Clinical Effect of Glucosamine and Chondroitin Contained Nutraceutical on Osteoarthritis in Dogs after Anterior Cruciate Ligament Rupture Surgical Repair

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Abstract

Glucosamine and chondroitin sulfates are key structural components of extracellular fluid in articular cartilage. From previous studies, glucosamine and chondroitin sulfates have several positive effects on cartilage homeostasis and are used in osteoarthritis patients. Although glucosamine and chondroitin sulfates have some positive effects on clinical treatment of osteoarthritis, clinical results still need to be investigated. The objective of this study was to investigate the clinical outcomes on lameness score, radiological score, serum WF6 level and serum HA level of glucosamine and chondroitin sulfates in osteoarthritic dogs, using anterior cruciate ligament rupture patients as study models of osteoarthritis (n=12). The results from this study show no statistical significance between the glucosamine/chondroitin (GsCn) (n=6) and placebo groups (Plab) (n=6) in weight bearing score, lameness score, serum level of hyaluronic (HA) and WF6 chondroitin epitope and radiological score throughout the 16-week study. In the GsCn group the serum WF6 level decreased more rapidly and the increase in serum HA level was slower, compared to the Plab group. Consequently, from these findings, glucosamine and chondroitin sulfate contained nutraceutical may provide some delay effects on the degradation process of cartilage and inflammation of synovial membrane. Moreover, glucosamine and chondroitin sulfates had a high safety index and did not show any adverse effect throughout the study period (16 weeks).

Keywords: anterior cruciate ligament rupture, chondroitin, glucosamine, osteoarthritis.

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Introduction

Osteoarthritis (OA) is an end stage joint disorder which is commonly found in senile patients and animals. OAs is characterized by destruction of articular cartilage, inflammation of synovial membrane and loss of functional movement of synovial joint. Etiopathogenesis of OA remains unclear but it may involve immunological induced inflammatory cascade, several pro-inflammatory cytokines and proteolytic enzymes (Kammermann et al., 1996; Hayami et al., 2006; Doom et al., 2008).

Anterior cruciate ligament (aCL) is a major stabilizing structure of the knee joint. Rupture of aCL is a common disorder in medium to large breeds of dogs, leading to injury of articular cartilage and meniscus, and finally osteoarthritis joint (Louboutin et al., 2009). From these reasons, aCL rupture is a described model in many osteoarthritis studies (Intema et al., 2010; Boulocher et al., 2011). The purpose of surgical treatment in aCL rupture dogs is to stabilize the knee joint but the process of OA still remains. Post-operative dogs may suffer from pain and joint stiffness from OA. Therefore, a long term management of aCL rupture dogs should focus on control of post-operative OA.

Glucosamine and chondroitin sulfates are key substrates for glycosaminoglycan (GAGs) and proteoglycans synthesis. Both glycosaminoglycan and proteoglycan are major composition in extracellular fluid (ECM) of cartilage and synovial fluid (Todhunter and Johnston, 2003). From previous clinical trial studies, glucosamine and chondroitin sulfates contained nutraceutical have several positive effects on clinical lameness scores and healing process of cartilage in osteoarthritis dogs compared with placebo group (McCarthy et al., 2007; Minami et al., 2011). Most nutraceuticals contain both glucosamine and chondroitin sulfates due to positive synergistic effects of these two drugs (Das et al., 2000; Yue et al., 2012).

Nevertheless, other clinical trials revealed that glucosamine and chondroitin sulfates might not have a positive outcome over placebo group (Aragon et al., 2007; Henrotin et al., 2011). Therefore, more investigations into the positive effects of glucosamine and chondroitin sulfates on the treatment of OA are required (Richette et al., 2004; Yang et al., 2004; Bana et al., 2006). One of the most important clinical diagnostic tools in OA patients is radiological imaging. Kellgren-Lawrence scoring system is based on osteophyte formation in radiological examination. Synovial and periosteal mesenchymal stem cells are major targets of TGF-β and BMP to form osteophyte as an endochondral ossification pathway (Van der Kraan et al., 2007).

Biomarker technique is an alternative diagnostic tool that measures a specific biological substrate as an indicator of biological response to diseases. WF6 chondroitin epitope is an anabolic biomarker of disintegrated cartilage which is specific to chondroitin-6-sulfate (main composite of articular cartilage). From previous studies, WF6 biomarker was significantly elevated in OA patients and dogs compared with normal patients (Nnganvongpanit et al., 2004; Pothacharon et al., 2006; Trakulsantit et al., 2010).

Hyaluronic acid (HA) is a polysaccharide which is composed of many of N-acetyl glucosamine and glucronic chains, normally found in connective tissue and synovial fluid. Major function of HA is to lubricate the synovial joint, from its hydrophilic property (Necas et al., 2008). Normally, HA is synthesized from synovioyte type II located in synovial membrane and having a molecular weight around 300-2,000 kDa. In OA process, pro-inflammatory cytokines can activate HA synthesis from synovioyte and reduce molecular weight leading to elevation of serum HA level in OA patient which can be used as an inflammatory biomarker in the OA process (Necas et al., 2008; Nganvongpanit et al., 2008).

The objective of this study was to investigate the clinical lameness score, radiological score, serum WF6 level and serum HA level to evaluate clinical effect of glucosamine and chondroitin sulfate in osteoarthritis dogs, using anterior cruciate ligament rupture patients as study models.

Materials and Methods

Animals: Twelve dogs of various breeds, weighting between 8-43 kg, were presented to Orthopedic Clinic, Small Animal Teaching Hospital Chulalongkorn University with complaint about unilateral hind limb lameness and were diagnosed with anterior cruciate ligament rupture. All protocols used in this study were approved by the Committee of the Ethical Care of Animal of Chulalongkorn University (approval No.12310064).

Surgical procedures: After premedication with 0.02-0.03 mg/kg acepromazine (Ventranquil™, Cevasante animal, Libourne, France) combined with 0.3-0.5 mg/kg morphine sulfate (FDA, Bangkok, Thailand) intramuscularly (pre-operative analgesic), all dogs were induced to surgical anesthetic level by 4 mg/kg propofol (Fresenius Kabi Austria GmbH Graz, Austria) intravenously and received crystalloid fluid intravenously at a rate of 5-10 ml/kg/hr through operation. Cefazolin (250 mg/ml) was administrated in the time of anesthetic induction as pre-operative antibiotic. All dogs were operated using extracapsular technique with CCL lateral suture system® (80 lbs for dog under 16 kg and 100 lbs for dog over 16 kg) (Todhunter and Johnston, 2003; Muir, 2010).

Groups: All dogs in this present study were divided into 2 groups by blind randomized method. In the first group (GaCr), 6 dogs received a compound of glucosamine sulfates (1,500 mg) and chondroitin sulfates (1,200 mg) per day. In the second group (Plab), six dogs received a placebo. All dogs received a treatment from the first day to 4 weeks post-operation.

Physical Examination and Lameness Scoring: Health status and clinical lameness score of all dogs were examined at 0, 2, 4, 8 and 16 weeks post-operation. Clinical lameness score system in this study was
modified from Mac Cathy’s scoring system. The scores range from I-V. In grade I, the dog must walk with a nearly normal gait. In grade II, the dog show slight lameness when it walks. In grade III, the dog shows moderate lameness when it walks. In grade IV, the dog show severe lameness when it walks. In grade V, the dog is reluctant to rise and does not walk more than five paces (Mac Cathy et al., 2007).

**Radiological Study:** Radiographs of all affected stifles were performed in anterior-posterior and mediolateral positions at 0, 2, 4, 8 and 16 weeks post-operation in order to the radiological osteoarthritis score using Kellgren-Lawrence score system. The scores range from 0-IV. In grade 0, osteophyte is not found in examination and the dog has a normal joint space. In grade I, osteophytic lipping is seen in examination with doubtful narrowing of joint space. In grade II, definite osteophytes are seen with possible narrowing of joint space. In grade III, moderate osteophytes are seen with some sclerosis and evidence of bone deformation. In grade IV, multiple large osteophytes are seen with marked narrowing of joint space and definite bone deformation (Takahashi et al., 2004).

**Serological Examination:** Five milliliters of blood were collected from cephalic or saphenous veins (1 ml of blood for complete blood count and blood chemistry analysis and 4 ml of blood for evaluation of serum WF6 biomarker and serum HA biomarker). The samples were collected at 0 (pre-operation), 2, 4, 8 and 16 weeks post operation.

**Serum WF6 Biomarker:** Serum level of WF6 was determined using enzyme-linked immunosorbent assay (ELISA) with mononuclear antibody WF6 from shark cartilage.

Serum samples were added to 1.5 ml plastic tubes and mixed with an equal volume of monoclonal antibody WF6. After incubation at 37°C for 1 h, the incubated samples were transferred to a microtitre plate which was pre-coated with shark skeletal aggre can. Then 1% BSA was added to the samples to block non-specific protein binding. The samples were reincubated for 1 h, washed with Tris-EDTA buffer (TE buffer, pH 7.3) and then peroxidase conjugated anti-mouse IgM antibody was added. The samples were reincubated again for 1 h, the amount of bound peroxidase was determined using O-Phenylene Diamine dihydrochloride substrate (o-PD substrate) and the plates were read at 492/690 nm. Concentration of the epitope WF6 in the samples was calculated from the standard curve. All samples were analyzed at Thailand Excellence Center for Tissue Engineering, Chiang Mai University (Pothacharoen et al., 2004).

**Serum HA Biomarker:** Serum HA level was determined by using enzyme-linked immunosorbent assay (ELISA) using bovine cartilage biotinylated HA-binding proteins (HABPs). Serum samples at different concentrations (19-10,000 ng/ml in 6% BSA-PBS pH 7.4) were mixed with an equal volume of bovine articular cartilage-biotinylated HABPs. Then the samples were incubated at room temperature for 1 h. The incubated samples were transferred to a microplate previously coated with human umbilical cord HA (Sigma-Aldrich, USA) and were blocked with 1% BSA. Reincubated samples were washed with PBS-Tween buffer and peroxidase conjugated antibi otin antibody was added (Zymed, USA). The plate was incubated at room temperature for 1 h and the amount of peroxidase bound was determined using o-PD substrate. The plates were analyzed at 492/690 nm. The amount of HA in the samples was calculated from the standard curve. All samples were analyzed at Thailand Excellence Center for Tissue Engineering, Chiang Mai University (Pothacharoen, 2006). All examination and scoring were performed by only an examiner using a blinded fashion.

**Statistical Analysis:** Signalement, health status and blood parameter of all dogs were analyzed with descriptive analysis. Serum WF6, serum HA level, clinical lameness scores and radiological scores of the GsCn and Plab groups were compared using Kruskal-Wallis one way ANOVA, of which p values ≤ 0.05 were accepted as significant difference. All data were analyzed within group using Wilcoxon sign rank test, of which p values ≤ 0.05 were accepted as significant difference.

**Results**

The average age and weight of the aCL rupture dogs in this study were 6.20±1.85 years and 19.66±10.87 kg, respectively. None of the dogs showed any significant side effects and clinical sign throughout the study period. The clinical lameness score of both GsCn and Plab groups gradually decreased from 1st to 16th weeks post-operation and significantly decreased in 8th week post-operation (p<0.05). Result from Kruskal-Wallis one way ANOVA showed no significant difference between the study groups in every week (p>0.05) (Fig 1).

Radiological osteoarthritis scores of both GsCn and Plab groups gradually increased from 1st to 16th weeks post-operation and significantly increased in 4th week post-operation (p<0.05). Result from Kruskal-Wallis one way ANOVA showed no significant difference between the study groups in every week (p>0.05) (Fig 2).

The serum WF6 levels of the GsCn group reached the highest peak at 2 weeks post-operation, significantly decreased at 4 weeks post-operation (p=0.046), and increased in 8 weeks post-operation (p>0.05). In the Plab group, the serum WF6 level reached the highest peak at 4 weeks post-operation (p=0.025) and decreased at 12 weeks post-operation (p>0.05). Result from Kruskal-Wallis one way ANOVA showed no significant difference between the study groups in every week (p>0.05) (Fig 3).

The serum HA level of the GsCn group reached the highest peak at 12 weeks post-operation (p<0.05) and gradually decreased in the last 2 weeks of the study period. In the Plab group, the serum HA level reached the highest peak at 8 weeks post-operation (p<0.05) and decreased gradually in the last...
In healthy knee joint, the continuous process of OA even in stable environment may not always decreased with time (Muir et al., 2006; Pothacharoen et al., 2006; Tharkal et al., 2008). According to the result of the present study, most aCL patients were in the middle portion of the gait, leading to activation of several inflammatory cytokines (Muir et al., 2006; Doom et al., 2008). According to the results of the present study, most aCL patients were in the middle ages (6.2±1.85 years) and had no traumatic injury. Intra-operative observations found that aCL in all knees were completely torn the middle portion on the ligament bands.

Discussion

Recently, aCL etiopathogenesis has been suggested to be a chronic degenerative change from the immunological cascade of collagen type I, which is the main composite in the aCL. In healthy knee joint, aCL is covered by synovial sheath which protects the aCL from immunological reaction. When aCL has microdamage from repetitive trauma, the collagen type I is exposed to synovial environment. Macrophage is a phagocyte which responds to the small particle of collagen type I and presents the antigen to T-lymphocyte, leading to activation of several inflammatory cytokines (Muir et al., 2006; Doom et al., 2008). According to the results of the present study, most aCL patients were in the middle portion of the gait, leading to activation of several inflammatory cytokines (Muir et al., 2006; Doom et al., 2008). Intra-operative observations found that aCL in all knees were completely torn the middle portion on the ligament bands.

From in vitro studies, GsCn could up-regulate the TGF-β producing genes and also the osteophyte formation (Tharkal et al., 2007). Therefore, Kellgren-Lawrence scoring system may not be suitable for monitoring the effect of GsCn in OA study. The gradual elevation of radiological score may indicate the continuous process of OA even in stabled knee after the operation. This may be due to the alteration of cartilage and subchondral bone homeostasis from immunological process (Pothacharoen et al., 2006; Karsdal et al., 2008; Kwan Tat, 2010). The degree of lameness in both groups constantly decreased throughout the study. It may be due to stabled knee without cranial translation of tibia in weight bearing phase of the gait. The clinical lameness scores were not significantly different between the GsCn and Plab groups. This finding indicates that GsCn may not have an analgesic effect on OA patients and support the negative results from previous studies (Dobenecker, 2006; Messier et al., 2007).

All dogs in this study did not show any adverse effects and had normal health status throughout the period of study. This may suggest that GsCn have a wide margin of safety in recommended dose (1,500 mg of glucosamine sulfate per day and 1,200 mg of chondroitin sulfate per day (Hatchcock et al., 2007). Nevertheless, one previous study found adverse effects such as diarrhea, nausea and vomit (Henrotin et al., 2004). Therefore, using glucosamine and chondroitin sulfates longer than 16-week period should be concerned.

WF6 chondroitin epitope is catabolic biomarker of disintegrated cartilage which is specific to chondroitin-6-sulfate. From the present study, the serum WF6 levels in both groups gradually increased in the first week, which might be due to the operative inflammation and previous OA. The serum WF6 level of the GsCn group decreased in the 8th week of study whilst that of the Plab group decreased in the 8th week of study. This result may reflect mild positive effect of GsCn on delaying the degradation of cartilage structure by inhibiting pro-inflammatory cytokines (IL-1β, TNF-α and MMPs) (Largo et al., 2003; Kim et al., 2007).

The serum HA levels of the GsCn group reached the highest level the 12th week of study whilst that of the Plab group reached the highest level in the 8th week of study. Although the serum HA levels in both groups did not have a significant difference,
slower increased serum HA levels in the GaCn patients reflect that glucosamine and chondroitin sulfates may have mild positive effects of delaying inflammatory process of synovial membrane. From previous studies, glucosamine and chondroitin sulfates were found to have inhibitory effects on pro-inflammatory cytokines such as IL-1β, TNF-α and the matrix-metalloprotease enzymes (MMPs) (Wollhein, 1999; Largo et al., 2003; Kim et al., 2007; Kapoor et al., 2011). 

From the results of this present study, glucosamine and chondroitin sulfates contained nutraceuticals have no significantly positive effects on the clinical outcome compared to the control group (Plab). All dogs in the glucosamine and chondroitin sulfates group (GsCn) had no significant improvement in clinical lameness score. Therefore, glucosamine and chondroitin sulfates may have no analgesic or anti-inflammatory effects on OA joints as in vitro study (Wen et al., 2010) but may delay the OA process (articular injury and synovitis) in a CI rupture dogs. Nevertheless, all dogs in the GaCn group had no side effects throughout the period of study. From these findings, it may be concluded that glucosamine and chondroitin sulfates contained nutraceuticals may be used in long-term management of OA joint in the light of high safety index and to slow down effect on OA process, but not as analgesia or anti-inflammatory drug.

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บทคัดย่อ

ผลของการใช้แกลลิชโลกามิและคอนดรอยตินต่อภาวะข้อเสื่อมในลูกสุนัขหลังเข้ารับการผ่าตัดแก้ไขเอ็นไขว้หน้าข้อเข่า

ปรากฏ หมายห์ร์พิษ กัมปนาท สุนทรวิภาต* นที เตชะอาภรณ์กุล

กลูโคซามีนและคอนดรอยตินซัลเฟตเป็นองค์ประกอบสำคัญของโครงสร้างนอกเซลล์ของกระดูกอ่อนข้อต่อดังนั้นจึงได้รับความสนใจและนำมาใช้ในการรักษาภาวะข้อเสื่อม โดยพบว่าสารทั้ง 2 ชนิดมีความสามารถในการรักษาสมดุลการสร้างและละลายกระดูกอ่อนข้อต่อ การนำมาใช้รักษาภาวะข้อเสื่อม ที่เกิดขึ้นจะช่วยให้เซลล์ที่มีการเจริญเติบโตในการรักษาภาวะข้อเสื่อมในทางการแพทย์และสัตวแพทย์อย่างแพร่หลายอย่างไรก็ตามยังไม่มีข้อมูลที่ก่อให้เกิดการรักษาภาวะข้อเสื่อมด้วยสารทั้ง 2 ชนิดอย่างทางคลินิก ดังนั้นการศึกษาจึงเน้นถึงผลการเปลี่ยนแปลงของอาการทางคลินิก คะแนนความเจ็บขณะเดิน คะแนนการเกิดภาวะข้อเสื่อมทางวิทยาและการเปลี่ยนแปลงของระดับการโดยูโรโปรตีน (HA) และ WF6-chondroitin epitope ในกระแสเลือดโดยใช้สุนัขที่เข้ารับการผ่าตัดแก้ไขภาวะเอ็นไขว้หน้าข้อเข่าขาด (n=12) เป็นตัวอย่างในการศึกษาภาวะข้อเสื่อม หลังจากนั้นทำการเปรียบเทียบระหว่างกลุ่มที่ได้รับกลูโคซามีน ซัลเฟต รวมกับคอนดรอยตินซัลเฟต (GsCn) (n=6) และกลุ่มที่ได้รับยาหลอก (Plab) (n=6) เป็นเวลา 16 สัปดาห์ พบว่าการรักษาด้วยกลูโคซามีน ซัลเฟต รวมกับคอนดรอยตินซัลเฟต ในกลุ่ม GsCn ระดับ WF6 ในกระแสเลือดลดลงเร็วกว่าและระดับ HA ในกระแสเลือดเพิ่มขึ้นช้ากว่ากลุ่ม Plab ซึ่งอาจเป็นผลจากกลูโคซามีนรวมกับคอนดรอยตินซัลเฟตอาจมีผลในการชะลอการทำลายกระดูกอ่อนข้อต่อและการเกิดภาวะข้อต่อยังเสียได้ดีกว่าโดยเฉพาะอย่างยิ่งในเวลา 4 สัปดาห์หลังการผ่าตัด นอกจากนี้ยังไม่พบอาการข้างเคียงและความเปลี่ยนแปลงทางหemaologyในระยะเวลากว่า 16 สัปดาห์ของการศึกษา

คำสำคัญ: ภาวะเอ็นไขว้หน้าข้อเข่าขาด คอนดรอยติน กลูโคซามีน ภาวะข้อเสื่อม

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