 3: (Partial weight-bearing standing and walking); 4: (Partial weight-bearing standing; non-weight-bearing walking); and 5: (Non-weight-bearing standing and walking). The degree of overall score of clinical condition was graded on a scale of 1-5 as follows: 1: (Not affected); 2: (Mildly affected); 3: (Moderately affected); 4: (Severely affected); and 5: (Very severely affected).

paragraph 4

Radiographic assessment

The dog is sedated with medetomidine hydrochloride (0.02 mg/kg intravenously), at the 8th week (T4) of puerarin administration, each dog was subjected to radiological evaluation of the knee joint in the anterior and lateral positions. The radiographs were scored for OA on a five-point discontinuous ordinal scale, with 0: representing no osteophytosis and 4: representing the most severe osteophytosis [47]. A single trained veterinary, blinded to treatment, analyzed all of the radiographic images based on the criteria previously described.

paragraph 5

Biomarkers immunoassays

At T1, T2, T3 and T4 time points, a sandwich enzyme-linked immunosorbent assay (ELISA) kit was used to detect the levels of IL-1 β , IL-6 and TNF- α in serum, and biomarkers of cartilage degradation in serum and joint synovial fluid CTX-II, COMP and CS 846 levels. The ELISA operation method is strictly in accordance with the manufacturer's instructions. Three replicate wells are set for each group of samples, and the OD value is averaged. Draw the linear regression curve of the kit standard, and introduce the OD value into the equation to calculate the concentration of each sample. All analyses were performed by the Heilongjiang Key Laboratory for Laboratory Animals and Comparative Medicine in Harbin.

paragraph 6

Statistical Analysis

All statistical analyses were performed using SPSS 19.0 software (Chicago, IL, USA). The results are displayed as mean + standard deviation (SD). Statistical analyses were performed using SPSS software (version 19.0 for Windows, SPSS, Chicago, IL, USA). All data were expressed as mean $\pm$ SD. Data were tested for Gaussian distribution by the Kolmogorov-Smirnov test. All data were normally distributed and were therefore analysed using parametric statistics (one-way ANOVA, two-way analysis of variance, paired two-tailed Student t test, unpaired twotailed Student t test). $P < 0.05$ was considered statistically significant.

Abbreviations

OA: Osteoarthritis; NSAIDs: Non-steroidal anti-inflammatory drugs; IL-1 β : Interleukin-1 β ; IL-6: Interleukin-6; TNF- α : Tumor necrosis factor α ; CTX-II: C-terminal telopeptides of collagen type II; COMP: Cartilage oligomeric matrix protein; CS 846: Chondroitin sulfate 846 epitope; ACLT: Anterior cruciate ligament transection; SF: Synovial fluid; ACL: Anterior cruciate ligament; COX-2: Cyclooxygenase 2;

Declarations

Ethics approval and consent to participate

The Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University (#NEAU-2017-06-0384-12) approved the animal surgery procedure in this study. Make every effort to minimize animal suffering and reduce the number of animals used.

Consent for Publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors were involved in conception and design of the study, or in acquisition analysis and interpretation of data, and in revising it critically for important intellectual content. The experiments were designed by TM, YW and LG. The experiments were performed by XS, HH, YL, MZ, HB, HC and LG collected and analyzed data. TM, YW and LG interpreted the data. TM wrote and edited the manuscript. All authors critically reviewed content and approved final version for publication.

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Figures

Figure 1

Ordinal scoring system to assess the severity of clinical symptoms after puerarin administration. A lameness; B joint mobility; C pain on palpation; D weight-bearing; E overall score of clinical condition. Data are presented as scatter dot plots (error bars denote mean $\pm$ SD) (n = 4).

Figure 2

X-ray images and radiographs score of the canine knee joint 6 weeks after puerarin administration. A-D Anterior position X-ray images of the right knee of each group of dogs. E-H Lateral position X-ray images

of the right knee of each group of dogs. A, E In the OA group, the gap between the tibial plateau and the lower end of the femur was narrowed (A, arrow), the patella gap was narrowed, and osteophytes formed (E, arrow). B, F 6 weeks after the administration of puerarin, the joint space was slightly narrow, the joint space was slightly narrow, the surface of the tibial plateau was rough, but the damage was not serious, there was less osteophyte formation, and the patella space was slightly wider than that in the OA group. C, G The joint space of the celecoxib group became smaller than that of the control group, and osteophytes formed. D, H In the control group, there was no osteophyte formation in healthy knee joint space (D, rectangle frame) and patella space (H, arrow). I radiographs score. Data are presented as scatter dot plots (error bars denote mean $\pm$ SD) (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the OA group; Δ P < 0.05, $\Delta\Delta$ P < 0.001 compared with the control group.

Figure 3

Effect of puerarin on IL-1 β , IL-6 and TNF- α inflammatory cytokines in canine OA model. A The level of IL-1 β in serum. B The level of IL-6 in serum. C The level of TNF- α in serum. Data are presented as scatter dot plots (error bars denote mean $\pm$ SD) (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the OA group; Δ P < 0.05, $\Delta\Delta$ P < 0.001 compared with the control group. NS no significant difference.

Figure 4

Effect of puerarin on serum CTX-II, COMP and CS 846 biomarkers in a canine OA model. A The level of CTX-II in serum. B The level of COMP in serum. C The level of CS 846 in serum. Data are presented as scatter dot plots (error bars denote mean $\pm$ SD) (n = 4). ***P < 0.001 compared with the OA group; $\Delta\Delta$ P < 0.01, $\Delta\Delta\Delta$ P < 0.001 compared with the control group. #P < 0.05, ##P < 0.01, ###P < 0.001 compared with the celecoxib group.

Figure 5

Effect of puerarin on synovial fluid CTX-II, COMP and CS 846 biomarkers in a canine OA model. A The level of CTX-II in synovial fluid. B The level of COMP in synovial fluid. C The level of CS 846 in synovial fluid. Data are presented as scatter dot plots (error bars denote mean $\pm$ SD). ($n = 4$) $**P < 0.05$, $***P < 0.001$ compared with the OA group; $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ compared with the control group. $\#P < 0.05$, $\#\#P < 0.01$ $\#\#\#P < 0.001$ compared with the celecoxib group.

Figure 6

ACLT surgery to induce canine OA model. A Make a 3-4 cm incision before the medial collateral ligament; B Shift the patella to determine the position of the ACL (arrow); C Cut off the ACL (rectangle frame), flush the joint cavity with saline.

Figure 7

Flow chart of the study design. It shows the number of animals, animal age, animal sex, sample collection time, administration time and dosage.

Supplementary Files

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