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Oral administration of undenatured native chicken type II collagen (UC-II) diminished deterioration of articular cartilage in a rat model of osteoarthritis (OA)

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SUMMARY

Objective: The aim of this study was to determine the ability of undenatured native chicken type II collagen (UC-II) to prevent excessive articular cartilage deterioration in a rat model of osteoarthritis (OA).
Methods: Twenty male rats were subjected to partial medial meniscectomy tear (PMMT) surgery to induce OA. Immediately after the surgery 10 rats received vehicle and another 10 rats oral daily dose of UC-II at 0.66 mg/kg for a period of 8 weeks. In addition 10 naïve rats were used as an intact control and another 10 rats received sham surgery. Study endpoints included a weight-bearing capacity of front and hind legs, serum biomarkers of bone and cartilage metabolism, analyses of subchondral and cancellous bone at the tibial epiphysis and metaphysis, and cartilage pathology at the medial tibial plateau using histological methods.
Results: PMMT surgery produced moderate OA at the medial tibial plateau. Specifically, the deterioration of articular cartilage negatively impacted the weight bearing capacity of the operated limb. Immediate treatment with the UC-II preserved the weight-bearing capacity of the injured leg, preserved integrity of the cancellous bone at tibial metaphysis and limited the excessive osteophyte formation and deterioration of articular cartilage.
Conclusion: Study results demonstrate that a clinically relevant daily dose of UC-II when applied immediately after injury can improve the mechanical function of the injured knee and prevent excessive deterioration of articular cartilage.

Introduction

Osteoarthritis (OA) is a complex multifactorial disease process involving over time all of the tissues within and surrounding the synovial lined joints. Progression of the disease leads to disability associated with joint pain and dysfunction[1,2]. Epidemiologic studies have determined that risk factors for the progression of OA include aging, over- or non-physiological loads, obesity, trauma, hormonal disorders or a combination of several factors. While the exact etiology of OA in not yet known, injury to the articular cartilage over time results in changes in both the chondrocyte and synoviocyte metabolism such that inflammatory cytokines that are produced impair the chondrocytes ability to restore the cartilage matrix[3]. The search for effective therapies that attenuate joint degradation, improve joint flexibility and relieve joint pain has been challenging and current therapies to treat OA include acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs)[4].

Because collagen is the most prevalent component of the solid phase of articular cartilage, collagen supplementation has been considered a key treatment option to prevent damage to the articular cartilage over time and support the healing process following the onset of OA. Several hypotheses have been proposed to elucidate the exact mechanisms by which collagen derivatives improve the health of the articular cartilage[5]. Currently, glucosamine and chondroitin are the two most commonly used nutraceuticals that provide medicinal, therapeutic, and health benefits to arthritic patients[6,7]. For example, treatment with collagen

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derivatives has been proposed to provide an adequate supply of nutrients required for cartilage repair and maintenance[8,9], improve and preserve the quality of the subchondral bone[10,11], and maintain the overall health of articular cartilage and subchondral bone[12,13]. Over the past several years, a novel nutraceutical undenatured type II collagen (UC-II) from chicken sternum cartilage has been studied in knee OA subjects[14–16]. *In vivo* animal studies have reported that UC-II acts via specific regulatory T cells (Tregs) in the gut that migrate and concentrate in areas of inflammation upon stimulation, where they modulate local immune responses in an antigen-specific manner[17,18]. Irrespective of the actual mechanism of action, collagen derivatives seem to improve the health of the articular cartilage and are safe for patients and therefore, should be considered for the prevention or treatment of OA as a sole therapy or in combination with other drugs[15,19]. UC-II is derived from chicken sternum cartilage and is being marketed as a powdered, shelf-stable ingredient that at daily dose of 40 mg demonstrated clinical benefit by improving joint comfort, flexibility and mobility in OA patients.[20,21]

Commonly used method to induce OA in rodents is unilateral medial meniscal tear (MMT) method resulting in rapid progression of degenerative changes in the articular cartilage of the medial tibial plateau including fibrillation of articular cartilage, osteophyte formation and a loss of chondrocytes[22,23]. The medial meniscotibial ligament anchors the medial meniscus to the medial tibial plateau to ensure high congruency between articular structures and the transfer of weight-bearing loads during locomotion. Because cartilage degeneration develops rather rapidly in rats, evaluating drugs aimed to protect articular cartilage using the MMT model is challenging. The partial medial meniscectomy tear (PMMT) method is deemed less invasive than the complete medial meniscectomy model and is thus considered a more suitable model to test the ability of UC-II products to prevent the deterioration of cartilage degeneration and improve the healing of damaged articular cartilage.[24]

The present study tested the ability of undenatured native chicken type II collagen administered orally at the time of cartilage injury imposed by PMMT to prevent the excessive deterioration and improve the healing of articular cartilage.

Method

Test article

UC-II (InterHealth, Benicia, CA) consists of undenatured native chicken type II collagen (collagen 263.0 mg/g, hydroxyproline 32.9 mg/g). UC-II was manufactured from chicken sternum cartilage in a GMP-certified facility using a patented, low-temperature manufacturing process that ensures a particular level of UC-II collagen. UC-II was formulated in 0.5% methyl cellulose suspension and administered orally at 0.66 mg/kg/day for a period of 8 weeks. The rat 0.66 mg/kg/day UC-II dose was chosen because it is equivalent to the 40 mg/day UC-II used in clinical studies for a 60 kg human. The vehicle (0.5% methyl cellulose) was dosed orally at 5 ml/kg/day 7 days per week.

Animals and management

Male, 4 months old Lewis rats (Charles River Laboratories, Portage, MI) weighing 350 g at the beginning of the experiments were used in this study. All *in vivo* procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and were performed in accordance with the US National Institutes of Health (NIH) Publication No. 85–23, revised 1996[25]. The rats were pair housed in a temperature- and humidity-controlled room on a regular 12 h light/dark cycle. Irradiated LabDiet™ 5053 (Purina, Richmond, IN) and water were provided *ad libitum*. The animals were acclimated for 1 week and were allocated to study groups based on their body weight the day before surgery. A group of 10 naïve rats were used as an intact control (Naïve), and another 10 rats received sham surgery (Sham). Additionally, 20 rats received the PMMT surgery and were allocated to receive vehicle treatment (PMMT/veh) or a UC-II (PMMT/UC-II) treatment. The ARRIVE guidelines was used to ensure the rigor of study conduct ad reporting of the data.

Surgery

Surgeries are performed in a dedicated rodent surgical facility at Pfizer consisting of an animal preparation room and recovery room, surgeon preparation room and a surgical suite. To minimize variations, only one surgical research specialist with extensive experience in performing the PMMT surgery was certified by the Academy of Surgical Research and have had his surgical skills and knowledge assessed by a designated subject matter expert (Global Trainer or Global Surgeon) approved to perform surgery. The rats were induced and maintained under anesthesia using isoflurane. One dose each of carprofen (Pfizer Animal Health, Florham Park, NJ) and sustained-release buprenorphine (Zoopharm, Windsor, CO) were administered prior to surgery to ensure analgesia. Rats in the surgery groups were subjected to a partial medial meniscal tear (PMMT) surgery[24]. Briefly, the medial meniscus was freed from its attachments to the margin of the medial tibial plateau prior to grasping the meniscus with forceps and transecting one-third of the medial collateral ligament and medial meniscus. In the sham surgery rats, the medial meniscus was visualized but not transected. The surgical incisions were closed in two layers using absorbable sutures.

Body weight, tissue collection and serum analyses

The body weight was recorded twice weekly throughout the study. At the end of the study rats were euthanized and the entire right hind limb was harvested and carefully cleaned of the soft tissue. The limbs were wrapped in saline-soaked gauze and frozen at −20°C for the *ex vivo* imaging and histological analyses of the tibial articular cartilage and bone. Blood was collected 8 weeks after surgery by jugular venipuncture under isoflurane anesthesia. The serum was stored at −20°C and used to run the standard chemistry panel and biomarkers of bone and cartilage metabolism[26] (see Supplemental Material for details).

Dynamic weight bearing (DWB)

DWB measurements were obtained before surgery, 6 days after surgery, 4 weeks after surgery and before euthanasia to assess the effects of surgery on the weight-bearing capacity of the hind and front legs. The DWB system (Bioseb, software 1.3.; Boulogne, France) is non-invasive method for measuring the weight and surface area of all four feet in a freely moving animal[27,28]. Zone parameters were set for the analysis as follows: ≥4 g for one sensor or a minimum of three adjacent sensors ≥2 g (in order to be considered a valid zone). For each time segment that was stable for more than one second, zones that meet the above criteria were validated and assigned as either right or left and front or rear. A mean value for the weight and area of each zone were calculated over the entire testing period, based on the length of time of each validated segment. For each testing period, the animals were placed into the chamber and allowed 20–30 s to explore prior to data collection. The following parameters were measured over a 3-min

period: body weight (g), percentage of weight (% weight) and surface area (mm^2) placed on the front left and right leg, both front legs combined, rear left and right leg and both rear legs combined.

Radiology

Following necropsy all knee joints were X-rayed with a Faxitron Model MX20 specimen scanner (Faxitron Bioptics LLC, Tucson, AZ) using an exposure time of 12–18 s at 31–35 kV. The radiographic images were used to inspect the bone samples for the presence of possible fractures or other bone abnormalities.

Micro-computed tomography (μCT)

The operated right knee joint was subjected to μCT utilizing a MicroCT 100® computed tomography system (Scanco Medical, Bassersdorf, Switzerland) to obtain a scout 3D image of the knee. The μCT images were used to ensure that the samples were reproducibly scanned and that the same region of interest (ROI) at the proximal tibial epiphysis and metaphysis for each specimen was analyzed[29].

The cancellous bone compartment of the metaphysis was analyzed 1 mm below the growth plate and extended 3 mm distally to include the metaphyseal secondary spongiosa. The cancellous bone was evaluated as previously described. In short, an ROI was drawn on 100 consecutive slices with a thickness of 1.0 mm that best represented the central segment of the tibia[28]. The cancellous bone parameters included bone mineral density, tissue volume (bone and bone marrow), bone volume, bone volume/tissue volume ratio, bone surface, bone surface/bone volume, trabecular number, trabecular thickness, trabecular separation, connectivity diameter, and structural model index.

For subchondral bone analysis a 2.0 mm × 0.5 mm ROI was drawn on the pre-contrast images to include the cortical and cancellous subchondral bone underlying the articular cartilage as described earlier[28] (for details see Supplemental Material).

Histopathology

After the knee joints were imaged with μCT, they were shipped to HistoTox Labs, Inc. (Boulder, CO, USA), for tissue processing. The knee joints were placed in SurgiPath Decalcifier I solution (Grayslake, IL, USA) for 10 days. Following decalcification, the knee joints were transected in the frontal plane to yield two approximately equal portions, embedded in paraffin, and serially sectioned at ~200 μm intervals into 5-μm-thick sections for staining. The slides were stained with hematoxylin and eosin (H&E) for general evaluation; toluidine blue (T-blue) for evaluation of the cartilage, safranin O was used to evaluate structural damage to the cartilage, and Cathepsin K was used to count number of dark-stained osteoclast below the growth plate on two consecutive slices. Only the histology of the medial aspect of the joint was analyzed because this region is the primary site of degeneration for this animal model. Slides were labeled in a coded manner. Two independent readers without knowledge of the treatment categories independently evaluated the histology. Results from the two slides per animal and from both readers were averaged for each section, and the average scores for gradable sections were then averaged for each rat. The following parameters were determined: cartilage degeneration score, osteophyte size, thickness of the cartilage matrix, cartilage matrix loss width, total cartilage degeneration width and significant cartilage degeneration width on four sections (two H&E and two T-blue) using an ocular micrometer as previously suggested[30]. The progression of cartilage matrix loss was measured along the surface (0% depth – where the cartilage on either side has intact superficial cartilage), the tidemark (100% depth – where the cartilage on either side shows complete loss of cartilage) and at the level of the mid-zone (50% depth between surface and tidemark) as recommended earlier[30]. The total cartilage degeneration with represents the total extent of the tibial plateau affected by any type of degeneration such as total loss or just fibrillation of matrix with or without chondrocyte death, thus this area is regularly larger than total cartilage loss parameter.

Statistical analysis

GraphPad Prism v.5.00 for Windows (GraphPad Software, USA, http://www.graphpad.com) was used for the statistical analysis. Data was expressed as mean ± 95% confidence interval where $n = 10$. Shapiro–Wilk test was used to test the normality of the data. One-way ANOVA followed by Dunnett's multiple-comparison post-test was performed for the comparison of group mean differences against the Naïve group of rats. Student's t test was done for unpaired comparison. Statistical significance was considered at $P \leq 0.05$.

Results

Animals and serum assays

After a transient loss of body weight due to sham-surgery and PMMT surgery, the body weight of all rats enrolled in the study increased by approximately 10% during the course of the study [Fig. 1(A)]. Rats in the PMMT/veh and PMMT/UC-II groups developed OA, as evidenced by X-ray, μCT and histology. Surgery or treatment with vehicle and UC-II did not affect the serum chemistry parameters or biomarkers of bone and cartilage metabolism, although the rats from the PMMT/veh group exhibited the highest level of cartilage degradation marker CTX-II ($P < 0.05$ vs Naïve and Sham), whereas the PMMT rats treated with UC-II exhibited significantly ($P < 0.05$) lower CTX-II values compared to PMMT/veh controls (Table 1, Supplemental Material).

DWB

All operated rats shifted weight bearing toward the front legs in order to reduce the weight bearing on the operated limb. The weight-bearing capacity of the operated right hind leg was significantly ($P < 0.05$) lower in PMMT/veh rats than in rats in the Naïve group during the entire study, and significantly ($P < 0.05$) lower relative to Sham and PMMT/UC-II rats at the end of the study [Fig. 1(B)–(D)].

Radiology

The radiological appearance of the right knee did not differ between the Naïve and Sham rats. Osteophytes were evident on the 2D images in all PMMT/veh and all PMMT/UC-II animals. Rats in the PMMT/veh group exhibited less cancellous bone at the proximal tibial metaphysis relative to rats in Naïve, Sham and PMMT/UC-II group (Fig. 2).

μCT evaluation

Bone parameters of the cancellous bone (secondary spongiosa) at the proximal tibial metaphysis were affected by the PMMT surgery. PMMT surgery in the vehicle treated rats resulted in slightly lower bone mineral density (BMD), bone volume and trabecular number, and higher trabecular separation parameter relative to Naïve, Sham and PMMT/UC-II treated rats, although the change was

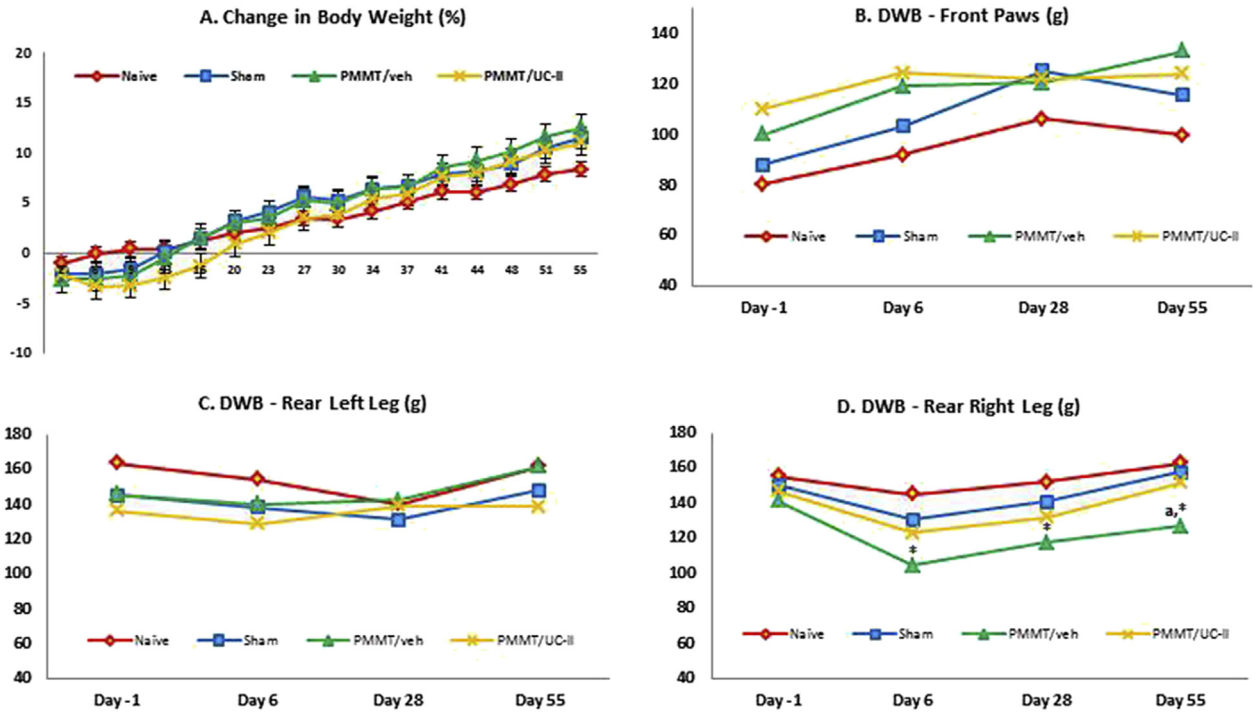

Fig. 1. **A** shows percent change in the body weight throughout the experiment. (**B**)–(**D**) show the change in the weight-bearing capacity and distribution of body weight placed on the front paws (**B**), rear left leg (**C**) and the rear right leg (**D**) at different time points during the 8-week experiment. Rats in the PMMT group showed a decreased weight-bearing capacity of the operated right hind leg relative to control groups and rats treated with the UC-II. $^{*}P < 0.05$ vs Naïve; $^{a}P < 0.05$ vs Sham and UC-II.

not significant (Fig. 2). In addition, Cathepsin K histochemistry showed more robust accumulation of the osteoclasts in the primary spongiosa bellow the growth plate cartilage of PMMT/veh rats relative to all other study groups which also indicate increased bone resorption and supports the μCT data (Fig. 3).

The subchondral bone parameters (bone area, bone volume and BMD) did not significantly differ between groups (Table 2, Supplemental Material). However, not statistically significant increase in the bone volume and BV/TV ratio indicated a mild thickening of the cortical layer in Zones 1 and 2 which is also visible in the 3D images of the tibial epiphysis of PMMT/veh and PMMT/UC-II rats. Additionally, osteophytes were clearly visible in both PMMT/veh and PMMT/UC-II rats, although their sizes varied (Fig. 4).

Cartilage damage and osteophytes were not evident in Naïve and Sham animals. However, significant articular cartilage damage was present in PMMT/veh rats relative to rats in Naïve and Sham group. Cartilage damage was less severe in PMMT/UC-II treated rats relative to Naïve and Sham controls, but also comparing to PMMT/veh group. In addition, the osteophytes in Zone 1 were significantly smaller in size in PMMT/UC-II rats compared to PMMT/veh rats (Fig. 5).

Cartilage histology

The thickness of the articular cartilage was similar in Naïve and Sham rats. PMMT/veh and PMMT/UC-II rats had damaged articular cartilage, with thickening of the cartilage in Zone 1 and a loss of cartilage matrix in Zone 2, but relatively intact cartilage in Zone 3 (Fig. 6). The 0.66 mg/kg dose of UC-II showed a modest effect in reducing damage to the cartilage as evidenced by less cartilage thickening in Zones 1, slightly thicker cartilage layer in Zone 2 and less variability in cartilage thickness in Zone 3. Also, rats in PMMT/UC-II group exhibited fewer fibrillations and less cartilage debris in the articular space relative to PMMT/veh rats (Fig. 6). In general, animals treated with the UC-II showed less variability in cartilage damage and better consolidated cartilage in Zones 1 and 3 relative to vehicle treated PMMT rats.

Loss of articular cartilage width was not evident in Naïve and Sham rats. However, width of articular cartilage loss was significantly lower in PMMT/veh rats relative to controls. Dosing with UC-II reduced cartilage damage in PMMT rats; however the efficacy of UC-II varied between animals (Fig. 7).

A loss of articular cartilage was not evident in the Naïve and Sham rats. As expected, articular cartilage loss in PMMT/veh rats was statistically significant relative to Naïve and Sham rats, and dosing of PMMT rats with UC-II attenuated this loss compared with the loss observed in the PMMT/veh rats not given the UC-II (Fig. 8).

Overall, the histological evaluation demonstrated that rats developed had PMMT-associated deterioration of their knee cartilage. Daily administration of UC-II reduced this PMMT-associated damage.

Discussion

This study was undertaken to assess the capacity of a UC-II to prevent the excessive deterioration of articular cartilage or to accelerate the recovery process following partial meniscectomy. The PMMT surgery results in the fractional displacement of the medial meniscus leading to shifts of the weight-bearing loads and to cartilage damage[22,23].

In our study, surgery resulted in transient decreases in body weight due to stress and postoperative pain. The overall increase in body weight was equal among all study animals, totaling 10% at the end of the 8-week study. Whereas the total weight bearing imposed on the rear legs in all rats slightly increased over time as the rats gained weight, the weight-bearing load placed on the right hind leg was lower in the PMMT rats relative to Naïve and Sham

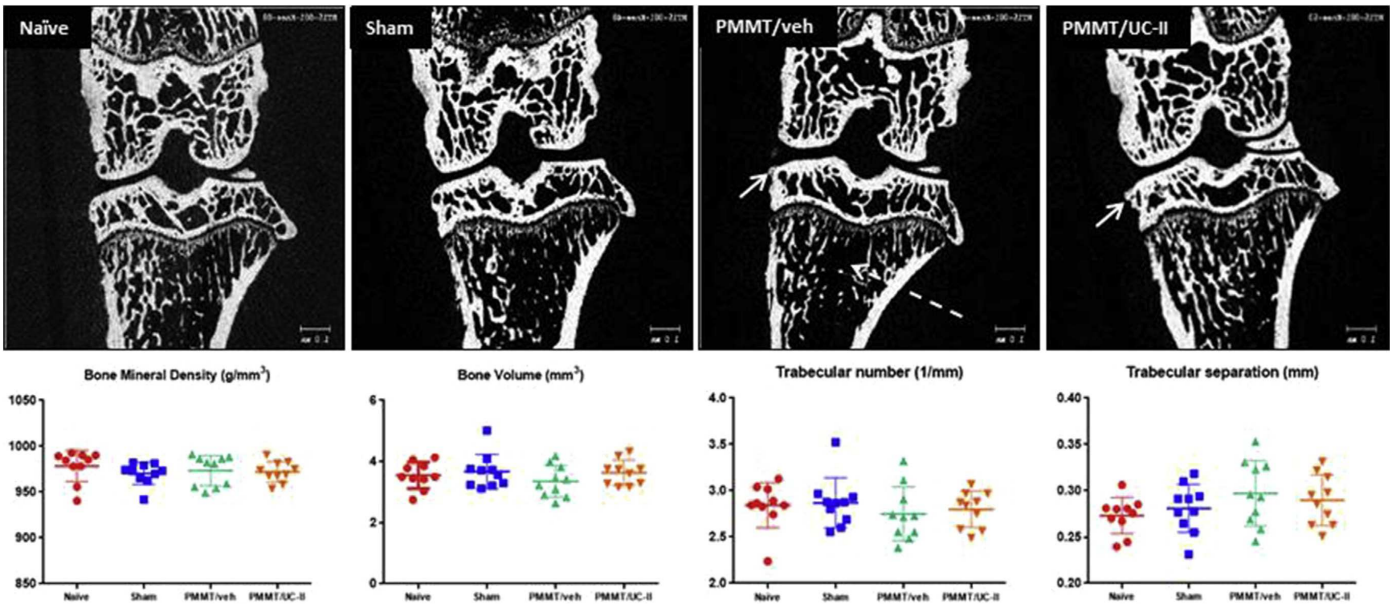

Fig. 2. Top row shows the 2D mCT images of the right knee. Solid arrows indicate osteophyte formation in the PMMT and UC-II rats. Dotted arrow indicates less cancellous bone at proximal tibial metaphysis in PMMT controls. Bottom row shows the structural analysis of the cancellous bone at proximal tibial metaphysis. Although the differences between groups were not significant, the PMMT rats exhibited a slightly lower trabecular bone volume, decreased trabecular number and increased trabecular separation relative to Naïve and Sham rats. Treatment with UC-II helped maintenance of cancellous bone.

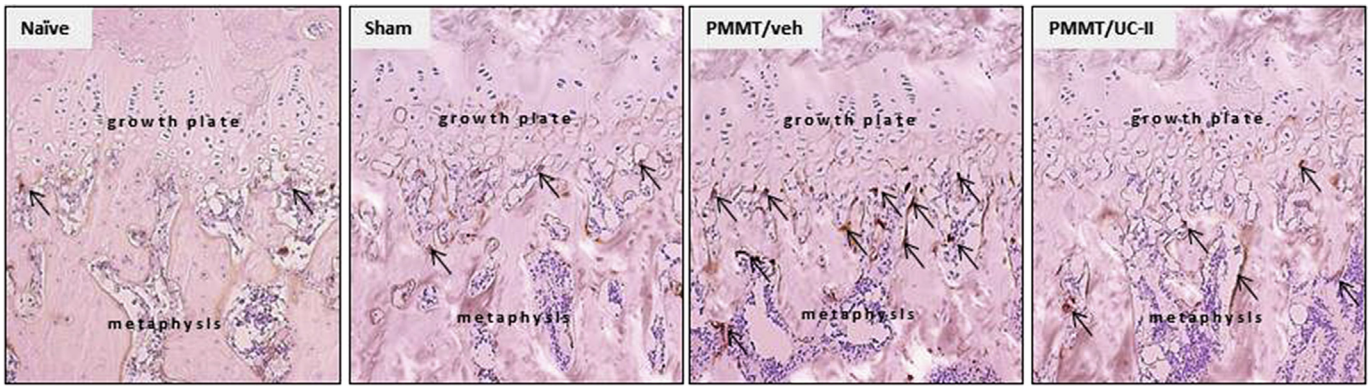

Fig. 3. Shows the Cathepsin K staining of the cancellous bone at the growth plate cartilage. The PMMT rats showed more intense bone resorption relative to rats in the Naïve, Sham, and UC-II groups, as evidenced by larger number and size of darkly stained osteoclasts.

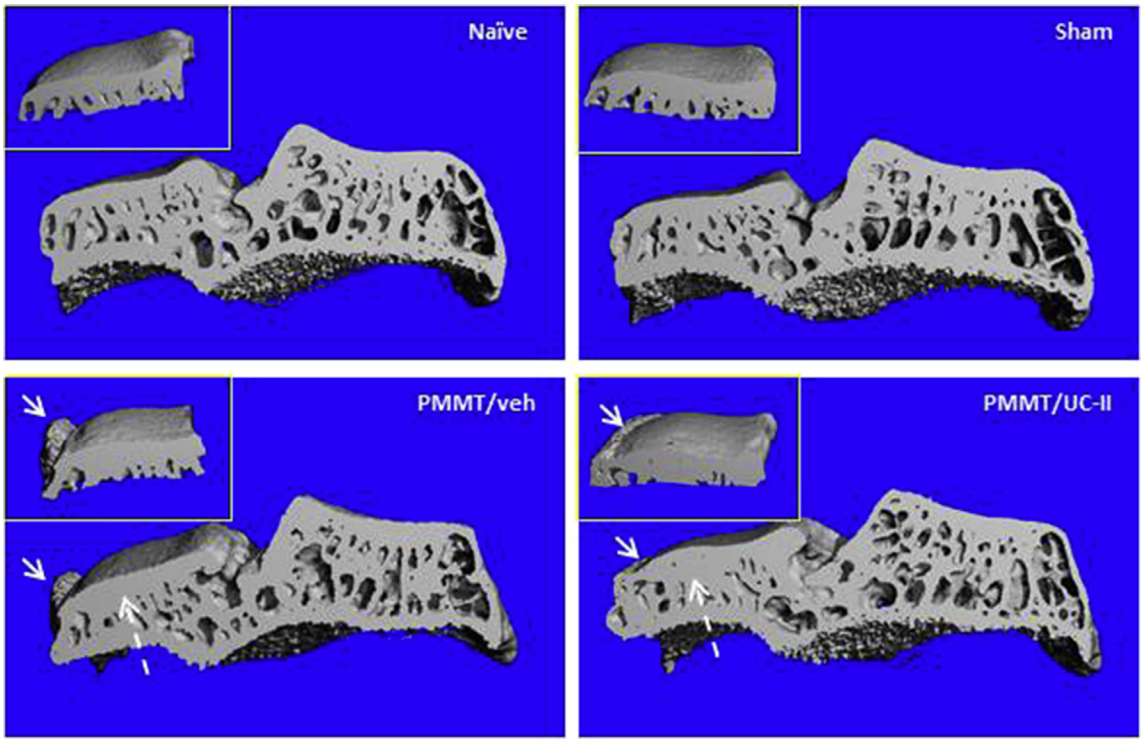

Fig. 4. Shows the 3D μCT images of the tibial epiphysis. Arrows indicate osteophyte formation in rats that received the PMMT surgery. The dotted arrow indicates the thicker subchondral bone in Zones 1 and 2 in PMMT and UC-II rats. The top view of the medial tibial plateau, depicted in the top left corner, shows the osteophyte formation.

controls. Other studies have previously shown that osteoarthritic rats reduce weight bearing on the injured limb and shift their weight distribution to the contralateral limb[31–33]. Patients with knee OA also exhibit gait asymmetries of the affected limb, such as reductions in the stance time and peak vertical force[34–36]. Our data confirmed earlier findings that in contrast to bipeds, in which only option is to shift the weight to the contralateral limb, rats (quadrupeds) tend to alleviate mechanical imbalances associated with pain by shifting at least part of the weight burden onto their front legs, rather than simply overloading their contralateral limbs[28]. Results from this study show that prompt treatment with UC-II at the time of surgery largely prevented the functional incapacity of injured limb to bear weight, allowing for subsequent close-to-normal biomechanics.

Physiologic mechanical loading plays a critical role in bone and cartilage physiology[37,38]. Mechanical loading is well known to drive changes in skeletal remodeling to adjust the bone mass and architecture to meet mechanical demands[39,40]. In rats, cancellous bone at the tibial metaphysis rapidly responds to changes in mechanical loading[41]. Despite sedentary lifestyle of caged laboratory rats, the partial underloading of the operated leg was expected to activate bone resorption and cause a mild loss of cancellous bone in the tibia of PMMT rats. Concurrent to the bone loss, the underloading of weight-bearing bones initiates degenerative changes in the articular cartilage[42,43]. Because the loss of bone in the operated limb was diminished by UC-II treatment, we hypothesized that the maintenance of knee functionality and load-bearing activity played a key role in preserving bone mass and structure in the tibia. Specifically, the maintenance of modest physical activity may indirectly help to limit damage of articular cartilage[44,45]. Although the mechanisms that regulate the maintenance of bone and cartilage are different, compelling evidence indicates that mechanical

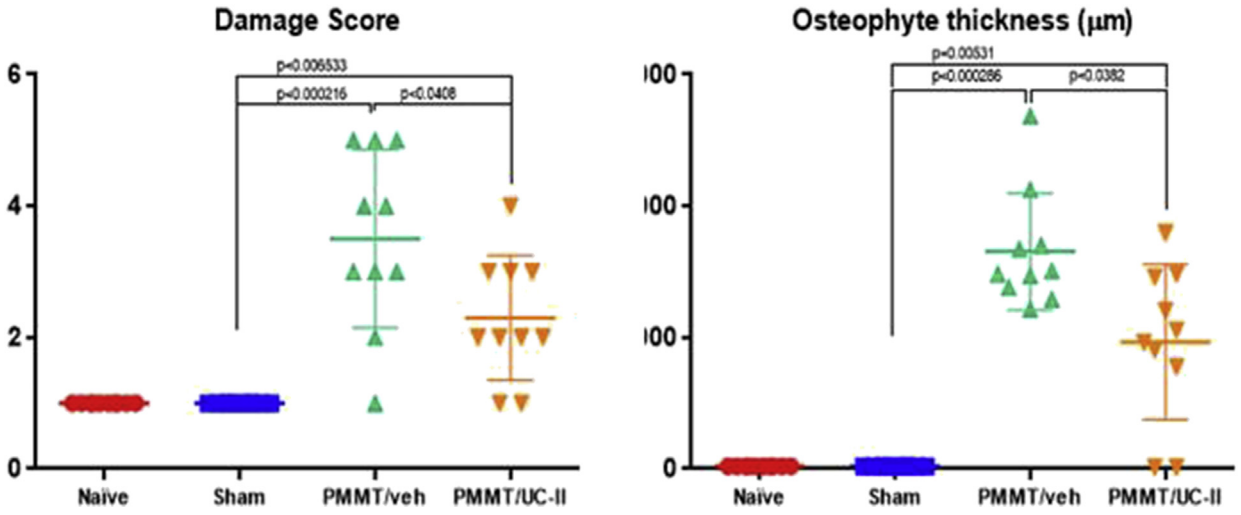

Fig. 5. Shows the damage score of the articular cartilage and size of the osteophytes. Cartilage damage and osteophyte formation were not present in Naïve and Sham rats. Dosing with 0.66 mg/kg of UC-II prevented excessive cartilage deterioration and growth of large osteophytes.

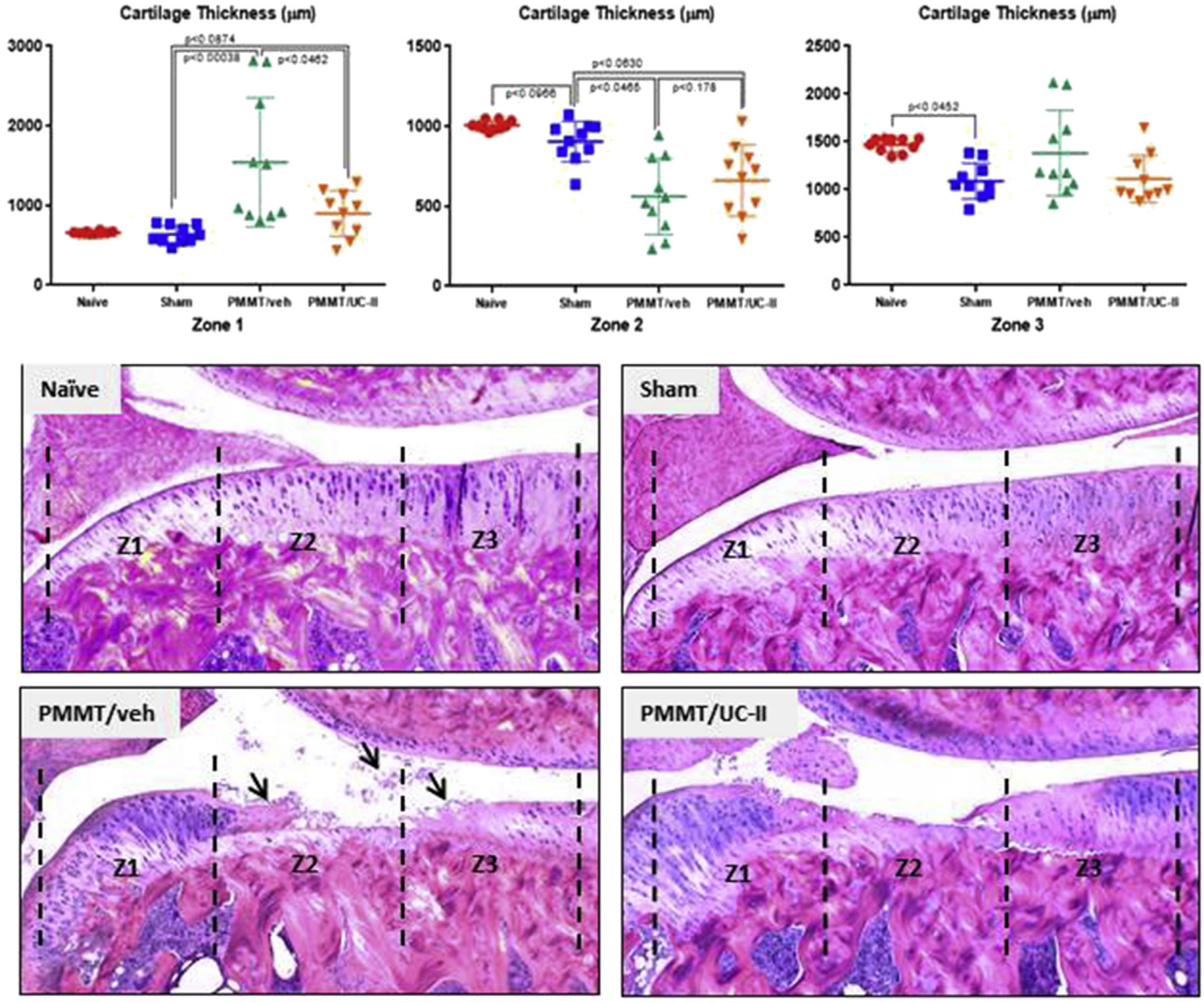

Fig. 6. Shows a zonal analysis of the cartilage thickness evaluated at the medial tibial plateau. The thickening of the articular cartilage in Zone 1 and deterioration of articular cartilage in the Zones 1 and 2 is visible in operated rats. Dosing with UC-II was moderately effective in preventing deterioration of articular cartilage caused by the surgery. In addition, more cartilage debris and fibrillations (indicated by arrows) are evident in PMMT rats compared to UC-II dosed rats.

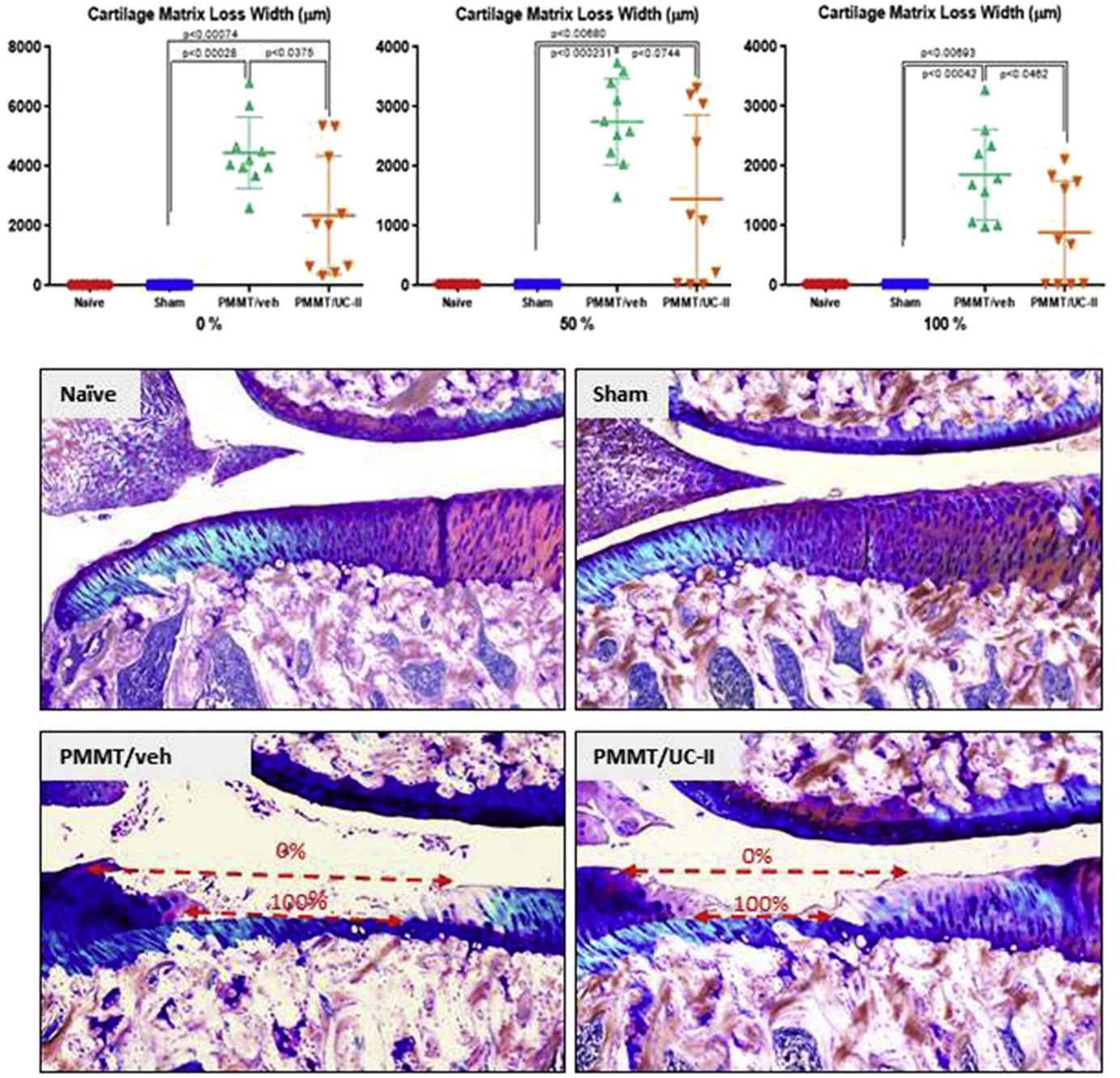

Fig. 7. Shows the loss of cartilage matrix width at the medial tibial plateau. The width of lesions was measured at 0%, 50% and 100% depths. Dosing with UC-II was moderately effective in preventing the deterioration of articular cartilage caused by PMMT. The width of the lesion at 0% and 100% depths are indicated by dotted arrows.

stimuli influence the crosstalk of signaling pathways, which plays a critical role in both cartilage and bone metbolism[46].

Changes in local mechanical loads triggered by the deterioration of articular cartilage resulted in the accumulation of cortical bone beneath the damaged cartilage and the formation of osteophytes. The formation of osteophytes is believed to be an adaptation of the skeleton aimed to stabilize inured joints, accommodate new mechanical needs and prevent the further deterioration of cartilage[47]. However, osteophytes can limit joint movements and cause pain, and their size is thought to be proportional to the severity of cartilage injury[48,49]. Combination of radiology and histology techniques revealed that treatment with UC-II limits osteophyte size that can potentially help joint mobility and functionality.

The damage score and zonal quantification of total cartilage thickness (i.e., calcified plus noncalcified) identified a significant loss of articular cartilage in Zones 1 and 2 in PMMT rats. The width of cartilage matrix loss (%) demonstrated the extent of cartilage damage, which was further emphasized by measurements of the significant cartilage degeneration width parameter. Spontaneous healing of the articular cartilage was not evident in the PMMT rats, nevertheless numerous fibrillations and cell debris were frequently found on the histology images. In contrast, dosing of PMMT rats with UC-II limited the extent of cartilage damage and produced signs of recovery. Specifically, cartilage thickening in Zone 1 was reduced while the calcified and noncalcified cartilage layers in Zones 2 and 3 were not different in rats given UC-II compared to

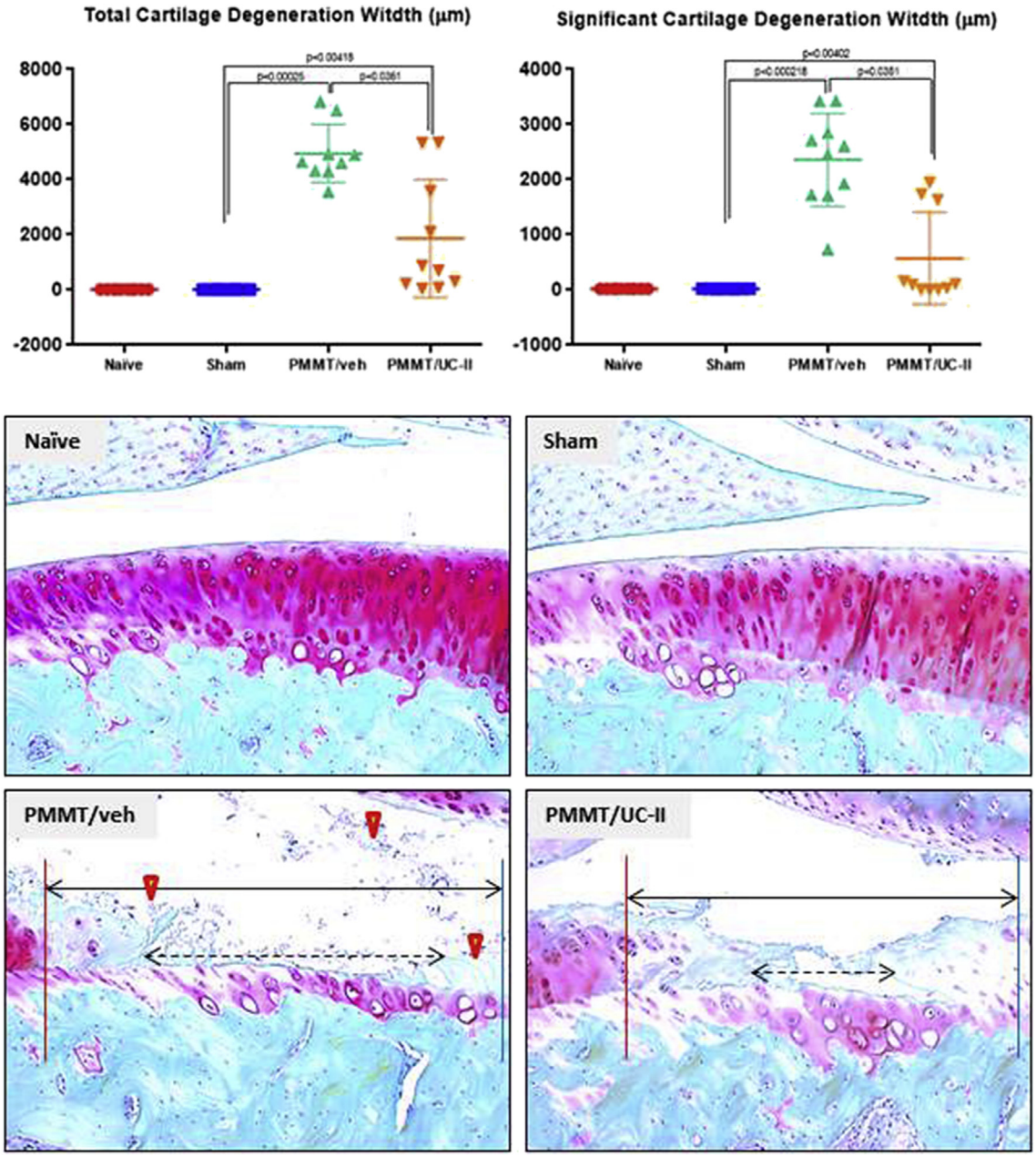

Fig. 8. Shows the total and significant cartilage degeneration width parameters assessed on the Safranin O-stained sections. The PMMT-associated loss of the articular cartilage was partially prevented by UC-II. Red lines indicate the outer border (osteophyte side), and blue lines indicate the inner border (normal cartilage). Solid arrows indicate the total cartilage degeneration width; dotted arrows indicate significant cartilage degeneration width; yellow arrowheads indicate fibrillated cartilage and debris, which were primarily evident in the PMMT rats given no UC-II.

PMMT controls. Likewise, the cartilage matrix loss width and cartilage degeneration width parameters were smaller in PMMT rats given UC-II compared to PMMT controls. Thus, the overall damage score index was favorable in PMMT rats given UC-II compound.

When applied at the time of injury, UC-II was moderately effective in preventing excessive degradation of the articular cartilage. A number of independent biomarkers (DWB, CTX-II, µCT and histology) showed that daily treatment with UC-II preserved joint functionality and curtailed excessive cartilage degradation. We hypothesize that several mechanisms most likely contribute to the efficacy of UC-II, including anti-inflammatory effects, the reduction of pain, the preservation of mechanical function and bone quality, and a supply of building material for cartilage repair. Our results support recent clinical data showing improved flexibility and pain reduction in arthritic patients receiving a 40 mg daily dose of UC-II[21]. Disease-modifying therapies for OA are not currently available, and approximately 75% of OA patients regularly receive more than one symptomatic treatment[50]. Other treatment modalities has been shown to reduce cartilage degradation, have no effect, or even to have a negative effect on articular cartilage in similar animal models of OA. Therefore, the complex nature of OA will most likely require simultaneous treatment with several lines of therapy to successfully treat the disease[4]. The modeling of treatments will depend on the severity and duration of OA but should include ingredients such as UC-II

that have been demonstrated to be safe and capable of improving joint flexibility, joint pain and the overall health of bone and cartilage.

In general, studies aimed to test drug efficacy and treat OA face common challenges including choice of the disease model, proper study design to accommodate extensive *in vivo* procedures such as mechanical loading, and availability of cartilage and bone tissues that need different processing to allow imaging, histological and molecular analyses. This study was also limited by availability of relevant tissues needed to adequately address important questions regarding the true mechanism of action of UC-II in the articular cartilage, so methods such as immuno-histochemical staining and gene expression of proteins related to cartilage metabolism including collagen type II and X, MMP-13, SOX9, CCN2 were not performed. However, results from this study helped design of the follow-up studies that will include use of exercise and gait analysis to better address joint functionality and impact of disuse and load bearing on cartilage metabolism, use of radiolabeled compound to assess metabolism and tissue distribution of UC-II and use of adequate immunological, histochemical and molecular methods to address some of the lingering questions regarding mechanism of action of "slow-acting" product such as UC-II.

Authors' contributions

CMB designed the study, interpreted the data and wrote the manuscript; EB did DWB, μCT and histology analyses consolidated all data and participated in writing the manuscript; ST contributed to study design, reviewed and helped interpretation of data, and participated in writing the manuscript, and NEL reviewed and helped interpretation of data, and participated in writing the manuscript.

Competing interest

The authors that are Pfizer employees have Pfizer stocks. Nancy Lane has no competing interest.

Role of the funding source

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2017.08.013.

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Efficacy and tolerability of an undenatured type II collagen supplement in modulating knee osteoarthritis symptoms: a multicenter randomized, double-blind, placebo-controlled study

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Abstract

Background: Undenatured type II collagen (UC-II) is a nutritional supplement derived from chicken sternum cartilage. The purpose of this study was to evaluate the efficacy and tolerability of UC-II for knee osteoarthritis (OA) pain and associated symptoms compared to placebo and to glucosamine hydrochloride plus chondroitin sulfate (GC).

Methods: One hundred ninety one volunteers were randomized into three groups receiving a daily dose of UC-II (40 mg), GC (1500 mg G & 1200 mg C), or placebo for a 180-day period. The primary endpoint was the change in total Western Ontario McMaster Universities Osteoarthritis Index (WOMAC) from baseline through day 180 for the UC-II group versus placebo and GC. Secondary endpoints included the Lequesne Functional Index (LFI), the Visual Analog Scale (VAS) for pain and the WOMAC subscales. Modified intent-to-treat analysis were performed for all endpoints using analysis of covariance and mixed model repeated measures, while incremental area under the curve was calculated by the intent-to-treat method.

Results: At day 180, the UC-II group demonstrated a significant reduction in overall WOMAC score compared to placebo ($p = 0.002$) and GC ($p = 0.04$). Supplementation with UC-II also resulted in significant changes for all three WOMAC subscales: pain ($p = 0.0003$ vs. placebo; $p = 0.016$ vs. GC); stiffness ($p = 0.004$ vs. placebo; $p = 0.044$ vs. GC); physical function ($p = 0.007$ vs. placebo). Safety outcomes did not differ among the groups.

Conclusion: UC-II improved knee joint symptoms in knee OA subjects and was well-tolerated. Additional studies that elucidate the mechanism for this supplement's actions are warranted.

Trial registration: CTRI/2013/05/003663; CTRI/2013/02/003348.

Keywords: Knee function, Osteoarthritis, T regulatory cell, Undenatured type II collagen

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Introduction

Osteoarthritis, which entails the destruction of joint cartilage and remodeling of the adjacent bone, is the most common form of arthritis affecting more than 25 million Americans [1]. Current therapies for OA include various over the counter analgesics, a number of nonsteroidal anti-inflammatory drugs (NSAIDs), intra-articular injections of corticosteroids or hyaluronic acid, plus tramadol and other opioid analgesics to relieve severe pain [2, 3]. While these therapies can alleviate symptoms in the near term, their ultimate impact on the pathophysiologic progression of OA is limited [4].

Previous studies reported UC-II to be efficacious for the treatment of arthritis [5, 6]. More recently, a statistically significant improvement in knee joint function over placebo was also reported in a clinical study comprising a group of healthy individuals, supplemented with UC-II, and who developed transient knee joint pain upon strenuous exercise [7]. These same individuals also took longer to experience pain after 120 days of supplementation. Based on these observations, the current study was designed to evaluate the efficacy of UC-II in knee OA subjects compared to placebo and to GC, which is a widely available supplement that is used for reducing joint pain.

Materials and methods

Investigational products

The study product UC-II® (Lot 1204004) was derived from chicken sternum. It was manufactured under current good manufacturing practice (cGMP) conditions using a patented process that preserved its native structure (Chick Cart Inc., Fort Smith, AR). Both glucosamine hydrochloride (GH) and chondroitin sulfate (CS) were purchased through Wilke Resources (Lenexa, KS). The Wellable group (Shishi City, Fujian) manufactured GH under cGMP and according to United States Pharmacopeia 26 specifications. Sioux Pharm (Sioux Center, IA) manufactured bovine-derived CS under cGMP. UC-II and GC were encapsulated in opaque, size "00" capsules with sufficient amounts of excipients (microcrystalline cellulose and silicon dioxide) such that they were sensory identical to placebo. InterHealth Nutraceuticals provided all study materials. All American Pharmaceutical (Billings, MT) verified the amount of active ingredients in the study capsules. Study materials were kept in a secure cabinet with access restricted to the site coordinator, the dispensing pharmacist, and the principal investigator.

Study design

The objective of this randomized, double-blind, placebo-controlled clinical study was to evaluate the ability of UC-II to improve knee symptoms in OA subjects, as measured by overall WOMAC score, compared to placebo and to GC. The trial was conducted at 13 centers in southern India. Because of a limitation in synovial fluid sampling procedures at multiple clinical sites, the study was conducted under two separate study protocols. Study protocols were approved by each center's Institutional Ethics Committee (IEC), and listed on the clinical trial registry of India as study protocols 003663 and 003348. Enrollment, randomization, and follow-up visits were identical for both protocols, and were carried out at days 1 (baseline), 7, 30, 60, 90, 120, 150 and 180 (Table 1). All investigators attended the same investigator meetings, used identical intake and data reporting forms, and were trained and monitored by the same group of clinical research associates.

Efficacy measurements were assessed at all visits and included WOMAC, VAS, and LFI indices. The knee flexion range of motion (ROM) test was performed at each visit. Subject diaries and study product were provided at all visits, except day 180 and were collected at all follow-up visits. Subjects were instructed to record daily the consumption of study product, use of rescue medication, as well as concomitant medications in the subject dairy for the entire duration of the study. Blood and urine were collected at screening and day 180. Pregnancy testing was done at screening and follow-up visits. Adverse events (AEs) were recorded using each subject's diary inputs plus site visit questionnaires administered by intake personnel at all study visits.

Clinical endpoints

The primary endpoint was defined as the change in total WOMAC score from baseline through day 180 for the UC-II group versus placebo and GC. Secondary clinical endpoints for both protocols were similar and included the change from baseline through day 180 versus placebo and GC for all endpoints including the following scores: (1) mean VAS; (2) mean WOMAC subscales; (3) LFI; and (4) knee flexion. Another endpoint included the change from baseline to day 180 for the serum biomarker cartilage oligomeric matrix protein (COMP). In protocol 003348, additional secondary endpoints included the change in serum biomarker, C-reactive protein (CRP) plus synovial fluid biomarkers interleukin (IL)-6, and matrix metalloproteinase (MMP)-3 from baseline to day 180.

Study subjects

A total of 234 subjects were screened and 191 randomized (Fig. 1). Study inclusion criteria were 40–75 years-old male and female subjects, a body-mass index (BMI) of 18–30 kg/m^2, moderate-to-severe OA by physical examination (crepitus, bony enlargements, joint swelling, etc.) in one or both knees, knee pain for at least

Table 1 Protocol Schedule and Activities

Procedures common to both protocols	Screening (Visit 1)	Study period		
		Day 1 (Baseline Visit 2)	Days 7, 30, 60, 90, 120, 150 (Visits 3, 4, 5, 6, 7, 8)	Day 180 (Visit 9)
Signed Informed Consent	X			
Inclusion/Exclusion Reviewed	X	X	X	
Medical/Surgical/Medication History	X			
Physical Examination	X			
Vital Signs	X	X	X	X
Height[a], Weight, BMI	X			X
Clinical Assessment for Knee Pain & Swelling	X	X	X	X
Knee Flexion Range of Motion		X	X	X
X-ray examination	X			
WOMAC Score	X	X	X	X
VAS Scale	X	X	X	X
LFI Score	X	X	X	X
Clinical Laboratory Tests (hematology, chemistry, urinalysis)	X			X
Urine Pregnancy Test (if applicable)	X		X	X
Serum biomarker analysis-COMP		X		X
Randomization Number Assigned		X		
Investigational Product Administration		X		
Dispense Subject Diary		X	X	
Collect/Review Subject Diary			X	X
Provide Directions for Concomitant Medication and Rescue Medication Use	X	X	X	
Dispense New Investigational Product		X	X	
Review Product Accountability			X	X
Assess use of Concomitant Medications		X	X	X
Adverse Events Assessed		X	X	X
Procedures Confined to Protocol 003348				
Synovial fluid biomarker—MMP-3 and IL-6		X		X
Serum biomarker analysis—CRP		X		X

[a]Height was measured only at Visit 1

3 months prior to the start of the study, an LFI score between 6 and 10 and a VAS score of 40–70 mm 7 days after withdrawal from excluded medications, plus a knee radiograph that was graded as Kellgren and Lawrence (K-L) radiograph score of either 2 or 3 [8]. All OA diagnoses were confirmed by each study site investigator and noted in the subject's case report form (CRF). In the case of bilateral knee involvement, the index knee used for the study was the one that presented with the most severe OA symptoms at baseline. Detailed inclusion–exclusion criteria are summarized in Table 2.

Ethics, consent and permissions

Subjects were recruited after they reviewed, understood the study details, and then signed the IEC-approved consent form. The study conformed to the Declaration of Helsinki (version 1996).

Randomization & blinding

Block randomization, consisting of nine individuals per block, was executed in a 1:1:1 ratio using random numbers generated by an independent statistician (SPSS version 16.0). Knowledge of the randomization code was limited to the statistician plus one QA monitor unrelated with the study. Each investigator was given opaque, sealed envelopes denoting single patient identity numbers, randomization codes, and supplementation regimen to be opened in case of an emergency. The code was broken after the clinical database was locked.

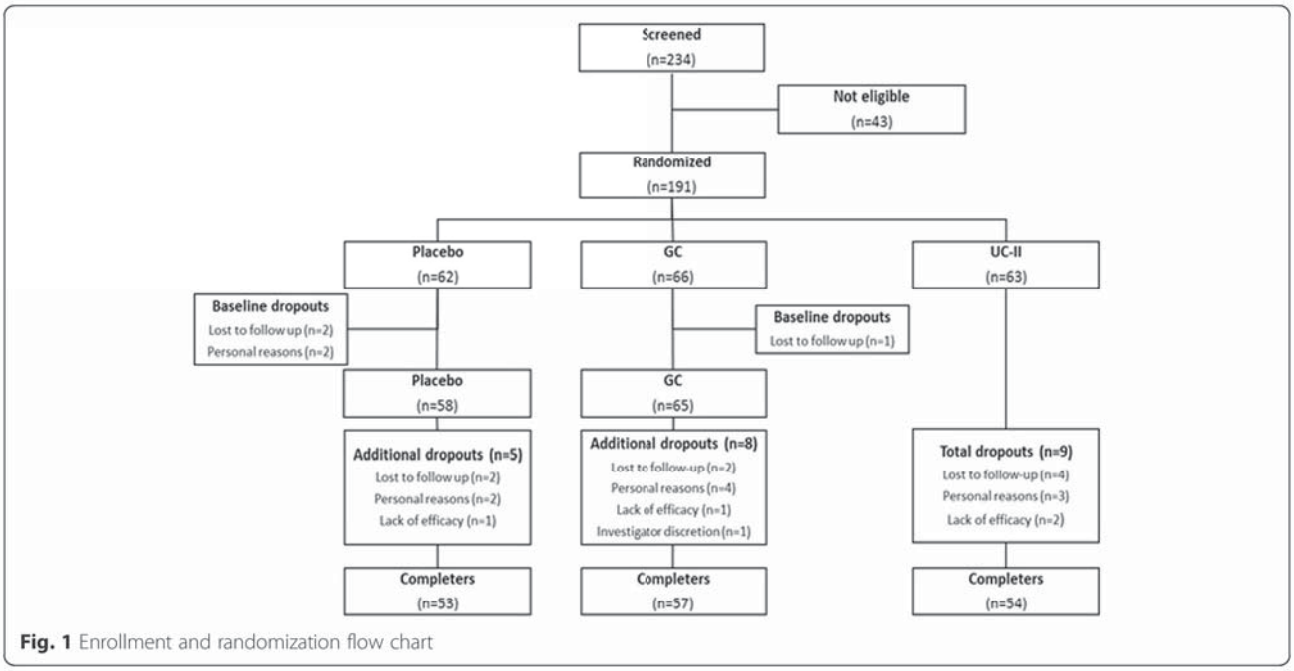

Fig. 1 Enrollment and randomization flow chart

Dosing regimen

Subjects ingested two blue pills in the morning with breakfast and two white capsules before bedtime. For the UC-II cohort, the two morning capsules were placebo, while the evening capsules contained 20 mg each of UC-II totaling 40 mg, which is identical to previously used clinical dose levels [5, 7]. This dose delivered 1.2 mg of undenatured type II collagen as determined by a newly developed and validated extraction-ELISA protocol (AIBiotech, Richmond, VA & Chondrex, Redmond, WA). For the GC group, the morning and evening doses delivered 750 mg of GH plus 600 mg of CS each totaling a daily dose of 1,500 mg of GH plus 1,200 mg of CS. The placebo group ingested identical numbers of blue and white capsules containing excipients only. Study bottles were labeled according to ICH-GCP and applicable local regulatory guidelines.

Prior and concomitant therapies

Prior medications were documented at the screening visit by the study investigator. At each visit, study personnel reviewed subject diaries and questioned each participant on the use of any concomitant medications including those on the prohibited list. Prohibited medications included ibuprofen, aspirin, other NSAIDS, or any other pain relievers (OTC or prescription), plus any dietary supplements (excluding vitamins) that could support joint health. All concomitant medications used during the study was documented in the subject's medical record by the study investigator then transcribed into their CRF by study personnel.

Rescue medications

Acetaminophen was allowed at a dose of 500 mg twice daily. Participants were instructed to not take this medication within 48 h of an evaluation visit. Usage levels and timing was entered at each visit into the subject's medical record by the study investigator. Study personnel transcribed this information into the subject's CRF.

Compliance and safety

Subjects were instructed to bring their bottles to each visit. Remaining capsules were counted and recorded in the subject's CRF and accountability log. As a secondary measure of compliance, subjects completed a diary indicating daily dosing of the study products. Safety assessments were performed at all visits by the site investigator and staff (see Table 9).

Study evaluations

WOMAC scores were determined using the WOMAC VA3.1 questionnaire containing 24 items grouped into three categories: pain, stiffness, and physical function (score range 0–2400). Each respective WOMAC subscale mean scores was determined by dividing the subscale score by the number of questions (5, pain; 2, stiffness; 17, physical function) it contained. The mean VAS score was determined using a VAS questionnaire containing 7 pain-related questions (score range 0–700), and then dividing the overall score by seven. LFI score was determined using an LFI questionnaire that assessed pain, walking distance, and activities of daily living,

Table 2 Inclusion-exclusion criteria

Inclusion

- Ambulatory, 40–75 years of age, with a BMI of 18 to 30 kg/m^2
- Females of childbearing age must agree to use a medically approved form of birth control and have a negative urine pregnancy test result throughout the study
- Female subjects of limited to no childbearing potential must be amenorrheic for at least 1 year or have had a hysterectomy, a bilateral oophorectomy, or both
- Unilateral or bilateral OA of the knee for greater than 3 months plus a Kellgren and Lawrence radiographic grade of 2 or 3
- VAS score during knee movement between 40–70 mm after 7 day withdrawal of excluded medications
- LFI score between 6–10 points after 7 day withdrawal of excluded medications
- Clinical laboratory results that are within normal range or considered not clinically significant by the Principal Investigator
- Be willing to participate in all scheduled visits, tests, and other trial procedures according to the clinical protocol
- Be willing to refrain from taking ibuprofen, aspirin or other NSAIDS, or any other pain reliever (OTC or prescription) during the entire trial other than acetaminophen (paracetamol) as rescue medication
- Provide a signed and dated informed consent indicating that the subject has been informed of all pertinent aspects and possible risks associated with participation in the trial

Exclusion

- History of hypersensitivity to the rescue medication or any of the products used in the study
- History of hypersensitivity to eggs, chicken or fowl, or shellfish
- History of inflammatory arthropathy, severe RA, OA (VAS score greater than 70), or Systemic Lupus Erythematosus
- Hyperuricemia (>440 μmol/L), past history of gout, or both
- Anticipation of surgery within the next 4 months
- Recent injury in the target knee (past 4 months)
- History of use for corticosteroid, indomethacin, glucosamine & chondroitin within 3 months of Visit 2; intra-articular treatments, including injections of corticosteroid or hyaluronic acid; consumption of Omega 3 fatty acids dietary supplements within 6 months preceding the treatment period (a 2-week washout period is allowed for subjects taking omega 3 fatty acid supplements)
- History of congestive heart failure
- Anticipated problems with product consumption
- Evidence or history of clinically significant hematological, renal, endocrine, pulmonary, gastrointestinal, cardiovascular, hepatic, neurologic diseases, or malignancies within the last 5 years
- High alcohol intake (>2 standard drinks per day) or use of recreational drugs (e.g., cocaine, methamphetamine, marijuana, etc.)
- Females who are pregnant or lactating or planning to become pregnant
- History of any mental illness that might impair the ability of subjects to provide a written informed consent
- Consumed acetaminophen (paracetamol), ibuprofen, aspirin or other NSAIDS, or any other pain reliever (OTC or prescription), or any natural health product, (excluding vitamins) within 7 days of first visit
- Participation in any clinical trials within 30 days prior to first visit

(score range 0–24). Knee flexion was measured using goniometry with the subject lying in the prone position and the leg to be tested positioned along the edge of the table [9].

Synovial fluid biomarkers

Synovial fluid (~0.5 mL) was aspirated from the knee joint using an appropriate sized needle (18–24 gauge, depending on joint size). Harvested fluid was stored frozen until tested. IL-6 and MMP-3 levels were determined using the corresponding Duoset ELISA kits (R&D Systems, Minneapolis, MN).

Serum biomarkers

COMP levels (Quantikine ELISA, R&D Systems) were determined in both study protocols. CRP levels (Latex COBAS INTEGRA, Roche Diagnostics GmbH, Mannheim) were assessed in protocol 003348. Serum was stored frozen until analyzed. Interassay and intrassay coefficients of variation for COMP and CRP were <5 %.

Statistics

We verified, using 2-way analysis of variance (ANOVA), that the results of the two protocols could be combined into a single analysis by demonstrating there was no group by study interaction and that the magnitude of the efficacy observed under the two protocols was similar.

A modified intent-to-treat (mITT) analysis was used to assess the efficacy and safety outcomes that was defined *a priori*. This included all subjects who were randomized, consumed study product, and had at least one completed post-baseline visit. An analysis of covariance (ANCOVA), that included supplementation as a fixed factor and the corresponding baseline value of the variable being tested as a covariate, was used for assessing the overall statistical significance of the primary and secondary endpoints. Following ANCOVA, the Tukey-Kramer multiple comparison test was used for determining pairwise statistical significance and calculating 95 % confidence intervals. Also, a mixed model repeated measures (MMRM) analysis of the primary endpoint was performed to account for the multiple assessments obtained during this study. In addition, the method of trapezoids was used to calculate incremental area under the curve (iAUC) for all study groups. For iAUC estimation, missing values were imputed using the expectation-maximization algorithm in SAS. Rescue medication usage between groups was compared using logistic regression followed by pairwise comparisons using the Tukey-Kramer test. In addition, a stratified analysis of the primary endpoint was performed according to baseline serum COMP levels above and below the median value for this biomarker.

Differences were considered significant if the resultant p-value was ≤0.05. An independent statistician performed the analyses and other calculations using SAS version 9.3 (Cary, NC).

Results

Demographics and baseline characteristics

Two hundred and thirty-four subjects were screened and 191 subjects who met the eligibility criteria were randomized to placebo ($n = 62$), GC ($n = 66$), or UC-II ($n = 63$) (Fig. 1). Per mITT criteria, 5 subjects were excluded from all analyses because they failed to present at any post-randomization visits resulting in an absence of clinical data. Table 3 summarizes the demographics of the remaining 186 subjects that were eligible for efficacy and safety analyses. Baseline subject characteristics, OA severity, serum CRP, COMP, IL-6 and other characteristics were similar among the three groups.

Subject dropouts

One hundred and sixty four subjects completed the study: 53, placebo; 57, GC; and 54, UC-II. The 27 dropouts, which included the five subjects mentioned previously, were allocated across the three cohorts as follows: 9, placebo; 9, GC; and 9, UC-II. The final dropout rate was 14 %. Subjects' dropout reasons are summarized in Fig. 1. No subject withdrew from the trial due to an adverse event attributable to any study product.

Study product compliance

Compliance with daily dosing of study capsules exceeded 90 % for all cohorts (data not shown).

Total WOMAC score

The UC-II supplemented group had statistically significant changes in the total WOMAC score compared to placebo (−551 vs. −414; 95 % CI −232 to −42; $p = 0.002$) and GC (−551 vs. −454; 95 % CI −190 to −3; $p = 0.04$) at day 180 (Fig. 2a, Table 4). When the total WOMAC results were analyzed, using MMRM, to account for treatment by time interactions, there remained a statistically significant difference between the UC-II and the placebo groups (−514 vs. −397; 95 % CI −210 to −24; $p = 0.0097$; Table 4). An iAUC analysis also yielded statistically significant differences between the UC-II group versus placebo (−2042 vs. -1479; 95 % CI −1012 to −113; $p = 0.0098$; Table 4). No significant changes were observed between the GC and placebo

Table 3 Demographic and baseline characteristics of the trial subjects

Characteristics	Placebo ($n = 58$)	GC ($n = 65$)	UC-II ($n = 63$)
Sex ((n) male + (n) female)	28M + 30F	28M + 37F	33M + 30F
Age (years)	53.1 ± 1.02	52.6 ± 1.02	53.5 ± 0.99
Height (cm)	162 ± 1.00	161 ± 1.12	161 ± 0.89
Body weight (kg)	64.5 ± 1.20	66.0 ± 1.13	65.5 ± 1.12
Body mass index (kg/m^2)	24.7 ± 0.40	25.5 ± 0.40	25.2 ± 0.37
Kellgren Lawrence radiographic score			
Grade 2 (n)	39	45	42
Grade 3 (n)	19	20	21
Lequesne's Functional Index	7.74 ± 0.12	8.02 ± 0.12	7.90 ± 0.13
Visual analog score (mm)	58.2 ± 0.97	59.1 ± 0.97	58.4 ± 0.99
Total WOMAC score	1382 ± 34.8	1396 ± 31.8	1398 ± 27.9
Mean WOMAC pain	56.9 ± 1.36	57.5 ± 1.33	58.1 ± 1.03
Mean WOMAC physical function	57.9 ± 1.51	58.5 ± 1.37	58.3 ± 1.24
Mean WOMAC stiffness	56.3 ± 1.63	57.3 ± 1.52	58.1 ± 1.32
Knee flexion ROM (°)	114 ± 1.62	114 ± 1.36	114 ± 1.57
Serum CRP (mg/L)[a]	5.29 ± 1.47	8.15 ± 1.79	3.35 ± 0.58
Serum COMP (ng/mL)[b]	325.2 ± 30.5	381.2 ± 44.1	334.6 ± 36.5
Synovial IL-6 (ng/mL)[c]	13.3 ± 4.73	13.9 ± 5.57	15.3 ± 6.04
Synovial MMP-3 (μg/mL)[d]	4.03 ± 1.20	2.54 ± 0.78	4.86 ± 1.74

Values presented as Mean ± SE

[a]Number of subjects used for analyses: 27, placebo; 29, GC; 29, UC-II

[b]Number of subjects used for analyses, 54, placebo; 58, GC; 55, UC-II

[c]Number of subjects used for analyses, 23, placebo; 24, GC; 21, UC-II

[d]Number of subjects used for analyses, 25, placebo; 27, GC; 23, UC-II

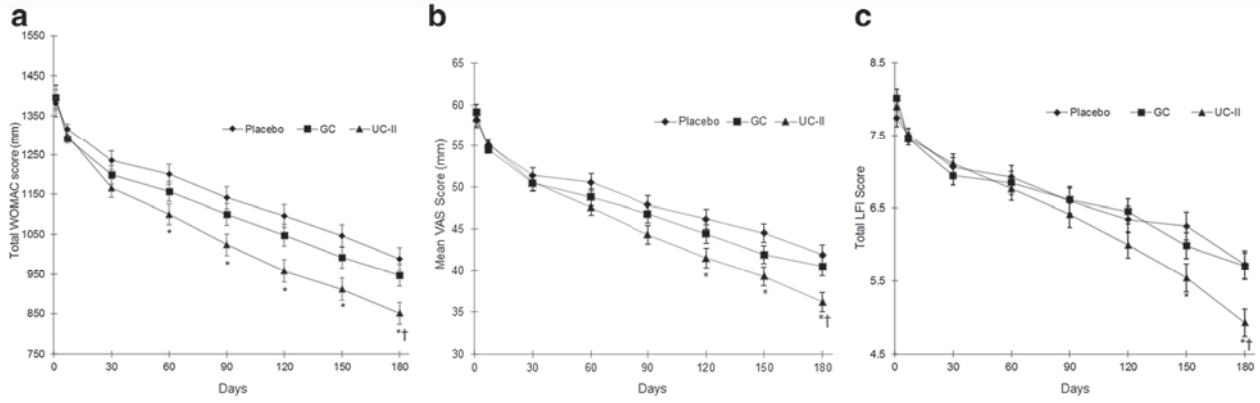

Fig. 2 Total WOMAC score (**a**), Mean VAS (**b**), Total LFI (**c**) in the UC-II, GC and placebo groups over the 180-day study period. Values are presented as mean ± SE. *Significant difference between the UC-II ($n = 54$) and the placebo ($n = 53$) group, $p < 0.05$. †Significant difference between the UC-II ($n = 54$) and the GC group ($n = 57$), $p < 0.05$

cohorts regardless of the type of analytical model used.

Total WOMAC score based on baseline COMP levels

We found that subjects supplemented with UC-II, and presented with baseline COMP levels ≥285 ng/mL (median), had a greater reduction in the total WOMAC score than both placebo and GC groups with similar COMP levels under all modeling conditions (Table 5). For study participants with baseline COMP levels <285 ng/mL, no significant differences between the study groups were noted. Interestingly, we did observe a smaller placebo effect among individuals with baseline COMP levels ≥285 ng/mL as compared to those with <285 ng/mL (28 % vs 32 %). Despite this, UC-II efficacy, as defined by a reduction in overall WOMAC score, was higher in subjects with COMP levels ≥285 ng/mL versus subjects with COMP levels < 285 ng/mL (43 % vs 36 %).

WOMAC mean subscores—pain, stiffness and physical function

At day 180, we observed significant reductions in all three WOMAC subscales for UC-II group compared to placebo (Table 6): pain (24.0 vs. 17.0; 95 % CI −11.1 to −2.8; $p = 0.0003$), stiffness (23.8 vs. 17.8; 95 % CI −10.4 to −1.6; $p = 0.004$), and physical function (22.5 vs. 17.3; 95 % CI −9.3 to −1.3; $p = 0.007$). The UC-II cohort also had significant reductions in WOMAC pain (24.0 vs. 19.2; 95 % CI −8.9 to −0.7; $p = 0.016$) and stiffness (23.8 vs. 19.4; 95 % CI −8.7 to −0.1; $p = 0.044$) at day 180 compared to GC.

Mean VAS score

The UC-II supplemented group had a significant decrease in mean VAS score at day 180 (Fig. 2b) versus both placebo (22.6 vs. 17.0; 95 % CI −9.5 to −1.8; $p = 0.002$) and GC (22.6 vs. 18.4; 95 % CI −8.0 to −0.4; $p = 0.025$). In contrast, the GC group was not significant compared to placebo at any time.

LFI score

A significant reduction was observed in the LFI score for the UC-II group at day 180 versus placebo (2.9 vs. 2.1; 95 % CI −1.4 to −0.2; $p = 0.009$; Fig. 2c). UC-II supplementation also has a greater improvement in LFI score versus GC (2.9 vs. 2.2; 95 % CI −1.4 to −0.2; $p = 0.008$). No significant change was observed between the GC and placebo cohorts. Improvement in the total LFI score for the UC-II group was attributed to a significant reduction in the LFI subscale for daily activities at day 180 ($p = 0.004$ vs. placebo; $p = 0.013$ vs. GC, data not shown).

Knee flexion

No significant differences were observed between the study groups (data not shown).

Serum biomarkers

A significant increase in the final CRP levels versus baseline occurred in all three cohorts ($p = 0.001$). However, no statistical difference between the three cohorts (Table 7; $p > 0.05$) was noted. The scientific reason behind this increase is not well understood. A significant decrease in serum COMP levels was seen in all groups versus baseline ($p = 0.04$) with no significant changes between groups.

Synovial fluid biomarkers

Similar non-significant decreases in IL-6 and MMP-3 levels were noted for all cohorts (Table 7).

Rescue medication usage

The number of subjects that used rescue medication was significantly lower in the UC-II group compared to

Table 4 Change in total WOMAC score from baseline

Analytical method	Type of analysis	Time point (Days)	Placebo ($n = 53$)	GC ($n = 57$)	UC-II ($n = 54$)	p value (95 % CI)			
						Overall[a]	GC vs PBO	UC-II vs PBO[b]	UC-II vs GC
ANCOVA	mITT	180	−414 ± 28.5	−454 ± 27.5	−551 ± 28.2	0.002	0.56 (−134 to 53)	0.002 (−232 to −42)	0.04[c] (−190 to −3)
MMRM	mITT	180	−397 ± 28.6	−452 ± 27.6	−514 ± 28.3	0.014	0.33 (−148 to 37)	0.0097 (−210 to −24)	0.25 (−153 to 30)
			($n = 58$)	($n = 65$)	($n = 63$)				
iAUC	ITT	1 to 180	−1479 ± 137	−1751 ± 130	−2042 ± 132	0.014	0.33 (−718 to 174)	0.0098 (−1012 to −113)	0.26 (−727 to 146)

Values presented as Mean ± SE

Abbreviations: *PBO* placebo

[a]Overall p value was obtained by comparing the mean changes among the three groups using ANCOVA

[b]Significant difference between the UC-II and the placebo groups using Tukey-Kramer test

[c]Significant difference between the UC-II and the GC groups using Tukey-Kramer test

Table 5 Stratified analysis for change in total WOMAC score based on baseline COMP levels

COMP (ng/mL)	Analytical method	Type of analysis	Time point (Days)	Placebo ($n = 27$)	GC ($n = 28$)	UC-II ($n = 27$)	p value (95 % CI)			
							Overall[a]	GC vs PBO	UC-II vs PBO	UC-II vs GC
≥285	ANCOVA	mITT	180	−368 ± 41.7	−396 ± 40.9	−574 ± 41.6	0.002	0.88 (−168 to 112)	0.002[b] (−347 to −65)	0.009[c] (−317 to −38)
	MMRM	mITT	180	−351 ± 44.1	−398 ± 41.1	−540 ± 44.2	0.006	0.71 (−188 to 94)	0.006[b] (−330 to −48)	0.048[c] (−282 to −1)
	iAUC[d]	ITT	1 to 180	−1351 ± 212	−1582 ± 204	−2384 ± 207	0.003	0.72 (−934 to 473)	0.002[b] (−1741 to −325)	0.02[c] (−1498 to −107)
				($n = 26$)	($n = 29$)	($n = 26$)				
<285	ANCOVA	mITT	180	−463 ± 38.8	−508 ± 36.6	−526 ± 38.7	0.48	0.67 (−173 to 82)	0.49 (−195 to 68)	0.94 (−145 to 109)
	MMRM	mITT	180	−442 ± 38.2	−493 ± 37.3	−521 ± 38.1	0.34	0.60 (−178 to 76)	0.32 (−208 to 50)	0.86 (−155 to 100)
	iAUC[e]	ITT	1 to 180	−1626 ± 185	−1908 ± 178	−1902 ± 185	0.49	0.52 (−896 to 333)	0.55 (−902 to 350)	0.99 (−607 to 618)

Values presented as Mean ± SE

[a]Overall p value was obtained by comparing the mean changes among the three groups using ANCOVA

[b]Significant difference between the UC-II and the placebo groups using Tukey-Kramer test

[c]Significant difference between the UC-II and the GC groups using Tukey-Kramer test

[d]Number of subjects used for analyses, 27, placebo; 29, GC; 28, UC-II

[e]Number of subjects used for analyses, 27, placebo; 29, GC; 27, UC-II

Table 6 Reduction in mean WOMAC subscores in placebo, GC and UC-II groups over 180 days

Parameter reduction	Day	Placebo ($n = 53$)	GC ($n = 57$)	UC-II ($n = 54$)	p value			
					Overall[a]	GC vs PBO	UC-II vs PBO[b]	UC-II vs GC[c]
WOMAC pain	7	3.21 ± 0.58	4.57 ± 0.54	3.88 ± 0.55	-	-	-	-
	30	6.61 ± 1.04	7.89 ± 1.00	9.18 ± 1.01	-	-	-	-
	60	8.17 ± 1.10	10.1 ± 1.07	12.7 ± 1.09	0.0149	-	0.011	-
	90	11.2 ± 1.17	12.7 ± 1.14	16.4 ± 1.16	0.0063	-	0.0059	-
	120	12.9 ± 1.28	15.6 ± 1.22	19.9 ± 1.26	0.0005	-	0.0004	0.040
	150	15.0 ± 1.21	17.5 ± 1.16	21.5 ± 1.20	0.0007	-	0.0006	0.047
	180	17.0 ± 1.25	19.2 ± 1.20	24.0 ± 1.23	0.0003	-	0.0003	0.016
WOMAC stiffness	7	3.47 ± 0.64	4.22 ± 0.61	4.24 ± 0.62	-	-	-	-
	30	6.81 ± 1.10	8.76 ± 1.05	9.28 ± 1.07	-	-	-	-
	60	9.36 ± 1.28	11.5 ± 1.25	13.1 ± 1.27	-	-	-	-
	90	11.3 ± 1.36	13.8 ± 1.32	17.0 ± 1.35	0.0158	-	0.010	-
	120	13.6 ± 1.40	15.0 ± 1.34	20.0 ± 1.39	0.0035	-	0.0039	0.029
	150	15.5 ± 1.32	17.7 ± 1.26	21.3 ± 1.31	0.0079	-	0.0058	-
	180	17.8 ± 1.31	19.4 ± 1.27	23.8 ± 1.30	0.0043	-	0.004	0.044
WOMAC physical function	7	3.17 ± 0.56	4.14 ± 0.53	3.91 ± 0.53	-	-	-	-
	30	6.30 ± 1.00	7.80 ± 0.96	9.26 ± 0.98	-	-	-	-
	60	7.75 ± 1.08	9.50 ± 1.05	11.9 ± 1.07	0.0278	-	0.020	-
	90	10.4 ± 1.17	12.1 ± 1.14	15.1 ± 1.16	0.0182	-	0.0136	-
	120	12.7 ± 1.20	14.5 ± 1.15	17.9 ± 1.19	0.0083	-	0.0064	-
	150	14.8 ± 1.19	16.9 ± 1.14	20.0 ± 1.18	0.0078	-	0.006	-
	180	17.3 ± 1.21	18.8 ± 1.16	22.5 ± 1.20	0.0068	-	0.007	-

Values presented as Mean ± SE
[a]Overall p value was obtained by comparing the mean changes among the three groups using ANCOVA
[b]Significant difference between the UC-II and the placebo groups using Tukey-Kramer test
[c]Significant difference between the UC-II and the GC groups using Tukey-Kramer test. '-'denotes a non-significant statistical outcome

placebo (Table 8; $p = 0.001$). Sixty individuals used rescue medications, at least once, during the study. Twenty-eight of these users were from the placebo group, 21 and 11 were from the GC and UC-II cohorts, respectively.

Safety assessments

No clinical or statistically significant changes were reported for any of the hematologic, blood biochemistry or vital signs results (Table 9). No significant changes were noted for the urinalyses results (data not shown).

A total of 45 AEs were reported during the 180-day study period: 9, placebo; 28, GC; and 8, UC-II (Table 10). The majority (62 %) of these occurred in the GC group. Fifteen of 45 events were classified as possibly related to supplementation, 14 of which belonged to the GC group and 1 to placebo. The 14 possible events linked to GC supplementation were primarily gastrointestinal in nature. The eight AEs noted for the UC-II cohort were deemed not related to supplementation. One individual in the GC group was removed from the study due to a respiratory tract infection (cough & fever). This infection was classified as an SAE. The event was investigated by

Table 7 Change from baseline to day 180 in serum and synovial fluid biomarkers

Matrix	Parameter reduction	Day	Placebo (n)	GC (n)	UC-II (n)
Serum	COMP (ng/mL)	180	−51.2 ± 31.3 (53)	−56.5 ± 36.0 (56)	−69.6 ± 40.8 (53)
	CRP (mg/L)	180	15.1 ± 6.33 (26)	9.09 ± 5.36 (28)	13.0 ± 4.64 (28)
Synovial	IL-6 (ng/mL)	180	−9.54 ± 4.83 (23)	−9.72 ± 5.28 (24)	−11.8 ± 5.37 (21)
	MMP-3 (μg/mL)	180	−2.24 ± 1.26 (25)	−0.93 ± 0.79 (27)	−2.67 ± 1.85 (23)

Values presented as Mean ± SE. Statistical analysis was performed on log transformed and baseline adjusted values. No significant differences were observed between the study groups ($p > 0.05$)

Table 8 Number of subjects reporting use of rescue medication

Day	Placebo	GC	UC-II
7	11/58	12/65	3/63
30	18/58	7/63	4/61
60	12/58	9/61	6/59
90	12/56	8/59	3/57
120	13/54	13/59	7/55
150	10/54	12/59	3/55
180	11/53	7/57	4/54
Entire study period	28/58	21/65	11/63[a]

The table summarizes the number of unique individuals reporting the use of rescue medication. Data presented as number of subjects using rescue medication / total number of subjects observed. [a]statistically significant versus the placebo ($p = 0.001$) based on pairwise Tukey-Kramer multiple comparison test. The overall group effect p-value was 0.002 using logistic regression

the attending physician and center staff and judged as not related to GC consumption.

Discussion

We assessed the ability of UC-II to improve joint symptoms in moderate-to-severe knee OA subjects. The results presented herein demonstrate that individuals consuming UC-II presented with better clinical outcomes versus those supplemented with placebo or GC. Analysis of the WOMAC subscales showed that reductions in all three WOMAC subscales contributed to the improvement in the overall WOMAC score observed in subjects supplemented with UC-II. In contrast, GC supplementation failed to induce a statistically significant improvement in the WOMAC, VAS or LFI scores versus placebo. These results confirm previous findings by Crowley et al. [5], which reported greater reduction in knee OA symptoms after 90 days of UC-II supplementation than what was observed with GC.

An interesting finding that emerged from this study is that stratification, according to baseline COMP levels, appears to have selected for individuals that responded better to UC-II supplementation. A greater reduction in knee OA symptom scores was observed among individuals with baseline serum COMP levels ≥285 ng/mL and supplemented with UC-II. This improvement was of sufficient magnitude that statistically significant outcomes for UC-II were observed versus both placebo and GC supplementation under all the statistical analyses we employed (ANCOVA, MMRM and iAUC). COMP, a cartilage turnover marker, is released into serum by chondrocytes and synovial cells [10–12]. Levels of this biomarker have been shown in several studies to have modest correlation with OA severity. However, serum COMP levels in groups of OA subjects overlap with levels observed in healthy populations, and this has limited the use of COMP as a prognostic marker for OA progression [12–14]. While the biologic or clinical significance to these findings remains to be understood, we find this preliminary observation an interesting one suitable for further investigation and confirmation.

The etiology behind UC-II's impact on OA symptoms is not known. However, undenatured type II collagen has been shown to protect animals against the onset of joint damage in both OA and RA experimentally induced arthritis models [15–18]. This protection is hypothesized to occur via the induction and migration of T regulatory cell (Tregs) to the area of inflammation and damage [19, 20]. The proposed role of Tregs may also have relevance to the moderation of OA symptoms, as *in vitro* studies have found that Tregs produce anti-inflammatory cytokines that stimulate chondrocytes to synthesize cartilage matrix components [21–23]. Additional studies that elucidate the precise mechanism through which UC-II mediates a reduction in knee OA symptoms are required.

The *in vivo* effects mentioned above may only be initiated by ingesting undenatured type II collagen as denatured (e.g., hydrolyzed) type II collagen fails to protect animals against the onset of arthritis [15]. This latter observation could explain why van Vijven and coworkers [24] concluded that there was insufficient evidence to support collagen for the treatment of OA as they combined data from all published clinical studies regardless whether native or denatured collagen was used in the trial.

We failed to observe any changes in knee ROM and distance walked regardless of supplementation. Improvements in these clinical outcomes are likely to be based not just on a symptomatic reduction in pain but also on physical improvements in the knee joint's overall functionality. Until we undertake trials of longer duration, it remains an open question as to whether a slow acting supplement like UC-II can impact the biomechanical status of the OA knee sufficiently to improve knee ROM.

In the current study, GC supplementation did not significantly improve the signs and symptoms associated with knee OA. The scientific literature supporting the efficacy of GC is mixed, but there are various published studies which suggest that GC may moderate OA symptoms [25–27]. The GAIT study found that GC, and each component of GC individually, failed to impact OA symptoms as measured by the WOMAC pain scale; however, the placebo effect in that study was nearly 60 % which resulted in an underpowered study to determine differences between the treatments [28]. In contrast, a significant difference in knee pain was observed in the GC subgroup with moderate-to-severe knee pain compared to the placebo treated group [28]. To confirm the observation that GC may be more efficacious in subjects with moderate-to-severe knee OA pain, Hochberg and coworkers [29] performed a study in which OA subjects with moderate-to-severe knee pain, were randomized

Table 9 Safety parameter assessment at baseline and day 180 in placebo, GC and UC-II groups

Parameter (Units)		Baseline						Day 180					
	Normal range	Placebo ($n = 58$)	GC ($n = 65$)	UC-II ($n = 63$)	p value GC vs PBO	p value UC-II vs PBO	p value UC-II vs GC	Placebo ($n = 53$)	GC ($n = 56$)	UC-II ($n = 53$)	p value GC vs PBO	p value UC-II vs PBO	p value UC-II vs GC
Hematology													
Hemoglobin (gm/dL)	12.1–17.2	12.1 ± 0.22	11.9 ± 0.21	12.1 ± 0.20	0.7613	0.9948	0.8095	12.7 ± 0.24	12.4 ± 0.20	12.7 ± 0.18	0.4454	0.9727	0.5851
ESR (mm/h)	0–29	21.1 ± 1.77	23.9 ± 2.18	17.5 ± 1.56	0.7629	0.1034	0.0144	15.1 ± 1.24	17.0 ± 1.91	13.6 ± 1.28	0.9424	0.5364	0.3387
RBC (million/mm^3)	4.7–6.1	4.29 ± 0.08	4.21 ± 0.08	4.33 ± 0.09	0.7747	0.9388	0.5498	4.32 ± 0.08	4.25 ± 0.08	4.37 ± 0.08	0.7935	0.8946	0.5129
WBC (/mm^3)	4500-10,000	7979 ± 234	8248 ± 222	7795 ± 249	0.7020	0.8483	0.3523	7984 ± 204	7981 ± 209	7769 ± 204	1.0000	0.7706	0.7639
Platelet count (x100000/mm^3)	1.5-4.5	2.77 ± 0.08	2.84 ± 0.08	2.78 ± 0.08	0.7837	0.9946	0.8319	2.77 ± 0.07	2.84 ± 0.07	2.77 ± 0.09	0.8304	0.9993	0.8113
Liver Function													
Albumin (gm/dL)	3.5–5.5	3.98 ± 0.06	4.02 ± 0.06	3.94 ± 0.06	0.8957	0.9089	0.6503	4.00 ± 0.05	4.03 ± 0.05	3.96 ± 0.04	0.8931	0.8902	0.6292
ALP (IU/L)	44–147	117 ± 5.74	118 ± 5.84	115 ± 5.57	0.9871	0.9838	0.9404	123 ± 5.72	116 ± 5.49	115 ± 5.59	0.5622	0.4847	0.9890
SGOT (U/L)	10–34	25.2 ± 0.93	24.0 ± 0.97	24.4 ± 0.60	0.5778	0.7796	0.9421	24.6 ± 0.73	23.9 ± 0.81	23.9 ± 0.65	0.7711	0.7930	0.9995
SGPT (U/L)	5–35	25.9 ± 1.23	25.0 ± 1.40	24.1 ± 0.95	0.5977	0.6004	1.0000	24.5 ± 0.94	24.3 ± 1.00	23.3 ± 0.99	0.9688	0.7119	0.8427
Total bilirubin (mg/dL)	0.3–1.9	0.78 ± 0.08	0.69 ± 0.03	0.72 ± 0.03	0.5376	0.9424	0.7343	0.72 ± 0.03	0.67 ± 0.03	0.78 ± 0.04	0.4243	0.6098	0.0718
Cardiac Function													
Systolic BP (mm Hg)	<120	125 ± 1.28	127 ± 1.35	127 ± 1.21	0.5980	0.7320	0.9752	127 ± 1.18	125 ± 1.33	128 ± 1.22	0.7263	0.8949	0.4409
Diastolic BP (mm Hg)	< 80	81.2 ± 1.19	80.2 ± 0.83	81.7 ± 1.02	0.7544	0.9283	0.5094	80.2 ± 1.03	80.5 ± 1.07	78.9 ± 0.96	0.9877	0.6233	0.5180
Pulse rate (beats/min)	60–100	80.0 ± 0.92	79.6 ± 0.98	80.3 ± 0.99	0.9149	0.9719	0.7956	80.0 ± 0.89	78.2 ± 0.82	79.2 ± 1.03	0.3201	0.8018	0.6989
Renal Function													
Creatinine (mg/dL)	0.6–1.3	1.00 ± 0.03	1.01 ± 0.04	0.96 ± 0.03	0.9995	0.5767	0.5778	0.96 ± 0.03	0.95 ± 0.02	0.96 ± 0.02	0.9904	0.9846	0.9508
BUN (mg/dL)	6–24	18.1 ± 1.08	18.0 ± 1.11	18.0 ± 1.15	0.9929	0.9878	0.9992	18.6 ± 1.11	17.8 ± 1.09	17.9 ± 1.02	0.7602	0.7953	0.9985

Results are presented as Mean ± SE. Normal ranges were obtained from Medline[a] and the Mayo Clinic[b]. Data was analyzed using ANCOVA followed by Tukey's multiple comparisons test ($p > 0.05$)

Abbreviations:

ESR erythrocyte sedimentation rate; *RBC* red blood cell; *WBC* white blood cell; *ALP* alkaline phosphatase; *SGOT* serum glutamic oxaloacetic transaminase; *SGPT* serum glutamic pyruvic transaminase; *BP* blood pressure; *BUN* blood urea nitrogen

[a]ADAM, Inc.: http://www.nlm.nih.gov/medlineplus/encyclopedia.html (accessed October 2015)

[b]Mayo Foundation for Medical Education and Research: Mayo Clinic. www.mayoclinic.org (accessed October 2015)

Table 10 Summary of analysis of adverse events in all subjects

	Study group		
	Placebo (*n* = 58)	GC (*n* = 65)	UCII (*n* = 63)
Severity			
Mild	7	21	5
Moderate	2	7	3
Severe	0	0	0
Relationship to Test Article			
Not related	8	14	8
Possible	1	13	0
Definite	0	1	0
Body System and AEs			
Gastrointestinal			
Acidity	2	3	2
Acute peptic disorder	1	0	1
Diarrhea	1	1	0
Epigastric burning	0	1	0
Febrile Enteritis	0	1	0
Heart burn	0	1	0
Vomiting	0	1	0
Nausea	0	1	0
Pain			
Arthralgia	0	1	0
Body pain	0	1	0
Low back pain	1	1	0
Neck Pain	0	1	1
Headache	2	4	0
Myalgia	0	1	0
Dermatology			
Itching	0	2	0
Xerotic skin	0	0	1
Pulmonary/Upper Respiratory			
Lower respiratory tract infection	0	0	2
Upper respiratory tract infection	0	1	0
Cough	0	2	0
Genitourinary			
Burning micturition	1	0	0
Burning sensation	0	0	1
Cardiovascular			
Palpitation	0	2	0
Constitutional Symptoms			
Fever	1	2	0
Insomnia	0	1	0
	9	28	8
Total Number of Adverse Events Experienced During Study			
Total Number of Subjects Experiencing Adverse Events: n (%)	7/58 (12 %)	20/65 (31 %)	8/63 (13 %)

to GC or celecoxib for a period of 6 months. The results showed that GC treatment reduced WOMAC measured knee pain by 50 %, comparable to the results obtained with celecoxib [28]. It is worth noting that results such as these are not consistent across a number of studies for reasons yet to be determined [25–27].

In recent years, interest has focused on developing various biomarkers for monitoring OA progression and drug development [12, 30]. We therefore assessed several biomarkers of inflammation (CRP, IL-6 and MMP-3) plus cartilage breakdown (COMP) and found no significant change for any of these biomarkers in this clinical trial. Since OA appears to impact the biology of several key components of the knee (e.g., synoviocytes, chondrocytes, etc.), the ability to achieve a significant change in any one biomarker could prove challenging for a slow acting supplement like UC-II. Also, multiple factors including ethnicity, physical activity, gender differences, and diurnal variation influence these biomarkers resulting in large variability in their levels [31–35]. Therefore, any change in these markers would have to occur as a result of a highly significant impact on the underlying pathophysiology of OA, given that the correlation between these biomarkers and OA pathophysiology are weak [12]. Such effects might be expected to occur more readily with a targeted agent [4, 36].

Conclusion

This study found that UC-II, a nutritional ingredient containing undenatured type II collagen, significantly improved knee function in OA subjects by day 180, compared to placebo and to GC, and was well-tolerated. Based on the data presented herein, we believe that additional research is warranted both to confirm and to define these findings more extensively.

Abbreviations

AEs: adverse events; ANCOVA: analysis of covariance; ANOVA: analysis of variance; cGMP: current good manufacturing practice; CI: confidence interval; COMP: cartilage oligomeric matrix protein; CRF: case report form; CRP: C-reactive protein; GC: glucosamine hydrochloride plus chondroitin sulfate; iAUC: incremental area under the curve; IEC: Institutional Ethics Committee; IL-6: interleukin-6; ITT: intent-to-treat; K-L: Kellgren and Lawrence; LFI: Lequesne functional index; mITT: modified intent-to-treat; MMP-3: matrix metalloproteinase-3; MMRM: mixed model repeated measures; NSAIDs: nonsteroidal anti-inflammatory drugs; OA: osteoarthritis; PBO: placebo; ROM: range of motion; Tregs: T regulatory cell; UC-II: undenatured type II collagen; VAS: visual analog scale; WOMAC: Western Ontario McMaster Universities Osteoarthritis Index.

Competing interests
JPL and ZMS are employees of InterHealth Nutraceuticals. NEL provided consulting services to InterHealth. This study was sponsored by InterHealth Nutraceuticals, Inc. Benicia, CA. The study was run and managed independently by Laila Pharmaceuticals Pvt. Ltd., India. Data collection was done by the clinical study staff at each respective site. Data analyses was performed by an independent statistician.

Authors' contributions
JPL and ZMS contributed in the conception and design of the study, data interpretation and manuscript preparation. NEL participated in data interpretation, manuscript drafting and revisions. All authors read and approved the final version of the manuscript.

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Research Paper

Safety and efficacy of undenatured type II collagen in the treatment of osteoarthritis of the knee: a clinical trial

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Abstract

Previous studies have shown that undenatured type II collagen (UC-II) is effective in the treatment of rheumatoid arthritis, and preliminary human and animal trials have shown it to be effective in treating osteoarthritis (OA). The present clinical trial evaluated the safety and efficacy of UC-II as compared to a combination of glucosamine and chondroitin (G+C) in the treatment of OA of the knee. The results indicate that UC-II treatment was more efficacious resulting in a significant reduction in all assessments from the baseline at 90 days; whereas, this effect was not observed in G+C treatment group. Specifically, although both treatments reduced the Western Ontario McMaster Osteoarthritis Index (WOMAC) score, treatment with UC-II reduced the WOMAC score by 33% as compared to 14% in G+C treated group after 90 days. Similar results were obtained for visual analog scale (VAS) scores. Although both the treatments reduced the VAS score, UC-II treatment decreased VAS score by 40% after 90 days as compared to 15.4% in G+C treated group. The Lequesne's functional index was used to determine the effect of different treatments on pain during daily activities. Treatment with UC-II reduced Lequesne's functional index score by 20% as compared to 6% in G+C treated group at the end of 90-day treatment. Thus, UC-II treated subjects showed significant enhancement in daily activities suggesting an improvement in their quality of life.

Key words: undenatured type II collagen, osteoarthritis, glucosamine, chondroitin, WOMAC, visual analog scale, Lequesne's Functional Index

INTRODUCTION

Arthritis afflicts approximately 43 million Americans or approximately 16.6% of the US population. The two most common types of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA). OA of the knee and hip is a growing health concern and is the most common forms of arthritis (1-3). Pain and disease can range from very mild to very severe (3). Patients with OA have pain that typically worsens with weight bearing, including walking and standing, and improves with rest (4). Other symptoms include morning stiffness and gelling of the involved joint after periods of inactivity. Currently, OA affects

nearly 21 million people in the United States, accounting for 25% of visits to primary care physicians, and half of all Non-Steroidal Anti-Inflammatory Drugs (NSAID) prescriptions. The diverse clinical patterns of OA are observed in approximately 10% of people older than 60 years thus compromising the quality of life of millions of Americans. In addition, OA costs the North American economy approximately $60 billion per year.

Current treatment of OA includes exercise, heat/cold therapy, joint protection, weight loss, physiotherapy/occupational therapy and medications (3-5). The most common medications include acetaminophen and NSAIDs. Although these drugs are effective for reducing pain associated with OA, they do not reverse the disease. In addition, there are considerable side effects associated with the use of these drugs. As a result, OA sufferers have turned to natural nutraceuticals to ease their pain and discomfort. These products are commonly used because they are well tolerated and considered safe. Nutraceuticals are defined as functional foods, natural products, or parts of food that provide medicinal, therapeutic, or health benefits, including the prevention or treatment of disease. Currently, glucosamine and chondroitin are the two most commonly used nutraceuticals in humans as well as in animals to alleviate pain associated with arthritis (6). However, recent randomized controlled trials and meta-analysis of these supplements have shown only small-to-moderate symptomatic efficacy in human OA (7). An emerging novel nutraceutical ingredient known as UC-II has received considerable attention in the treatment of OA. UC-II is a novel undenatured type II collagen derived from chicken sternum cartilage. Previous studies have shown that undenatured type II collagen is effective in the treatment of RA (8-11), and preliminary human (12) and animal (13) trials have shown it to be effective in treating OA. Obese-arthritic dogs given 4 mg or 40 mg daily dose of UC-II for 90 days showed significant declines in overall pain, pain during limb manipulation and lameness after physical exertion (14). Greater improvement was observed with the 40 mg dose. No adverse effects or significant changes in serum chemistry were noted. Following UC-II withdrawal for a period of 30 days, all dogs experienced a relapse of overall pain, exercise-associated lameness and pain upon limb manipulation. Studies have also shown that small doses of orally administered undenatured type II chicken collagen inhibit killer T-cell attack (15). The present clinical trial evaluated the safety and efficacy of UC-II in the treatment of the knee in OA patients.

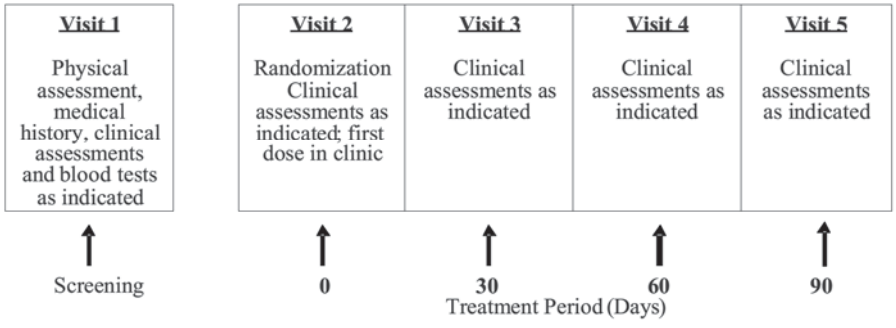


Figure 1. UC-II clinical study design. The study was a two-site, randomized, double-blind study conducted in London, Ontario and Corunna, Ontario, Canada.

Materials and Methods

Study Design

This clinical trial (Human Clinical Trial Approval #06UOHI) was managed by KGK Synergize Inc. (London, ON, Canada). The study was conducted at two sites: 1) KGK Synergize Inc., and 2) Corunna Medical Research (Corunna, ON, Canada). Figure 1 illustrates the study design while Table 1 lists the procedures and observations at each time point.

Briefly, at screening (Visit 1) the consent form was discussed, signed and a complete physical examination was performed. Activity level, diet history, medication/supplement use and medical history were recorded. The VAS score, the WOMAC Index and Lequesne scores were obtained. Urine was collected for a pregnancy test for women of childbearing potential. A blood sample was taken for determination of uric acid, CBC count and differentiation, albumin, total protein, sodium, potassium, chloride, BUN, creatinine, ALT, AST, bilirubin, erythrocyte sedimentation rate (ESR) and rheumatoid factor. Upon review of blood test results, eligible subjects were instructed to get an X-ray of the affected knees to confirm diagnosis. A total of 52 subjects were recruited using the inclusion and exclusion criteria outlined in Table 2. At the first treatment visit (Visit 2), selected subjects were randomly assigned to receive UC-II (n = 26) or glucosamine HCl plus chondroitin sulfate (n = 26, G+C). On each test day (day 0, 30, 60, 90), subjects were required to come to the clinic for clinical assessment. The clinical assessments included WOMAC, Lequesne's functional index and 100-mm VAS pain scores. A subject treatment diary was completed by each patient throughout the study period to determine side effects, medication use, and product compliance.

Table 1. Schedule of observations and procedures

Procedure	Visit 1 Screening	Visit 2 Day 0	Visit 3 Day 30	Visit 4 Day 60	Visit 5 Day 90
Informed consent	X				
Review inclusion/exclusion	X	X	X	X	X
Medical history including activity level and diet history	X				
Physical examination	X				
Biometric measurements: Weight, height*, heart rate and blood pressure.	X	X	X	X	X
Urine pregnancy test	X				
Concomitant medications	X	X	X	X	X
Blood samples: Uric acid, CBC count and differentiation, albumin, total protein, sodium, potassium, chloride, BUN, creatinine, ALT, AST, bilirubin, ESR, rheumatoid factor	X				X
WOMAC, VAS and Lequesne scores	X	X	X	X	X
X-ray	X				
Randomization		X			
Blood sample: ALT, AST, bilirubin, albumin.			X†	X†	
Knee flexion, Time to walk 50m, Swelling in the knee joint, Time for climbing 10 steps		X	X	X	X
Physician's Global Assessment		X	X	X	X
Subject's Global Assessment		X	X	X	X
Investigational Product dispensed		X	X	X	
Subject Treatment Diary dispensed		X	X	X	
Investigational Product returned Compliance calculated			X	X	X
Subject Treatment Diary returned			X	X	X
Adverse Events			X	X	X

* height was only measured at visit 1

† If acetaminophen use was greater than 2 g/day for more than 7 days

Table 2. Inclusion and exclusion criteria

Inclusion Criteria
Males and females 40-75 years old
Females of childbearing potential must agree to use a medically approved form of birth control and have a negative urine pregnancy test result
Unilateral or bilateral OA of the knee for greater than 3 months (American College of Rheumatology criteria) confirmed by radiologist's report, i.e. X-rays showing osteophytes, joint space narrowing or subchondral bone sclerosis (eburnation)
Erythrocyte sedimentation rate (ESR) < 40 mm/hr
Moderate OA as indicated by Lequesne's functional index score of 4.5-7.5 after 7 day withdrawal of usual medications
Able to walk
Availability for duration of study period (3-4 months)
Subject using other therapies for OA, such as exercise, heat/cold therapy, joint protection and physiotherapy/occupational therapy agrees to continue these therapies as normal avoiding changes in frequency or intensity and to record therapies in the study diary
Subject agrees not to start any new therapies for OA during the course of the study
Able to give informed consent
Exclusion Criteria
History of underlying inflammatory arthropathy; septic arthritis; inflammatory joint disease; gout; pseudogout; Paget's disease; joint fracture; acromegaly; fibromyalgia; Wilson's disease; ochronosis; haemochromatosis; heritable arthritic disorder or collagen gene mutations or rheumatoid arthritis
History of asthma, history of diabetes (Type I or Type II)
Hyperuricemia (urate, males > 480 umol/L, females > 450 umol/L)
Expectation of surgery in the next 4 months
Recent injury in the area affected by OA of the knee, i.e. meniscal tear (past 4 months)
Cartilage reconstruction procedure in the target knee
Severe OA as indicated by Lequesne's functional index score of 8 or greater, after 7 day withdrawal of usual medications
Intra-articular corticosteroid injections in the target knee within the last 3 months
Viscous injections in the target knee within the last 6 months
Hypersensitivity to NSAIDs
Abnormal liver or kidney function tests (ALT or AST > 2 times the upper limit of normal; elevated creatinine, males > 125 umol/L, females > 110 umol/L)

Abnormal findings on complete blood count
History of coagulopathies, history of peptic ulceration and upper GI hemorrhage
Uncontrolled hypertension
History of congestive heart failure, history of allergic reaction to chicken and/or eggs
History of allergic reaction to local anesthetic or to any ingredients in the test product including shellfish
Hyperkalemia (potassium > 6.2 mmol/L)
Anticipated problems with product consumption
History of cancer as well as gastrointestinal, renal, hepatic, cardiovascular, hematological, or neurological disorders
High alcohol intake (>2 standard drinks per day)
Pregnant, breastfeeding or planning to become pregnant during the study
History of psychiatric disorder that may impair the ability of subjects to provide written informed consent
Use of other natural health products, including glucosamine and chondroitin, one month prior to study and during the study, other than multivitamin and mineral supplements containing vitamins and minerals as the sole medicinal ingredients
Use of concomitant prohibited medication (narcotics, oral NSAIDs, topical NSAIDs) within four weeks of randomization
Use of acetaminophen or ibuprofen within 7 days of randomization
Subject is unwilling to stop taking pain medication other than the study medication (for arthritis or other types of pain) or is unwilling to stop taking other medications for the treatment of OA
Any other condition that, in the opinion of the investigator, would adversely affect the subject's ability to complete the study or its measures

Supplements

Each UC-II (InterHealth Nutraceuticals, Inc., Benicia, CA) capsule contained 20 mg UC-II standardized to 5 mg of bioactive undenatured type II collagen. Subjects in the UC-II group were instructed to take two "sugar pills" in the morning to protect blinding and two UC-II capsules in the evening accounting for a daily dose of 40 mg UC-II containing 10 mg of bioactive undenatured type II collagen.

Each G+C capsule contains 375 mg of glucosamine HCl (USP Grade) and 300 mg of chondroitin sulfate (USP Grade). The subjects were instructed to take two G+C capsules in the morning and two in the evening for a daily dose of 1500 mg glucosamine and 1200 mg chondroitin.

Removal of Patients from Therapy or Assessment

The criteria for removal of patients from the study included:

Adverse events

For any adverse event, patients were examined and appropriately managed or the patients would be referred to another medical professional for proper evaluation and treatment. If medical problems were attributed to the trial compounds, then the trial drugs were discontinued and the toxicities were reported.

Personal reasons

As stated in the Consent Form, subjects were able to withdraw from the study for any reason at any time.

Clinical judgment of physician

Subjects were withdrawn from the study (without penalty) if, in the opinion of the treating physician, it was not in the patient's best interest to continue. For instance, if during the course of the study a patient became pregnant, she would be withdrawn from the study because it was not known how the study compounds/medications might affect an unborn child.

Protocol violation

Any subject found to have entered this study in violation of the protocol or failed to follow the study protocol were discontinued from the study at the discretion of the Principal Investigator. Subjects were withdrawn for protocol non-compliance if they adhered to the dosing schedule less than 75% of the time.

Method of assigning patients to treatment groups

Patients were assigned to treatment groups (order of treatments) using computer-generated randomization tables. Patients were not stratified or assigned using any other specific method and were not randomized after stratification or blocking procedures.

Selection of doses in the study

The justification for the daily dose of 40 mg UC-II in capsules (providing 10 mg of undenatured collagen II) is based on efficacy demonstrated in earlier studies (8,9).

Blinding

In order to protect blinding, subjects were given bottles containing product labeled with "AM" or "PM" to distinguish the time in which treatment was to be taken. Each bottle contained descriptions of all potential products to ensure blinding was protected. Additionally, each bottle was labeled with a randomization number. In the event that an adverse effect was considered serious and related to the investigational product, the blind would be broken for

that individual subject.

Neither the patient, nor investigator, nor research staff, were aware which test compound the subject was assigned. Interim analysis was performed in order to write a preliminary report and thus preliminary unblinding occurred by an individual unrelated to the study conduct. Personnel related to analysis, statistics, and report writing remained blinded.

Prior and concomitant therapy

Uses of medications such as narcotics, oral NSAIDS, topical NSAIDS within four weeks of randomization and during the study, were not allowed.

Treatment compliance

Compliance was assessed by capsule count at visits 3, 4, and 5 and review of subject diary.

Efficacy and Safety Variables

Efficacy and safety measurements assessed

Adverse events

During the study, subjects recorded adverse effects in their subject diary. At each visit, the subjects were asked if they experienced problems or difficulties. Any adverse events were documented and recorded in the study record and was classified according to the description, duration, severity, frequency, and outcome. The investigator assessed the adverse events and decided causality. Classifications were as per the Coding Symbol Thesaurus of Adverse Reaction Terms (COSTART) U.S. Food and Drug Administration (16).

Blood tests

Blood samples were taken from all subjects during screening (visit 1) and at end of study (visit 5). Blood samples (approximately 15 ml) were taken from subjects at day 30 and day 60 (visits 3 and 4) for the determination of ALT, AST, bilirubin, and albumin if the subjects had been taking acetaminophen greater than 2 g/day for more than 7 days. All blood samples were analyzed by MDS Laboratory Services (London, Ontario, Canada).

Appropriateness of Measurements

The efficacy and safety assessments used in this study were standard for OA and are widely used and recognized as reliable, accurate, and relevant.

WOMAC scores were determined, at screening, and baseline, as well as at days 30, 60 and 90 as described in Bellamy et al (17). Other objectives also performed at days 0, 30, 60 and 90 included determination of Lequesne's functional index, VAS pain scores, knee flexion, time to walk 50 m, time to climb 10 steps, physician's and subject's global assessment. The Lequesne's functional index is described in Lequesne et al. (18).

Statistical Methods

Sample size of 25 subjects per group was based on the subject number used in Braham et al. (1). To compare UC-II with G+C group, a linear contrast was included in the analysis of variance. Data missing subsequent to 30 days were imputed using the last-observation-carried forward technique. Furthermore, comparisons between the UC-II and G+C groups were made at each visit using analysis of variance, using the baseline visit as a covariate. SAS version 9.1 has been used to perform the statistical analysis. Probability values less than 0.05 were considered statistically significant for between-group comparisons.

Results

Baseline Statistics and Compliance of Trial Subjects

Demographic and baseline characteristics of patients are summarized in Table 3. Overall, the patient profiles with respect to age, sex, height, weight, blood pressure, heart beat and target knee were similar between both treatment groups. Table 4 shows treatment compliance of the trial patients. There were no significant interaction terms or between-group differences for compliances. When compliances were compared at each visit, there were no overall between-group differences among the two treatment groups.

Table 3. Demographic and baseline characteristics of the trial subjects

	UC-II (N=26)	G + C (N=26)
Age (years)	58.9 ± 9.79	58.7 ± 10.3
Sex: male/female (%)	13/26 (50%)	17/26 (65%)
Height (cm)	167.7 ± 9.90	167.0 ± 8.73
Weight (kg)	84.3 ± 17.4	86.6 ± 21.0
Systolic Blood Pressure (mm)	128.2 ± 9.36	126.3 ± 12.5
Diastolic Blood Pressure (mm)	81.9 ± 7.43	79.7 ± 8.60
Heart Rate (bpm)	68.2 ± 7.72	67.4 ± 8.47
Target knee		
Left; n (%)	16 (61.5%)	13 (50%)
Right; n (%)	10 (38.5%)	13 (50%)
Where applicable, values are expressed as mean ± SD		

Table 4. Treatment compliance as assessed during specified visits

Visit	Treatment Group	
	UC-II	G + C
AM Capsule Compliance		
Visit 3	[25] 90.5 ± 19.2	[25] 93.6 ± 11.5
Visit 4	[24] 93.2 ± 9.66	[26] 94.5 ± 11.8
Visit 5	[23] 98.5 ± 5.15	[26] 93.3 ± 11.0
PM Capsule Compliance		
Visit 3	[25] 88.1 ± 18.7	[25] 92.5 ± 12.5
Visit 4	[24] 92.8 ± 8.97	[26] 91.6 ± 12.3
Visit 5	[22] 95.3 ± 9.92	[26] 89.7 ± 12.6

There were no significant interaction terms and between-group differences for compliances. When compliances were compared at each visit, there were no overall between-group differences among the five treatment groups. Values are expressed as [n] mean ± SD.

WOMAC Score

The interaction between visit and treatment was significant in UC-II treated group for "pain walking on flat surface" (p=0.034), "difficulty walking on flat surface" (p=0.038) and "performing heavy domestic duties" (p=0.031) as compared to G+C treated group. There was evidence that UC-II treatment has a significant effect for "ascending stairs" (p=0.013) as compared to G+C treatment. Additionally, when groups were compared at each visit, UC-II was significantly better than G+C for "ascending stairs at 30 days and 60 days" (p=0.019 & 0.040 respectively), "at night while in bed" (p=0.015) at 60 days and difficulty walking on flat surface at 90 days (p=0.035). There were no further statistically significant differences for any other individual WOMAC components or summary scores. Treatment with UC-II was most effective and reduced the WOMAC scores by 33% compared to 14% in (G+C)-treated groups after 90 days. Within-group analysis indicated that treatment with UC-II for 90 days significantly ($p<0.05$) improved WOMAC scores at all treatment time points measured. In contrast, subjects received G+C did not show any statistical significant change in WOMAC scores at Day 90 of treatment (Fig. 2).

VAS Score

The interaction between visit and treatment was non-significant for all VAS components and summary scores. However there was evidence that UC-II treatment had a significant effect for "pain during climbing up and down stairs", "night pain" and "resting pain" (p=0.035, 0.030 and 0.024 respectively). When groups were compared at each visit, UC-II was significantly better than G+C for "night pain" (p=0.040) and "resting pain" (p=0.020) at 60 days and "pain during climbing up and down stairs" (p=0.014) and "resting pain" at 90 days (p=0.034). There were no between-group differences for any of the VAS components or summary scores. Although both the treatments reduced the VAS score, UC-II was found to be more effective with a 40% decrease after 90 days of treatment compared to a 15% decrease in G+C treated groups.

Within-group analysis indicated that subjects on UC-II showed a significant reduction in total VAS scores at Day 60 and Day 90 as compared to baseline. However, subjects on G+C showed a significant reduction in total VAS scores at Day 30 and no significant difference was observed at either Day 60 or Day 90 as compared to baseline (Fig. 3).

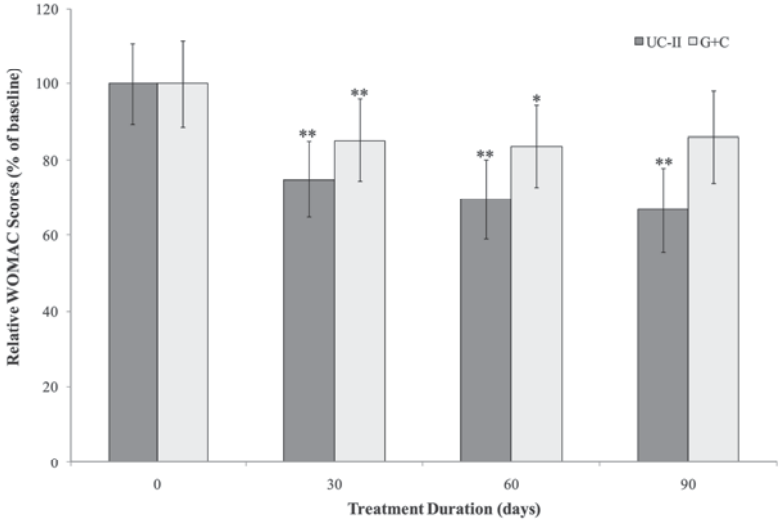


Figure 2. Changes in WOMAC scores at Day 90 from baseline. WOMAC scores from each treatment group were compared to baseline value at specified time points. Each bar presents mean ± SEM. *p<0.05, **p<0.005 indicate significantly different from baseline.

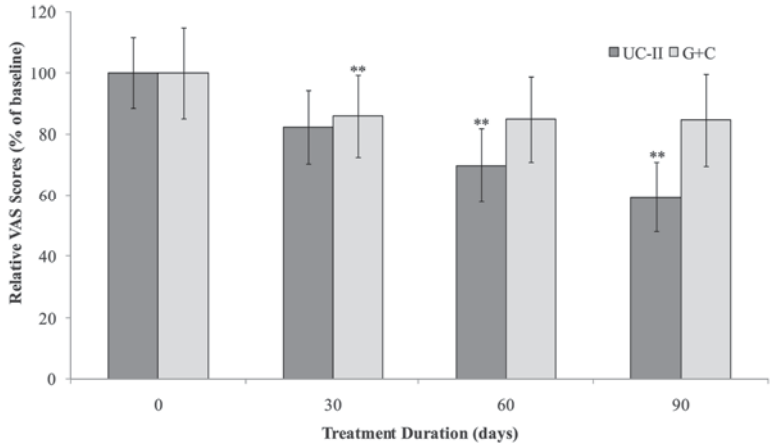


Figure 3. Changes in VAS score at Day 90 from baseline. VAS scores from each treatment group were compared to baseline value at specified time points. Each bar presents mean ± SEM. **p<0.05 indicates significantly different from baseline.

Lequesne Score

The Lequesne's functional index was used to determine the effect of different treatments on pain during daily activities. The interaction between visit and treatment was non-significant for all Lequesne's components and summary scores. Furthermore, there were no between-group differences for any of the Lequesne's components or summary scores. However there was evidence that visit has a significant effect in UC-II treated group for "pain while up from sitting" and "maximum distance walked" (p=0.036 and 0.002 respectively) as compared to G+C treated group.

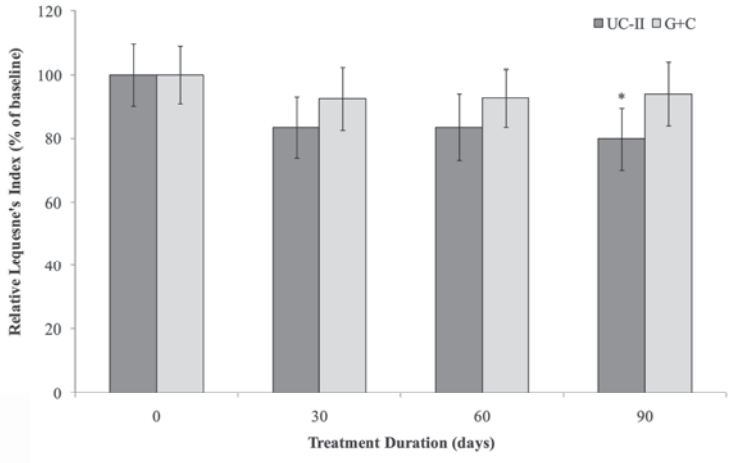
There was as a strong trend toward UC-II efficacy. UC-II treatment effectively reduced Lequesne's functional index score by 20.1% as compared to 5.9 % by G+C treatment.

Within-group analysis suggested that subjects on UC-II demonstrated a significant reduction in total Lequesne's index of severity score from baseline to Day 90, whereas no significant difference from baseline was observed for subjects on G+C at any treatment time points evaluated (Fig. 4).

Figure 4. Changes in Lequesne's functional index at Day 90 from baseline. Lequesne's functional index from each treatment group was compared to baseline value at specified time points. Each bar presents mean ± SEM. *p<0.05 indicates significantly different from baseline.

Adverse Events

Adverse effects that occurred during the 90-day trial period are summarized in Table 5. Overall, there were 58 adverse events noted in the subjects receiving G+C treatment, whereas, only 35 adverse events were observed in UC-II group. In terms of severity, 60% of mild and 38% of moderate adverse events were experienced by subjects on G+C in comparison to 43% and 54% by subjects on UC-II. In relationship to test product a higher number of subjects (23%) on G+C demonstrated adverse events possibly related to product as compared to 11.4% of subjects on UC-II. For UC-II the possible adverse events related to products were constipation and headaches (intermittently). For G+C the possible adverse events related to products were bloating, stomach pain, rash, water retention (edema around eyes and scars), hives on face and chest, and headache. However, there was no significant difference in the occurrence of adverse effects between the two treatment groups.



Rescue Medication

A greater percentage of subjects used rescue medication while on G+C as compared to UC-II at every time point assessed. From baseline to Day 30 a total of 8 subjects (33.3%) on UC-II used rescue medication as compared to 23 subjects (88.5%) on G+C. From Day 30 to Day 60, 13 subjects (54.2%) on UC-II used rescue medication as compared to 21 subjects (80.8%) on G+C. Fourteen subjects (63.6%) on UC-II used rescue medication as compared to 19 subjects (79.2%) on G+C from Day 60 to Day 90.

Table 5. Summary of analysis of adverse events in all subjects

	Treatment Group	
	UC-II (n=26)	**G + C (n=26)**
Severity (n)		
Mild	15	35
Moderate	19	22
Severe	1	1
Relationship to Test Article (n)		
Not related	17	20
Unlikely	14	30
Possible	4	8
Probable	0	0
Most Probable	0	0
Body System (n)		
Pain	10	17
Gastrointestinal	5	15
Musculoskeletal/Soft Tissue	7	5
Neurology	0	2
Pulmonary / Upper Respiratory	2	1
Hemorrhage/Bleeding	2	1
Blood/Bone Marrow	2	1
Dermatology/Skin	2	3
Allergy / Immunology	0	1
Infection	1	3
Lymphatics	0	1
Hepatobilary / Pancreatic	0	0
Renal / Genitoruinary	0	0
Constitutional Symptoms	2	3
Syndromes	1	1
Auditory/Ear	0	1
Ocular / Visual	0	1
Metabolic / Laboratory	1	2
Total Number of Adverse Events Experienced During Study (n)	35	58
Total Number of Subjects Experiencing Adverse Events: n (%)	16/26 (61.5%)	20/26 (76.9%)

Discussion

OA is the most common form of arthritis, and it is often associated with significant disability and an impaired quality of life. Clinical and radiographic surveys have found that the prevalence of OA increases with age from 1% in people <30 years to 10% in those <40 years to more than 50% in individuals >60 years of age (19). Although there are no curative therapies currently available for OA, individualized treatment programs are available to help relieve pain and stiffness, and to maintain and/or improve functional status.

In the last few years, various nutritional supplements including chondroitin, glucosamine, avocado/soybean unsaponifiables and diacerein have emerged as new treatment options for osteoarthritis (20). In this study, the efficacy of UC-II was studied in patients identified with moderate to severe OA. The objective of this study was to determine the effect of UC-II on disease specific measures and blood measures of OA of the knee compared to G+C. It was hypothesized that UC-II would reduce symptoms of OA of the knee to a greater extent than G+C.

A meta-analysis of 20 randomized control studies (2570 patients) comparing the effects of glucosamine (glucosamine sulphate, GS or glucosamine HCl, GH) vs. placebo was done. Of these only eight studies met the required controlled conditions for adequate

allocation concealment and received a quality score of 4 or higher (rated on the JADAD scale). These studies failed to show the benefit of glucosamine (GS or GH) for pain and WOMAC function. When all 20 studies were included in the meta-analysis, the results favored glucosamine with improvement in pain and functionality; however, the results were not uniformly positive and the parameters for WOMAC pain, daily function and stiffness did not reach statistical significance. Combinations of glucosamine and chondroitin have been studied in the "GAIT" study. These authors reported that glucosamine HCl and chondroitin sulphate alone or in combination did not reduce pain significantly in patients with OA of the knee. However in a subgroup of patients with moderate to severe knee pain the combination of compounds were found to be effective. Limitations to this study included a high rate of response to placebo (60.1%) and the fact that 78% of the participants were in the mild pain subgroup (21).

Previous studies have shown that UC-II is effective in the treatment of RA (8-11), and preliminary human (12) and animal (13-15) trials have shown it to be effective in treating OA. In obese-arthritic dogs given 4 mg or 40 mg per day UC-II for 90 days, significant declines in overall pain, pain during limb manipulation and lameness after physical exertion were noted (15). Greater improvement was observed with the 40 mg dose. No adverse effects or significant changes in serum chemistry (creatinine, blood urea nitrogen, alanine aminotransferase, and aspartate aminotransferase) were noted. Following UC-II withdrawal for a period of 30 days, all dogs experienced a relapse of overall pain, exercise-associated lameness and pain upon limb manipulation.

In a recent investigation, efficacy of UC-II was evaluated in arthritic horses (22). In this study, groups of horses were orally administered with a daily dose of placebo, UC-II at 320, 480 or 640 mg, or a combination of glucosamine (5.4 g) and chondroitin (1.8 g) for 150 days. Horses receiving placebo did not show any improvement in arthritic condition, while those receiving a daily dose of 320, 480 or 640 mg of UC-II exhibited significant reduction in arthritic pain. Although G+C treated group showed significant reduction in pain compared to baseline values, the efficacy was less as compared to that observed with UC-II treatment. In fact, UC-II at 480 or 640 mg/day was found to be more effective than G+C in treatment of arthritic pain in horses. Clinical conditions (body weight, body temperature, respiration rate, and pulse rate), and liver (bilirubin, GGT, and ALP) and kidney (BUN and creatinine) functions were not affected by UC-II treatment, suggesting that UC-II is well tolerated and does not cause any adverse effects (22).

In a preliminary trial of subjects with OA, taking a single oral daily dose of 40 mg UC-II on an empty stomach prior to bedtime for 42 consecutive days, an average of 26% reduction of pain was noted in four of five subjects in the study. No side effects were associated with treatment (12). The precise biochemical mechanism involved in UC-II induced pharmacological anti-arthritic effects in humans, dogs or horses is not clearly established. Type II collagen is the primary form of collagen contained in cartilage. Type II collagen extracts contain the amino acids found in the framework of human cartilage. In addition, these amino acids are required for the synthesis and repair of connective tissue throughout the body. These products reportedly aid in reducing the destruction of collagen within the body, may provide anti-inflammatory activity, and may improve joint flexibility (8-12).

The current study indicated that both treatments reduced the WOMAC scores, which measures the difficulty in physical function, stiffness and pain in the knee. However, treatment with UC-II was found to be more effective in reducing the WOMAC scores by 33% as compared to 14% in G+C treated groups after 90 days. Similar results were observed for VAS scores. Although both the treatments reduced the VAS score, UC-II was found to be more effective with 40% decrease after 90 days of treatment as compared to 15.4% in G+C treated groups. The Lequesne's functional index was used to determine the effect of different treatments on pain during daily activities. Treatment with UC-II reduced Lequesne's functional index by 20.1% as compared to 5.9 % in G+C treated groups. Thus, UC-II supplementation showed improvement in daily activities suggesting an improvement in overall quality of life in the patients receiving UC-II.

Acknowledgement

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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Undenatured type II collagen (UC-II®) for joint support: a randomized, double-blind, placebo-controlled study in healthy volunteers

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Abstract

Background: UC-II contains a patented form of undenatured type II collagen derived from chicken sternum. Previous preclinical and clinical studies support the safety and efficacy of UC-II in modulating joint discomfort in osteoarthritis and rheumatoid arthritis. The purpose of this study was to assess the efficacy and tolerability of UC-II in moderating joint function and joint pain due to strenuous exercise in healthy subjects.

Methods: This randomized, double-blind, placebo-controlled study was conducted in healthy subjects who had no prior history of arthritic disease or joint pain at rest but experienced joint discomfort with physical activity. Fifty-five subjects who reported knee pain after participating in a standardized stepmill performance test were randomized to receive placebo (n = 28) or the UC-II (40 mg daily, n = 27) product for 120 days. Joint function was assessed by changes in degree of knee flexion and knee extension as well as measuring the time to experiencing and recovering from joint pain following strenuous stepmill exertion.

Results: After 120 days of supplementation, subjects in the UC-II group exhibited a statistically significant improvement in average knee extension compared to placebo (81.0 ± 1.3° vs 74.0 ± 2.2°; p = 0.011) and to baseline (81.0 ± 1.3° vs 73.2 ± 1.9°; p = 0.002). The UC-II cohort also demonstrated a statistically significant change in average knee extension at day 90 (78.8 ± 1.9° vs 73.2 ± 1.9°; p = 0.045) versus baseline. No significant change in knee extension was observed in the placebo group at any time. It was also noted that the UC-II group exercised longer before experiencing any initial joint discomfort at day 120 (2.8 ± 0.5 min, p = 0.019), compared to baseline (1.4 ± 0.2 min). By contrast, no significant changes were seen in the placebo group. No product related adverse events were observed during the study. At study conclusion, five individuals in the UC-II cohort reported no pain during or after the stepmill protocol (p = 0.031, within visit) as compared to one subject in the placebo group.

Conclusions: Daily supplementation with 40 mg of UC-II was well tolerated and led to improved knee joint extension in healthy subjects. UC-II also demonstrated the potential to lengthen the period of pain free strenuous exertion and alleviate the joint pain that occasionally arises from such activities.

Keywords: UC-II, Undenatured type II collagen, Joint function, Knee extension, Stepmill, Joint pain

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Introduction

The impact of strenuous exercise on knee joints may cause localized pain and stiffness, which are hallmark features of pathologic inflammatory disease [1]. It has been shown that when dogs undergo a strenuous running regimen, significant losses in articular cartilage and glycosaminoglycans occur [2]. Such studies suggest that strenuous exercise may activate some of the same physiological processes that occur in arthritic disease [2-4]. In fact, *in vitro* studies have shown that many of the cytokines implicated in the onset and progression of both rheumatoid arthritis (RA) and osteoarthritis (OA) also appear to regulate the remodeling of the normal knee extracellular matrix (ECM) following strenuous exertion [5].

When normal chondrocytes undergo strenuous mechanical stimulation under static conditions, their physiology shifts towards ECM breakdown, as indicated by the upregulation of several metalloproteinases (MMPs), such as MMP-13 as well as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and various aggrecanases [5,6]. This *in vitro* catabolic response is mediated by changes in the phosphorylation, expression, or translocation of several transcription factors to the cell nucleus such as NF-κB, p38 MAPK, Akt, and ERK [7,8]. By contrast, normal chondrocytes produce the anti-inflammatory cytokine IL-4 when mechanically stimulated under moderate and dynamic conditions [9]. The secretion of this autocrine molecule not only helps in shifting chondrocyte metabolism towards the synthesis of aggrecan and type II collagen, it also downregulates production of nitric oxide (NO) and various MMPs and aggrecanases [10-12]. This conclusion is corroborated by the finding that pretreatment of strenuously compressed normal chondrocytes with IL-4 attenuates their catabolic response [11]. This suggests that IL-4 plays a key role in downregulating remodeling functions, restoring articular cartilage homeostasis, as well as decreasing chondrocyte apoptosis following strenuous mechanical loading [12,13].

Mechanically stressed chondrocytes also produce a number of other molecules known to participate in inflammatory responses, including prostoglandin E2, NO, and vascular endothelial growth factor [14]. These are proinflammatory molecules that, in conjunction with TNF-α, IL-6 and IL-1β, result in a localized, and transitory inflammatory-like response that is part of the normal repair process occurring in knee joints, serves to moderate remodeling events [3]. Ostrowski et al. [15] showed that healthy individuals express up to 27-fold greater concentrations of the anti-inflammatory cytokine IL-10 in blood following a marathon run when compared to IL-10 blood levels at rest. This finding is not surprising given that these same individuals also show marked increases in the proinflammatory cytokines TNF-α, IL-1β, and IL-6. It therefore appears that in healthy subjects undergoing strenuous exertion, the induction of proinflammatory cytokines is offset by the synthesis of anti-inflammatory agents as part of the recovery process. This view is supported by the observation that IL-10 reduces the catabolic impact of IL-1β and TNF-α on cartilage explants from healthy volunteers, and this effect is enhanced by combining IL-10 with IL-4 [13].

Another protein released by dynamically compressed chondrocytes is transforming growth factor (TGF)-β [16-18]. This factor is secreted by many cell types and is known to interfere with the cell cycle and arrest differentiation [19]. With regard to chondrocytes, TGF-β induces cell proliferation *in vitro* and slows terminal differentiation into hypertrophic cells [20]. Numerous studies have shown that TGF-β reverses the *in vitro* catabolic effect of various proinflammatory cytokines on normal chondrocytes as well as chondrocytes harvested from RA and OA donors [21-23].

The overall findings discussed above point to a new, unifying view of joint physiology. It suggests that many of the biological processes occurring in knee joints affected by RA and OA also participate in the maintenance of healthy knees [1,4,5]. It therefore seems appropriate to test the efficacy of natural supplements or ingredients, which have been shown to moderate joint pain in RA and OA, as possible candidates for treating the joint discomfort that occasionally results from strenuous exercise in healthy individuals.

UC-II is a natural ingredient which contains a glycosylated, undenatured type-II collagen [24]. Previous studies have shown that small doses of UC-II modulate joint health in both OA and RA [24-26]. Tong et al. [27], using an *in vivo* model of collagen induced arthritis (CIA), demonstrated that ingesting microgram quantities of undenatured type II collagen significantly reduces circulating levels of inflammatory cytokines, potentially serving to decrease both the incidence and the severity of arthritis [28]. The ability to alter immunity via the ingestion of a food, or an antigen, is called oral tolerance. This is an ongoing normal physiological process that protects the alimentary tract against untoward immunological damage [29,30]. Research into its mechanism of action has revealed that several distinct types of T regulator cells mediate this phenomenon by releasing IL-10 and TGF-β [30]. It has also been shown that this effect is transitory in nature requiring that the food, or antigen, be consumed continuously in order to maintain the tolerogenic state [30]. Given these findings, plus our current understanding of the role of various cytokines in normal joint physiology, it was hypothesized that supplementation with UC-II might relieve joint discomfort and restore joint function in healthy subjects.

The aim of this randomized, double blind, placebo-controlled study was to assess the impact of UC-II on knee function in otherwise healthy subjects with no prior history of arthritic disease who experienced knee pain upon strenuous physical exertion. The primary efficacy variable for assessing knee function included measurements of flexibility using range of motion (ROM) goniometry.

Methods

Investigational product

The investigational study product UC-II is derived from chicken sternum. It is manufactured using a patented, low-temperature process to preserve its native structure. For the clinical study, 40 mg of UC-II material (Lot 1109006), which provides 10.4 ± 1.3 mg of native type-II collagen, was encapsulated in an opaque capsule with excipients. Placebo was dispensed in an identical capsule containing only excipients (microcrystalline cellulose, magnesium stearate and silicon dioxide). Both study materials were prepared in a good manufacturing practice (GMP)-certified facility and provided by InterHealth Nutraceuticals, Inc. (Benicia, CA). Subjects were instructed to take one capsule daily with water before bedtime.

Recruitment of subjects

One hundred and six subjects were screened for eligibility using the inclusion–exclusion criteria defined in Table 1. Only healthy adults who presented with no knee joint pain at rest and no diagnosable markers indicative of active arthritic disease, as outlined by the American College of Rheumatology (ACR) guidelines [31,32], were admitted into the study. To accomplish this, all potential subjects were screened by a board certified clinician. Subjects presenting with any knee pain at rest and at least 3 of 6 clinical classification criteria, which included age greater than 50 years, morning stiffness in the joint lasting 30 minutes or less, crepitus on knee joint manipulation, body tenderness, bony enlargements, knee swelling or presence of excess fluid, and palpable warmth, were excluded. Potential subjects reporting the occasional use of NSAIDs, other pain relief medication, or anti-inflammatory supplements underwent a 2-week washout period before randomization.

Subjects were required to undergo a 10 minute period of performance testing using a standardized stepmill test developed and validated by Medicus Research (Udani JK, unpublished observation). It involved exercising at level 4 on a StepMill® model 7000PT (StairMaster® Health & Fitness Products, Inc., Kirkland, WA) until one or both knees achieved a discomfort level of 5 on an 11 point (0–10) Likert scale [33]. This pain threshold had to be achieved within a 10 minute period otherwise the subject was excluded. Once the requisite pain level was achieved the subject was asked to continue stepping for an additional two minutes in order to record the maximum pain level achieved before disembarking from the stepmill. The following knee discomfort measures were recorded from the start of the stepmill test: (1) time to onset of initial joint pain; (2) time to onset of maximum joint pain; (3) time to initial improvement in knee joint pain; (4) time to complete recovery from knee joint pain. Subjects who experienced a pain score of 5 (or greater) within one minute of starting the stress test were excluded. Out of 106 screened candidates, 55 subjects were enrolled in the study. Each subject voluntarily signed the IRB-approved informed consent form. After enrollment, the subjects were randomly assigned to either the placebo or the UC-II group.

Study design and trial site

This randomized, double blind, placebo-controlled study was conducted at the Staywell Research clinical site located in Northridge, CA. Medicus Research (Agoura Hills, CA) was the contract research organization (CRO) of record. The study protocol was approved by Copernicus Group IRB (Cary, NC) on April 25th 2012. The study followed the principles outlined in the Declaration of Helsinki (version 1996).

Randomization and blinding

Simple randomization was employed using a software algorithm based on the atmospheric noise method (www.random.org). Sequential assignment was used to determine group allocation. Once allocated, the assignment was documented and placed in individually numbered envelopes to maintain blinding. Subjects, clinical staff, plus data analysis and management staff remained blinded throughout the study.

Study schedule

The study duration was 17 weeks with a total of 7 visits that included screening, baseline, days 7, 30, 60, 90 and 120 (final visit). Table 2 summarizes the study visits and activities. Figure 1 depicts the sequence of study procedures that subjects underwent during each visit. All subjects completed a medical history questionnaire at baseline and compliance reports during follow-up evaluations at 7, 30, 60, 90 and 120 days. Subjects were assessed for anthropometric measures, vital signs, knee range of motion (flexion and extension), six-minute timed walk, as well as the onset and recovery from pain using the Udani Stepmill Procedure. A Fitbit (San Francisco, CA) device was used to measure daily distance walked, steps taken and an average step length for study participants. Subjects were also asked to complete the KOOS survey as well as the Stanford exercise scales.

Table 1 Inclusion–exclusion criteria

Inclusion

- Subject must be ≥30 and ≤65 years of age
- Body mass index (BMI) must be ≥18 and ≤35 kg/m^2
- Knee joint criteria: (1) no knee joint discomfort at rest; (2) must achieve a knee joint discomfort score of at least 5 on an 11-point Likert scale within 10 minutes of initiating the stepmill protocol
- Maintain existing food and physical activity patterns throughout the study period
- Judged by Investigator to be in general good health on the basis of medical history
- Subject understands the study procedures and provides signed informed consent to participate in the study and authorizes the release of relevant health information to the study investigator
- Females of child bearing age must agree to use approved birth control methods during the study

Exclusion

- Subjects with any indicators of arthritis, joint disorders, or history of immune system or autoimmune disorders
- Daily use of NSAIDs; however, daily use of 81 mg of aspirin for cardioprotection is allowed
- Daily use of anti-inflammatory or omega-3-fatty acid dietary supplements or using supplements to maintain joint health 30 days prior to screening
- Subjects with a history of knee or hip joint replacement surgery, or any hip or back pain which interferes with ambulation
- Use of any immunosuppressive drugs in the last 12 months (including steroids or biologics)
- Glucocorticoid injection or hyaluronic acid injection in affected knee within 3 months prior to enrollment
- History of surgery or significant injury to the target joint within 6 months prior to study enrollment, or an anticipated need for surgical or invasive procedure that will be performed during the study
- Subjects with a chronic pain syndrome and in the judgment of the Investigator is unlikely to respond to any therapy
- Participation in a clinical study with exposure to any non-registered drug product within 30 days prior
- Subjects who have any physical disability which could interfere with their ability to perform the functional performance measures included in this protocol
- Any significant GI condition that would potentially interfere with the evaluation of the study product
- Clinically significant renal, hepatic, endocrine (including diabetes mellitus), cardiac, pulmonary, pancreatic, neurologic, hematologic, or biliary disorder
- Subjects with vascular condition which interferes with ambulation
- Known allergy or sensitivity to herbal products, soy or eggs
- Vegetarian or Vegan
- History or presence of cancer in the prior two years, except for non-melanoma skin cancer.
- Individual has a condition the Investigator believes would interfere with his or her ability to provide informed consent, comply with the study protocol, which might confound the interpretation of the study results or put the person at undue risk
- Untreated or unstable hypothyroidism, an active eating disorder, or evidence of any neurological disorders
- Recent history of (within 12 months) or strong potential for alcohol or substance abuse
- Females who are pregnant, lactating, or unwilling to use adequate contraception during the study

Knee range of motion measurements

Knee extension was measured by goniometry. Briefly, subjects were instructed to sit in an upright position on a table edge with their backs straight (knee position defined as 90°). The axis of a goniometer was placed at the intersection of the thigh and shank at the knee joint. Subjects were asked to bring their knees to full extension without changing the position of the pelvis and lumbar spine. The extended knee joint angle was measured and recorded. For knee flexion measurement, subjects were asked to actively flex their knees while lying in a prone position with their shins off the end of the table. The range of knee flexion motion was then measured and documented.

Timed joint discomfort measurements

Briefly, a stopwatch was started when subjects began climbing the stepmill. Time to onset of pain was recorded at the first sign of pain in the target knee. The baselines at each time point were normalized to account for dropouts. Percent change in time to complete recovery from pain was measured as follows: a new stopwatch was started when the subjects disembarked from the stepmill and the time to complete recovery from pain was recorded. The baselines at each time point were normalized to account for dropouts then compared against the reference interval which was defined as the percentage change between the study baseline and day 7.

KOOS knee survey & Stanford exercise scales

The KOOS survey is a validated instrument consisting of 42 questions that are classified into sub-scales such as symptoms, stiffness, pain, daily activities, recreational activities and quality of life [34]. It measures the subjects' opinion about their knees and their ability to perform daily activities during the past week. The Stanford exercise behavior scale comprises 6 questions designed to assess exercise behaviors during the previous week [35].

Six minute timed walk

Subjects were instructed to walk up and down a hallway for 6 minutes as rapidly as possible without causing any pain. A measuring wheel (RoadRunner Wheel, Keson Industries, Aurora, IL) was used to measure distance travelled in 6 minutes.

Table 2 Protocol summary

Protocol activities	V1 Day −7 Screen	V2 Day 0 Baseline	V3 Day 7	V4 Day 30	V5 Day 60	V6 Day 90	V7 Day 120 End
Informed consent	x						
Inclusion/Exclusion	x						
Medical history and physical exam	x						
Vital signs/anthropometric measures	x	x	x	x	x	x	x
Urine pregnancy test	x	x					
Administer and review scales/questionnaires/diaries	x	x	x	x	x	x	x
Stressor (Udani Stepmill protocol)	x	x	x	x	x	x	x
Functional measures (6-min timed walk)	x	x	x	x	x	x	x
Goniometry (range of motion)		x	x	x	x	x	x
Review concomitant therapies	x	x	x	x	x	x	x
Intercurrent medical issues review		x	x	x	x	x	x
Compliance assessment (including phone calls)		x	x	x	x	x	x
Randomization		x					
Study supplement preparation & dispensing		x	x	x	x	x	

Rescue medication

No rescue medications were allowed during the course of the study. At all study visits, subjects were given a list of the 43 prohibited medications and supplements (Table 3). Changes in overall medication history, or the use of these substances, were then recorded by the study coordinator. Subjects found to have used any of these prohibited substances were excluded from further participation in the study as per protocol.

Statistics

Outcome variables were assessed for conformance to the normal distribution and transformed as required. Within group significance was analyzed by non-parametric Sign

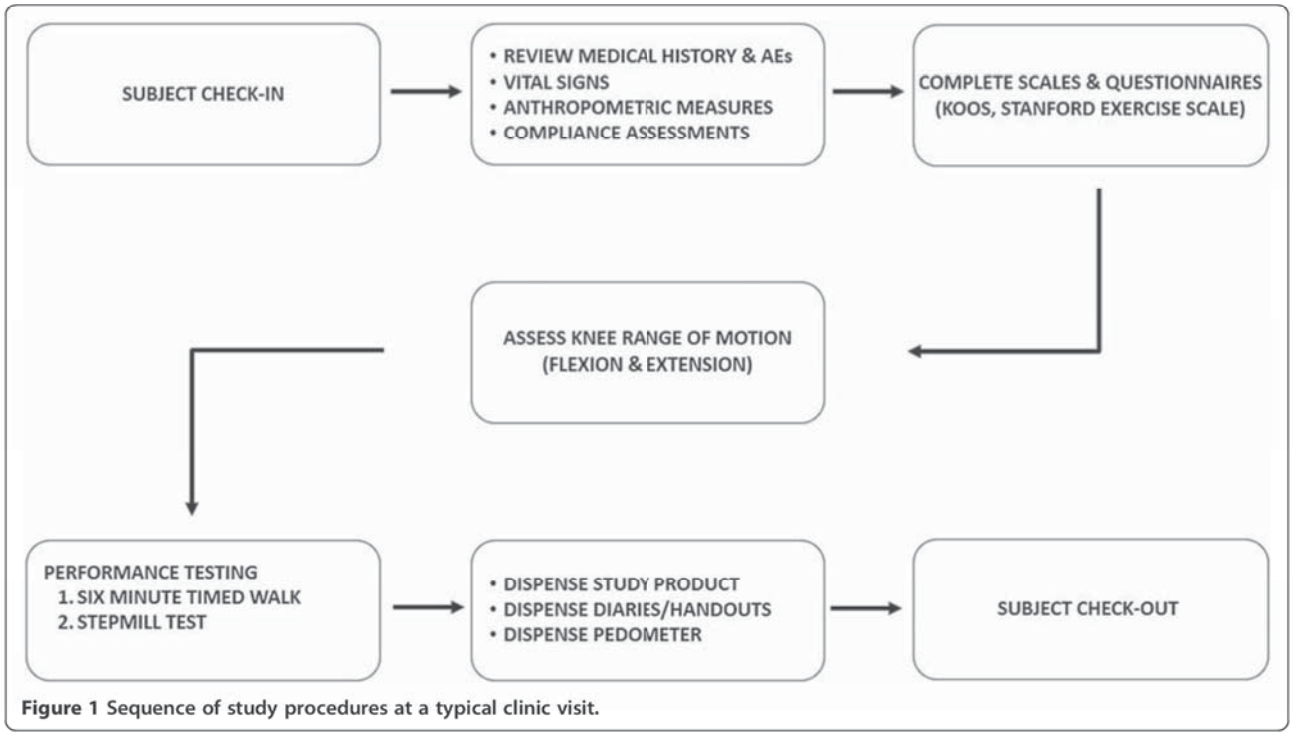

Figure 1 Sequence of study procedures at a typical clinic visit.

Table 3 Representative list of prohibited medications* by category

Category	Medications
Joint supplements (Omega-3, Omega-6 plus others)	Alpha-Linolenic acid
	Docosapentaenoic acid
	Docosahexaenoic acid
	Eicosatrienoic acid
	Eicosatetraenoic acid
	Eicosapentaenoic acid
	Hexadecatrienoic acid
	Heneicosapentaenoic acid
	Stearidonic acid
	Tetracosapentaenoic acid
	Tetracosahexaenoic acid
	Glucosamine (all forms)
	Chondroitin (all forms)
	Other herbal ingredients
NSAIDs (OTC and prescription)	Aspirin
	Diflunisal
	Diclofenac
	Celecoxib
	Etodolac
	Fenoprofen
	Flurbiprofen
	Ibuprofen
	Indomethacin
	Ketoprofen
	Meclofenamate
	Mefenamic acid
	Meloxicam
	Nabumetone
	Naproxen
	Oxaprozin
	Piroxicam
	Rofecoxib
	Sulindac
	Tolmetin
	Valdecoxib

*Selected from a list of 43 prohibited medications and supplements.

test or by non-parametric Wilcoxon Signed Rank test, while Wilcoxon Mann–Whitney test was used to analyze between groups significance. The Fisher Exact test was used to evaluate the complete loss of pain between study cohorts whereas the binomial test was used to assess the likelihood of complete loss of pain at each visit. P-values equal to or less than 0.05 were considered statistically significant. All analyses were done on a per protocol basis using SPSS, v19 (IBM, Armonk, NY). Results were presented as mean ± SEM.

Results

Baseline demographics

A total of 55 individuals met the eligibility criteria and were randomized to the placebo (n = 28) or to the UC-II (n = 27) group. Baseline demographic characteristics for subjects in both groups were similar with respect to age, gender, height, weight and BMI (Table 4). A total of nine subjects, three in UC-II group and six in placebo group, were lost to follow-up. The results presented herein encompass 46 total subjects, 22 subjects in the placebo group plus 24 subjects in the UC-II group. It should be noted that the average age of the study participants was approximately 46 years which is about 16 years younger than the average age observed in many OA studies [36-38].

Knee extension and flexion

Figure 2 summarizes the average knee extension changes over time for subjects supplemented with either UC-II or placebo. The UC-II supplemented cohort presented with a statistically significant greater increase in the ability to extend the knee at day 120 as compared to the placebo group (81.0 ± 1.3^{o} vs 74.0 ± 2.2^{o}, $p = 0.011$) and to baseline (81.0 ± 1.3^{o} vs 73.2 ± 1.9^{o}, $p = 0.002$). The UC-II group also demonstrated a significant increase in knee extension at day 90 (78.8 ± 1.9^{o} vs 73.2 ± 1.9^{o}, $p = 0.045$) compared to baseline only. An intent to treat (ITT) analysis of these data also demonstrated a statistically significant net increase in knee extension at day 120 versus placebo (80.0 ± 1.3^{o} vs 73.7 ± 1.8^{o}, $p = 0.006$). No statistically significant changes were observed in the placebo group at any time during this study. With respect to knee flexion, no significant changes were noted in either study group ($p > 0.05$). The power associated with the former per protocol statistical analyses was 80%.

Time to onset of initial joint pain

Supplementation with UC-II resulted in statistically significant increases in the time to onset of initial joint pain at day 90 (2.75 ± 0.5 min, $p = 0.041$) and at day 120 (2.8 ± 0.5 min, $p = 0.019$) versus a baseline of 1.4 min for each visit. No statistically significant differences were noted for either the placebo group or between groups (Figure 3).

Five individuals in the UC-II group and one in the placebo group reported no onset of pain by the end of study (see below and Table 5). Given this unexpected finding, an additional analysis was undertaken which included these individuals in the time to onset of initial pain analysis. The 10 minute limit of the stepmill procedure was used as the lower limit to pain onset. Under

Table 4 Demographic and baseline characteristics of enrolled subjects

Characteristics	UC-II	Placebo
Total number of subjects	27	28
Number of males	11	12
Number of females	16	16
Age (years)	46.1 ± 1.5	46.6 ± 1.8
Weight (kg)	75.5 ± 2.9	77.5 ± 3.1
Height (cm)	167.1 ± 2.0	168.4 ± 2.0
BMI (kg/m^2)	26.8 ± 0.8	27.1 ± 0.7

Values are expressed as Mean ± SEM.

these conservative assumptions, supplementation with UC-II yielded statistically significant increases in time to onset of pain at day 90 (3.65 ± 0.7 min, p = 0.011) and day 120 (4.31 ± 0.7 min, p = 0.002) versus a baseline of 1.4 min for each visit. The between-group comparison at day 120 approached the statistical level of significance favoring the UC-II cohort (p = 0.051).

Time to onset of maximum joint pain

A statistically significant difference between groups was noted at day 60 (6.39 ± 0.5 min vs 4.78 ± 0.5 min; p = 0.025) favoring the UC-II cohort. This significance did not persist during the remainder of the study suggesting that this was a random occurrence.

Time to initial improvement in knee joint pain

The time to offset of joint pain was recorded immediately upon the subject stepping off the stepmill. Both groups began to recover from pain with the same rate resulting in no significant differences between groups in the time to initial offset of joint pain (p > 0.05).

Time to complete recovery from knee joint pain

The time to complete recovery from joint pain showed significant reductions at days 60, 90 and 120 compared to baseline for both the UC-II group as well as the placebo group (Figure 4). Percent changes in times were calculated after normalizing the baselines against the reference range of baseline to day 7. The UC-II group exhibited average reductions of 31.9 ± 11.7% (p = 0.041), 51.1 ± 6.1% (p = 0.004) and 51.9 ± 6.0% (p = 0.011) at days 60, 90 and 120, respectively. By contrast, the reductions for the same time points for the placebo cohort, 21.9 ± 10.2% (p = 0.017), 22.2 ± 15.5% (p = 0.007) and 30.0 ± 11.8% (p = 0.012), were of lower magnitude but nonetheless statistically significant versus baseline. None of these between group differences achieved statistical significance.

Time to complete loss of knee joint pain

During the course of this study it was noted that a number of subjects in both the placebo and the supplemented cohorts no longer reported any pain during the stepmill protocol. For the UC-II group, 5 subjects (21%) no longer reported pain by day 120, whereas only 1 subject (5%) in placebo group reported complete loss of pain (Table 5). This effect did not reach statistical significance between groups but there was an evident trend in the data towards a greater number of subjects losing pain in the UC-II cohort (p = 0.126). A binomial analysis for complete loss of pain at each visit demonstrated a statistical significance for the UC-II group by day 120 (p = 0.031). It is important to note that the complete loss of knee pain was not a random event. The pattern among the subjects indicates that loss of knee pain appeared to be a persistent phenomenon that spanned multiple visits (Table 5). A detailed review of the clinical report forms showed that none of these individuals consumed pain relief medication prior to their visits.

Six-minute timed walk & Daily number of steps

No significant differences were observed between the study groups for the six-minute time walk or the daily number of steps taken (p > 0.05). The distance walked in six-minutes by the UC-II (range = 505 to 522 meters) and the placebo (range = 461 to 502 meters) groups were within the reference range previously reported [39] for healthy adults (399 to 778 meters, males; 310 to 664 meters, females). Similarly, the average step length calculated from Fitbit data for both study groups (0.69 to 0.71 meters) also agreed with previously published results for normal adults [40].

KOOS knee survey & Stanford exercise scales

No significant differences were seen between the study groups for either the KOOS survey or the Stanford exercise scale (p > 0.05).

Use of analgesics and NSAIDs

Review of the clinical report forms showed that no subject in either study cohort consumed any of the 43 prohibited medicines or supplements during the study.

Safety assessments

A total of eight adverse events, equally dispersed between both groups, were noted (Table 6). None of the adverse events was considered to be associated with UC-II supplementation. All events resolved spontaneously without the need for further intervention. No subject withdrew from the study due to an adverse event. Finally, no differences were observed in vital signs after

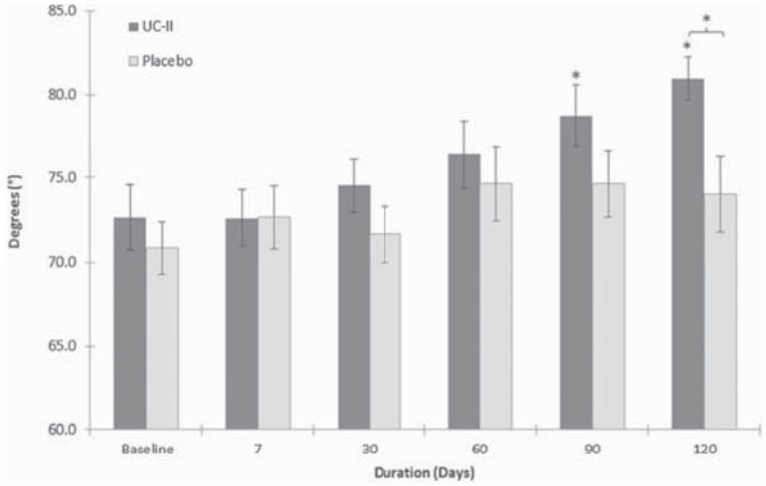

Figure 2 Knee extension as measured by goniometry. Values are presented as Mean ± SEM. *p ≤ 0.05 indicates a statistically significant difference versus baseline or placebo. Number of completers: n = 24 in UC-II group (n = 3 dropouts); n = 20 in placebo group (n = 6 dropouts; n = 2 did not participate in ROM assessment).

seventeen weeks of supplementation, and no serious adverse events were reported in this study.

Discussion

In this study, the UC-II supplement, consisting of undenatured type II collagen, was investigated for its ability to improve joint function in healthy subjects who develop joint pain while undergoing strenuous exercise. The rationale behind this approach centered on the hypothesis that strenuous exercise might uncover transient joint changes due to daily physical activities that are not attributable to a diagnosable disease. In the same way that nominally elevated blood levels of lipids, glucose plus high blood pressure and obesity can be predictive of future progression to diabetes and heart disease [41], the development of joint pain upon strenuous exercise may be indicative of possible future joint problems.

At study conclusion, we found that subjects ingesting the UC-II supplement experienced a significantly greater forward ROM in their knees versus baseline and placebo

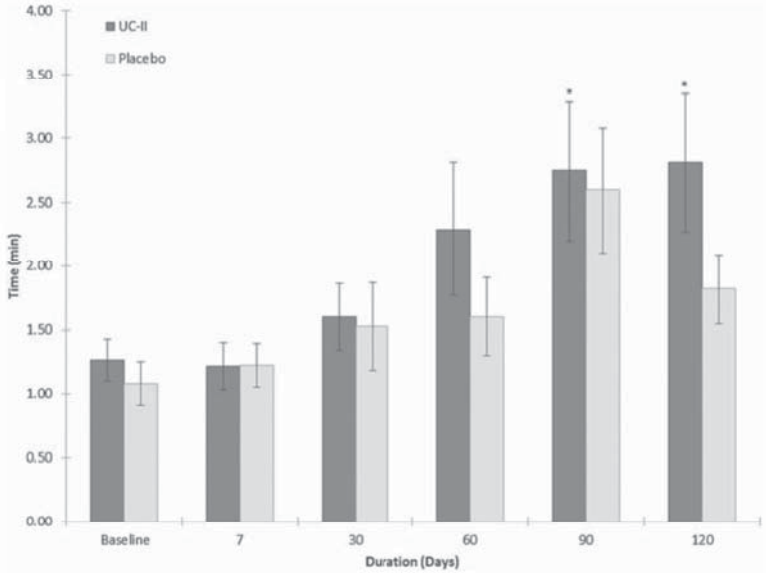

Figure 3 Impact of stepmill procedure on the onset of pain. Values are presented as Mean ± SEM. *p ≤ 0.05 indicates a statistically significant difference from baseline. Number of completers: n = 19 in UC-II group (n = 3 dropouts; n = 5 did not have pain); n = 20 in placebo group (n = 6 dropouts; n = 1 did not have pain; n = 1 did not use stepmill).

Table 5 Subjects reporting complete loss of knee pain on stepmill test

Visit	UC-II			Placebo		
	No. of pain free subjects (%)	Continuity of pain loss[#]	P value (Binomial test)	No. of pain free subjects (%)	Continuity of pain loss[#]	P value (Binomial test)
Baseline	0.0 (0)	0	NA	0.0 (0)	0	NA
Day 7	0.0 (0)	0	NA	0.0 (0)	0	NA
Day 30	1.0 (4)	1N	0.5	0.0 (0)	0	NA
Day 60	3.0 (13)	1R, 2N	0.125	0.0 (0)	0	NA
Day 90	3.0 (13)	2R, 1N	0.125	1 (5)	1N	0.5
Day 120	5.0 (21)	3R, 2N	0.031[†]	1 (5)	1R	0.5

Values denote number of subjects while parenthesis provides the percent of total subjects who did not have any pain on stepmill. Continuity indicates the number of subjects in whom the absence of pain was maintained across visits. [†]Significant at $p \leq 0.05$ based on independent binomial testing of each visit using the null hypothesis that the probability of a subject experiencing no joint pain is equal to zero. There was no statistical difference between groups. [#]*R* Repeat subject (i.e. same subject who reported no pain in previous visit), *N* New subject who reports no pain for the first time.

as measured by knee extension goniometry. Knee extension is necessary for daily function and sport activities. Loss of knee extension has been shown to negatively impact the function of the lower extremity [42,43]. For example, loss of knee extension can cause altered gait patterns affecting ankles and the hip which could result in difficulty with running and jumping [42,43]. Studies have further shown that a permanent loss of 3-5° of extension can significantly impact patient satisfaction and the development of early arthritis [44].

By contrast, when knee flexion, another measure of knee function, was assessed via goniometry, no differences in clinical outcomes were observed between the two study cohorts. From a structure-function perspective this outcome is not surprising. During the earliest characterized phases of OA there is an apparent preferential loss of knee extension over knee flexion, and this loss has been shown to correlate with WOMAC pain scores [45,46]. In addition, MRI imaging of the early osteoarthritic knee has shown that initial changes in knee structure appear to center on articular cartilage erosions (fibrillations) about the patella and other weight bearing regions of the knee [47]. Such changes might favor a loss in knee ROM that preferentially affects extension over flexion. The pathophysiology of the early osteoarthritic knee, we believe, provides insight regarding the effect of daily physical activities on the healthy knee insofar as it helps explain the discordance in clinical outcomes between knee extension and flexion.

Both the time to onset of initial joint pain as well as time to full recovery were measured in this study. For

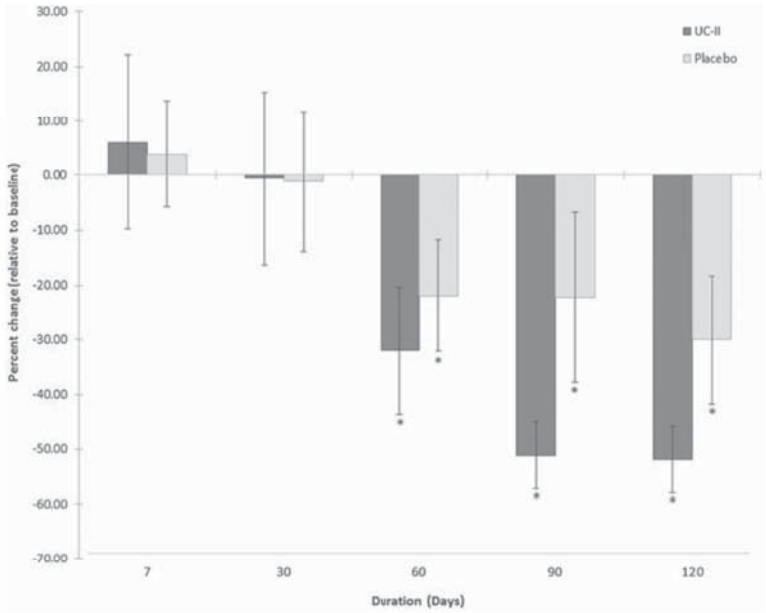

Figure 4 Percent change in time to complete recovery from pain. Values are presented as Mean ± SEM. *$p \leq 0.05$ indicates a statistically significant difference from baseline. Number of completers: n = 18 in UC-II group (n = 3 dropouts; n = 5 did not have pain; n = 1 time to complete recovery from pain was not achieved); n = 20 in placebo group (n = 6 dropouts; n = 1 did not have pain; n = 1 did not use stepmill).

Table 6 Summary of analysis of adverse events (AEs) in all subjects

Study groups	Adverse event (Body system)	Number of AEs
UC-II	Upper respiratory infection (Pulmonary)	3
	Food poisoning (Gastrointestinal)	1
Total number of AEs		**4**
Total number of subjects reporting AEs: n		**4/27**
Placebo	Bilateral ankle edema (Musculoskeletal)	1
	Right ankle fracture (Musculoskeletal)	1
	Sinusitis (Ears/Nose/Throat)	1
	Skin infection right ankle (Dermatological)	1
Total number of AEs		**4**
Total number of subjects reporting AEs: n		**2/28**

each of these measures the clinical outcomes favored the UC-II supplemented cohort versus their baseline status. The ability of UC-II to modulate knee extension may relate to its ability to moderate knee joint pain. Crowley et al. [26] and Trentham et al. [25] demonstrated that UC-II effectively enhances joint comfort and flexibility thereby improving the quality of life (QoL) in both OA and RA subjects, respectively. This effect may be attributable to the finding that microgram quantities of undenatured type II collagen moderate CIA in both the rat and the mouse via the induction of T regulator cells [27,28,48]. The induction of these T regulators takes place within gut associated lymphatic tissues (GALT), including mesenteric lymph nodes, in response to the consumption of undenatured type II collagen [27]. Studies have shown that these regulatory cells produce IL-10 and TGF-β [30,49]. A special class of $CD103^+$ dendritic cells, found almost exclusively in the GALT, facilitates this process [48,50]. Once activated, T regulator cells appear to downregulate a wide range of immunologic and proinflammatory activities resulting in the moderation of the arthritic response initiated by undenatured type II collagen [27]. The phenomenon of oral tolerance has also been demonstrated in humans, and appears to involve a similar set of T regulators [30,51-53].

The above description of how UC-II might modulate joint function is most easily understood in the context of RA given that the CIA animal model resembles this disease most closely [27,28,54]. However, the case for T regulators and immune cytokines having a moderating effect on healthy or OA knee joint function appears less apparent. This view has changed in recent years due to a growing body of evidence suggesting that both OA and normal chondrocyte biology appears to be regulated by some of the same cytokines and chemokines that regulate inflammation [5,6,55]. For example, Mannelli and coworkers [56] recently reported that feeding microgram amounts of native type II collagen (porcine) prevents monoiodoacetate-induced articular cartilage damage in this rat model of osteoarthritis, as measured by pain thresholds and by circulating levels of cross linked c-telopeptides derived from type II collagen. This finding corroborates the efficacy of undenatured type II collagen in improving joint comfort in osteoarthritic conditions [26].

In the present study, we show for the first time that UC-II can improve joint function in healthy subjects undergoing strenuous physical exercise. This observation, when considered in context with normal chondrocyte physiology, suggests that activated T regulator cells, specific for undenatured type II collagen, home to an overstressed knee joint where their release of the anti-inflammatory cytokines, IL-10 and TGF-β reverse the catabolic changes caused by strenuous exertion [13,21,57]. In addition, the IL-10 and TGF-β produced by these T regulators may tilt the T_H balance in the knee joint towards T_H2 [30,58] responses which preferentially result in IL-4 production further fostering a shift in chondrocyte metabolism towards ECM replenishment.

Several additional tests were used in this study to assess overall joint function, QoL, and physical activity. The additional parameters and tests measured included a six minute timed walk plus the Stanford exercise scale and KOOS survey. With respect to the KOOS survey, both cohorts were statistically significant versus baseline for symptoms, pain, daily function, recreational activities and QoL but were not significant from each other. This is not an unexpected finding given that this study was carried out with healthy subjects who do not present with any joint issues at rest. It is only when the knee is stressed via the stepmill do subjects report any joint discomfort. Under these conditions, and as indicated above, the UC-II group appears to experience less joint discomfort and greater joint flexibility. No difference in clinical outcomes between groups was seen in the six minute timed walk, the daily distance walked, or the Stanford exercise scale questionnaire. Once again we are not surprised by these results given that these tests and questionnaires are designed and clinically validated to assess the severity of arthritic disease in unhealthy populations.

No clinical biomarkers associated with arthritic diseases were assessed in this study. Healthy subjects would not be expected to present with significant alterations in their inflammatory biomarker profile as they lack clinical disease [59]. In addition, it should be noted that the joint discomfort measured in this study is acute pain induced by a stressor rather than due to an ongoing inflammatory event. Therefore, any elevation in inflammation markers that might occur in these healthy subjects may simply be due to the physiological impact of strenuous exercise.

There are two study limitations to consider when reviewing these results. The first, time to onset of initial pain, was limited to a 10-minute interval. The current study design did not address the possibility that subjects might cease to experience pain on the stepmill. Future studies should allow for an extension of the exertion interval in order to gauge how much longer a subject can exercise before reporting pain. In this way better defined parameters can be placed upon the degree to which UC-II supplementation results in the cessation of joint pain due to strenuous exercise in healthy subjects.

The second limitation that merits consideration is the possibility that study subjects may have early signs of arthritis that do not meet the ACR criteria. This possible limitation was addressed by performing an extensive medical examination for signs and symptoms of OA and by excluding volunteers who experienced pain levels of 5 or greater within one minute of using the stepmill.

UC-II is a unique ingredient that supports healthy joints. Previous studies have focused on the efficacy of this ingredient in OA subjects. By including healthy subjects in this study, and using non-disease endpoints as a measure of efficacy, it is believed that the benefits that derive from UC-II usage now extends to include healthy individuals. Further, this ingredient appears to be safe for human consumption based on an extensive series of *in vivo* and *in vitro* toxicological studies as well as the absence of any adverse events in this and in previous human studies [24,26,60]. In conclusion, daily supplementation with 40 mg of UC-II supports joint function and flexibility in healthy subjects as demonstrated by greater knee extension and has the potential both to alleviate the joint pain that occasionally arises from strenuous exercise as well as to lengthen periods of pain free exertion.

Abbreviations

RA: Rheumatoid arthritis; OA: Osteoarthritis; ECM: Extracellular matrix; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; IL-4: Interleukin 4; IL-10: Interleukin-10; MMP: Matrix metalloproteinase; NF-κB: Nuclear factor-kappa-light-chain-enhancer of activated B cells; MAPK: Mitogen activated protein kinase; ERK: Extracellular receptor kinase; NO: Nitric oxide; TGF-β: Transforming growth factor-beta; CIA: Collagen induced arthritis; KOOS: Knee injury and osteoarthritis outcome score; ROM: Range of motion; MRI: Magnetic resonance imaging; GALT: Gut associated lymphatic tissue; QoL: Quality of life; MIP-1β: Macrophage inflammatory protein-1 beta; IP-10: Interferon gamma-induced protein 10; T_H: T helper cell; WOMAC: Western Ontario and McMaster universities osteoarthritis index; ACR: American College of Rheumatology.

Competing interests

Medicus Research received research grants from InterHealth Nutraceuticals, Inc., Benicia, California. Dr. Udani has provided consulting services to InterHealth Nutraceuticals, Inc. Drs. JPL, ZMS, and FCL are employees of InterHealth Nutraceuticals, Inc. Medicus Research does not endorse any brand or product nor does it have any financial interests with any supplement manufacturer or distributor.

Authors' contributions

JKU was the principal investigator and together with JPL, JKU, ZMS, FCL JPM, MNP and ANS contributed to the writing, data analyses and data interpretation that are a part of this manuscript. All the authors read and approved the final draft of the manuscript.

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EFFECTS OF ORALLY ADMINISTERED UNDENATURED TYPE II COLLAGEN AGAINST ARTHRITIC INFLAMMATORY DISEASES: A MECHANISTIC EXPLORATION

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Summary: *Arthritis afflicts approximately 43 million Americans or approximately 16.6% of the US population. The two most common and best known types of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA). A significant amount of scientific research has been done in attempts to explain what initiates forms of arthritis, how it is promoted and perpetuated and how to effectively intervene in the disease process and promote cartilage remodeling. Current pharmacological strategies mainly address immune suppression and antiinflammatory mechanisms and have had limited success. Recent research provides evidence that alterations in the three-dimensional configuration of glycoproteins are responsible for the recognition/response signaling that catalyzes T-cell attack. Oral administration of autoantigens has been shown to suppress a variety of experimentally induced autoimmune pathologies, including antigen-induced RA. The interaction between gut-associated lymphoid tissue in the duodenum and epitopes of orally administered undenatured type II collagen facilitates oral tolerance to the antigen and stems systemic T-cell attack on joint cartilage. Previous studies have shown that small doses of orally administered undenatured type II chicken collagen effectively deactivate killer T-cell attack. A novel glycosylated undenatured type II collagen material (UC-II) was developed to preserve biological activity. The presence of active epitopes in the UC-II collagen is confirmed by an enzyme-linked immunosorbent assay test and distinguishes this form from hydrolyzed or denatured collagen. Oral intake of small amounts of glycosylated UC-II presents active epitopes, with the correct three-dimensional structures, to Peyer's patches, which influences the signaling required for the development of immune tolerance. UC-II has demonstrated*

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the ability to induce tolerance, effectively reducing joint pain and swelling in RA subjects. A pilot study was conducted for 42 days to evaluate the efficacy of UC-II (10 mg/day) in five female subjects (58-78 years) suffering from significant joint pain. Significant pain reduction including morning stiffness, stiffness following periods of rest, pain that worsens with use of the affected joint and loss of joint range of motion and function was observed. Thus, UC-II may serve as a novel therapeutic tool in joint inflammatory conditions and symptoms of OA and RA.

Introduction

Arthritis represents a group of debilitating diseases of the joints, bones, tendons, muscles and eventually organs. It afflicts approximately 43 million Americans, imposing a cost in excess of $65 billion annually (1). The two most common types are osteoarthritis (OA) and rheumatoid arthritis (RA), traditionally defined as age-related "wear-and-tear" arthritis and "autoimmune" arthritis, respectively (1, 2). However, inflammatory response has been identified to be a common mediator in both types of arthritis (1, 2).

Understanding of RA pathogenesis has changed over the years. RA is characterized by attack of killer T-cells on type II joint collagen, which results in damage to cartilage, joint swelling, pain and inflammation (3-6). The body's attempts to remodel joint cartilage are outpaced by immune mediated attack on and degradation of joint cartilage (3-6). Collectively, these events have been characterized as an out-of-control autoimmune response (3). Extensive research has explored the multifaceted dynamics of recognition, response and compensatory homeostatic mechanisms in an effort to understand, manage and maintain immune competence. Research in transgenic mice points to the possibility that B-lymphocytes and immunoglobulins outside the joint indirectly provoke RA pathogenesis *via* a self-reactive T-cell receptor in the joint (7). However, our understanding of autoimmunity still presents unresolved challenges that may require a paradigm shift in research for the development of effective and safe therapies.

It has been proposed that mechanisms involved in host defense, protection and maintenance of self-integrity are counteracting forces in which tolerance mechanisms efficiently suppress immune attack on self to a required threshold. An evolutionary perspective alleges that a tendency toward autoimmune malfunction should theoretically be higher during years when young immune systems are aggressively protecting the reproductive potential of the host. Misrecognition of self would be a predictable deficiency of the system (8). Autoimmune disorders are less prevalent in the young, increasing with advancing age and decline of reproductive potential. Indeed, there is a clear relationship between advancing age and an increased incidence of arthritis. To explain this, one rationale theorizes that RA disease must result from a deteriorating function of the immune system, which provides ideal conditions for a breakdown in self-tolerance (8). Decreased recognition and up-regulated self-attack is a logical conclusion consistent with an age-related decline in immune efficiency (9). However, explanations regarding "autoreactivity" of the immune system in RA disease favor an emphasis on functional flaws in surveillance, recognition and response (and their symptomatic manifestations) rather than the possibility that structural flaws in immune system complexes, and possi-

bly the target tissues, may be etiological catalysts. Recent strategies for therapeutic management of RA, therefore, focus on methods of inhibiting symptom manifestation to reduce the severity of the end-stage of this disease (10).

Etiological and therapeutic research faces the challenge of explaining how arthritic processes originate and progress (2). Most of the past and current work on rheumatoid diseases examine strategies to intervene or halt "out of control" immunologic and/or inflammatory events associated with autoimmune disease (10). The traditional paradigm proposes that RA is an immunological disorder for an as yet unidentified arthrogenic antigen. Various immunological factors are involved, such as CD4-inducer lymphocytes, CD4 cells, macrophages, neutrophils and tumor necrosis factor- (9, 10). This conventional view has procuded pharmacological therapies that favor manipulation of cyclooxygenase-2 events and immune suppression, with less than ideal results. Almost all of the biomolecules responsible for innate and adaptive immune response are glycoproteins (11). However, little attention is directed at the possibility that impaired glycosylation affects the configuration of glycoproteins, including IgG and type II collagen. These may alter recognition and response signaling during immune surveillance, inciting attack on the body's own joint collagen (11-19).

This view suggests that the term hyperreactive "immune abnormality" may be a misnomer for RA, as the immune system is behaving appropriately against host tissues ultimately identified as foreign pathogenic antigens (10). Altered glycosylation could produce a number of identification errors responsible for up-regulating self-attack. Among the possibilities are misidentification of type II joint collagen as antigenic by aberrant IgG, possible binding of hypogalactosylated IgG with certain rheumatoid factors leading to significant levels of immune complexes characteristic of RA and/or appropriate glycomic identification markers may be missing from the joint collagen and immune complexes. This perspective provides insight into how the immune system incurs a loss of self-tolerance and explores the possibility of flaws in glycosylation/galactosylation. This phenomenon is at the root of impaired immunological recognition and response activities for the hyper-autoreactive immune self-destruction of joint collagen in the pathogenesis of RA (19, 20). Hence, alterations in glycosylation/ galactosylation are hallmark characteristics of RA. This also provides a possible explanation as to who orally ingested native type II collagen produces tolerance, down-regulating autoimmune aggression (3, 4).

Impaired galactosylation affects glycoprotein synthesis, altering the requisite three-dimensional conformations of glycoproteins such as type II collagen and IgG, producing the loss of self-recognition. Lang and Yeaman (20) demonstrated that removal of carbohydrate moieties from antigens resulted in a loss of antibody binding. In RA patients, decreased levels of β1-4 galactosyltransferase activity in peripheral blood B- and T-lymphocytes correlates with the decreased galactosylation of serum IgG (13).

Immunoglobulins are by definition glycoprotein molecules produced by plasma cells in response to an immunogen, which function as antibodies (11). In RA, immune complexes that consist exclusively of immunoglobulin are present, indicating a role as both antibody and antigen. Both cartilage and immune system complexes are, for the most part, made of glycoprotein structures in which glycoprotein synthesis requires the necessary substrate and competent glycosylation (16). Impaired glycosylation/galactosylation intersects at a number of junctures contributing to the initiation, promotion and progression stages of RA (11-19).

Comparisons of the *N*-glycosylated pattern of serum IgG isolated from healthy individuals with that of RA patients demonstrates that differences ob-

served in RA patients are due to changes in the relative extent of glycosylation compared with normal individuals. In RA, an increased number of oligosaccharide structures lack the terminal galactose residue (19). This suggests that RA may be a glycosylation disease, reflecting changes in the intracellular processing, or post-secretory degradation of *N*-linked oligosaccharides (12, 19). Other research has reported a decrease in galactose residues in the oligosaccharide chains of the serum IgG of RA patients, which was presumed to affect the three-dimensional structure of the CH_2 domain. Galactose- depleted IgG reduced Clq binding and Fc receptor binding, which implies an important biological function of the glyconutrient moiety of IgG (16). Rademacher *et al.* (17) clearly demonstrated that galactose-deficient IgG glycoforms are directly associated with pathogenicity in collagen-induced rheumatoid arthritis in mice. Nonpathogenic autoantibodies were made pathogenic by altering their glycosylation state (17).

Immunization with undenatured type II collagen (antigen) has been shown to induce arthritis (21). However, orally ingested undenatured native antigens interact with gut-associated lymph tissue (GALT), resulting in an entirely opposite effect. Oral tolerization, using small doses of glycosylated undenatured type II collagen (UC-II), has demonstrated its effectiveness in turning off T-cell attack on type II joint collagen, inducing immunological hyporesponsiveness, and reducing pain and inflammation (3-6). In contrast, while denatured collagen may provide a nutritional source of substrate for joint cartilage synthesis, research demonstrates that it does not induce immunological hyporesponsiveness and has not demonstrated an effect on reducing pain and inflammation (6). Although the same amino acids are present in both forms, the tertiary and quaternary structures in the denatured form may be completely destroyed and the galactose moiety is degraded (Fig. 1), not allowing epitope recognition in the Peyer's patch (3, 10, 22). Furthermore, the hydrolyzed or denatured form may be pharmacologically ineffective because of the loss of conformation. Interestingly, the effects of oral tolerance do not appear to be confined to RA diseases alone, but confer appreciable benefits in some cases of OA as well. A pilot

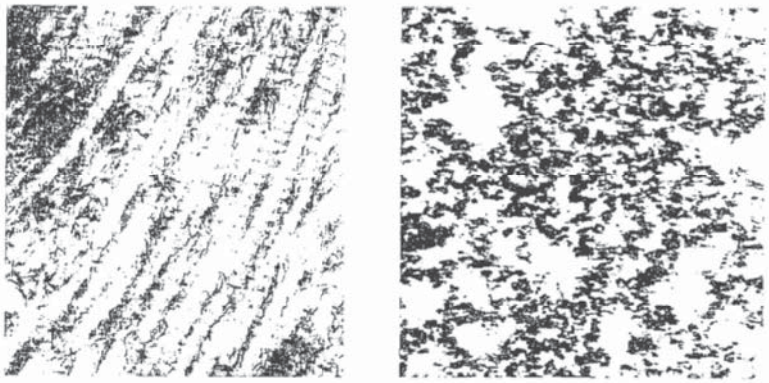

Fig. 1 Electron micrograph (magnification x 50.000) of undenatured type II collagen vs. denatured type II collagen. Undenatured type II collagen (on left) shows intact tertiary and quaternary glycoprotein integrity allowing for epitope recognition and hyporesponsive immune stimulation. Denatured type II collagen (on right) contains no tertiary and quaternary glycoprotein integrity.

study provides preliminary evidence that 10 mg/day of a commercial enzyme-linked immunosorbent assay (ELISA) verified undenatured glycosylated type II collagen (UC-II InterHealth Nutraceuticals Incorporated, Benicia, CA, USA) administered orally reduced sensory pain by 26% in four out of five women, aged 58-78 years old, for 42 days. Two of the women were previously diagnosed with OA and the remaining three exhibited similar symptoms but had no clinical diagnosis. There were no adverse effects associated with the intake of UC-II (Table I).

Peyer's patches are relatively large aggregates of lymph tissue located in the GALT of the small intestine (10, 22). The overlying "dome" epithelium contains large numbers of intraepithelial lymphocytes. Some of the epithelial cells have complex microfolds in their surfaces, known as M-cells. M-cells are important in the transfer of antigen from the gut lumen to the Peyer's patch (10). Peyer's patches then facilitate the generation of an immune response within the mucosa. An antigen in the Peyer's patch stimulates B-cell precursors and memory cells (10). Cells pass to the mesenteric lymph nodes where the immune response, if needed, is amplified. Activated lymphocytes pass into the blood stream *via* the thoracic duct. Oral tolerance occurs only after the correct three-dimensional conformation of UC-II antigen is identified as nonpathogenic (10, 22).

Materials and methods

Chemicals. Pepsin (Catalog number I.U.B. 3.4.23.1) was purchased from Worthington Biochemical Corporation (Freehold, NJ, USA). Unless otherwise stated, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

UC-II. UC-II was obtained from InterHealth Nutraceuticals. The presence of glycosylated "active" epitopes in the UC-II collagen matrix was confirmed by a validated ELISA test. Furthermore, electron microscopic analysis of UC-II was conducted to demonstrate the conformational integrity of the triple helical structure.

For electron microscopic analysis, a small amount of UC-II powder was fixed with Karnovsky fixative for 2 h, rinsed with cacodylate buffer for 20 min, placed in 1% osmium tetroxide for 2 h, rinsed with distilled water for 1 min and placed overnight in 0.5% uranyl acetate. The sample was then dried using ethanol and placed into propylene oxide for 30 min and finally placed in 50:50 propylene oxide:SPURR (embedding material) for 2 h and then into 100% SPURR overnight. It was then placed into a 70 °F oven overnight. A section was taken using ultra microtome, stained with uranyl acetate for 4 min, rinsed with distilled water, stained with lead citrate for

Table I *Measurement of pain level following a 42-day study of oral administration of undenatured type II collagen (UC-II)*

Subject #	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Reduction in Pain (%)
1	3	3	3	3	3	3	3	0
2	5	5	5	5	5	2	2	22
3	5	5	4	4	3	3	5	22
4	6	6	5	5	3	2	2	22
5	7	8	5	5	4	3	1	34

Administered dose: A single, daily oral dose of 10 mg glycosylated undenatured type II collagen (UC-II). Pain index: 10 = unbearable, 1 = tolerable.

2 min and rinsed again with distilled water and dried. The transmission electron microscope procedure was conducted in an EM JEOL 100CX (Peabody, MA, USA). An electron micrograph of undenatured type II collagen *vs.* denatured type II collagen is shown in Fig. 1. Undenatured type II collagen (on left) shows intact tertiary and quaternary glycoprotein integrity allowing for epitope recognition and hyporesponsive immune stimulation. Denatured type II collagen (on right) contains no tertiary or quaternary glycoprotein integrity. Epitopes of healthy undenatured type II collagen contain the correct composition and structural conformation of galactose-dependent glycoprotein, as evidenced by ELISA analysis (Fig. 2).

Time-dose measurements of UC-II activity in simulated human gastric fluid. Five samples of UC-II were analyzed for collagen activity *via* ELISA analysis. Samples were digested in pepsin, simulating an artificial stomach. The pepsin solution was made using 995 ml distilled water, 3.73 g KCl, 4 g HCl and 30 mg pepsin. Five collagen samples of 14.7 g each were incubated individually for 0, 15, 30, 60 and 90 min in 100 ml pepsin solution at 32 °C and pH 2.0. The digestion process was stopped by increasing the pH to 6.0 using 0.5 M NaOH solution. Both the solid material (insoluble collagen) and the supernatant (soluble collagen) were collected and analyzed for native type II collagen using a commercially available Capture ELISA kit supplied by Chondrex LLC (Redmond, WA, USA). The quantity of UC-II (mg%) was determined in both supernatant soluble type II collagen and insoluble type II collagen following incubation for 0, 15, 30, 60 and 90 min at 32 °C and pH 2.0.

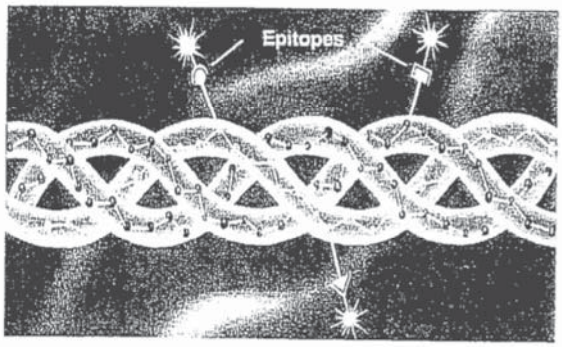

Fig. 2 Undenatured type II collagen triple helix molecule exhibiting epitope positions.

Pilot study to evaluate the efficacy of UC-II in human subjects. An open label pilot study was performed in five human subjects (women aged 58-78 years) suffering from significant joint pain, using a commercial ELISA-verified undenatured type II collagen (UC-II, InterHealth Nutraceuticals). To be eligible, patients had to meet the American College of Rheumatology criteria. Patients were excluded from the study if they had myocardial insufficiency, renal insufficiency (serum creatine > 2.0 mg/dl), disturbance of liver function, alkaline phosphatase > 300 units/liter, serum glutamic oxaloacetic transaminase > 50 units/liter, or bilirubin > 1.5 mg/dl), malignancy or a considerably reduced general state of health as determined by the physician. The five subjects enrolled in this study presented a history of osteoarthritis more than rheumatoid symptomology. These subjects reported early morning stiffness, stiffness following periods of rest, pain that worsened with use of the affected joint and loss of joint range of motion and function. Weather changes from warm to cold or dry to moist were also reported as pain-enhancing factors. All patients were required to sign an informed patient consent form prior to participation. The subjects were also given a questionnaire with detail protocol procedures, possible risks and benefits, *etc.* Two of the five subjects who suffered from osteoarthritis symptoms were clinically diagnosed 3 years prior to participation in this study. The remaining three subjects reported similar symptomology. Measurements included weekly diary-format

observations and qualitative feedback. Each subject received a single oral daily dose of 10 mg UC-II on an empty stomach prior to bedtime for 42 consecutive days. Each subject was asked to rate their respective pain level on a scale of 1-10, with a score of 10 representing "unbearable" and a score of 1 denoting "tolerable," prior to participation and immediately following completion of 7 days of treatment.

Results

Time-dose measurements of UC-II activity in gastric fluid. Following ingestion, the UC-II glycoprotein encounters hydrochloric acid and pepsin. Dose- and time-dependent studies were conducted to determine whether these monomers were still in the triple helical form, which we confirmed by ELISA assay. Figure 3 demonstrates the time-dose measurements of UC-II activity in simulated human gastric fluid at 32 °C and pH 2.0. Figure 3 clearly exhibits the UC-II activity in supernatant soluble type II collagen and insoluble type II collagen over a period of time (0-90 min). Thus, these results demonstrate that following incubation of UC-II for 90 min, approximately 50% of soluble UC-II is available to the epitopes.

Pilot study to evaluate the efficacy of UC-II in human subjects. An open label pilot study was conducted in five female subjects (aged 58-78 years) demonstrating the symptoms of significant joint pain. These subjects received a single oral daily dose of 10 mg UC-II on an empty stomach prior to bedtime for 42 consecutive days. All subjects rated their respective pain level on a scale of 1-10 (a score of 10 representing "unbearable" descending to a score of 1 denoting "tolerable"). The subjects rated their pain level before trial dose application and during treatment once every 7 days. Measurement of pain level in these human subjects following 42-day supplementation of UC-II is shown in Table I. Subject 1 perceived no reduction in her pain status throughout the open label trial. Subject 2 perceived a reduction in pain during the sixth week of the study, while under these same conditions Subjects 3, 4 and 5 reported a reduction in their pain level during the third week of treatment. Thus, a trial dose of 10 mg UC-II was associated with a ~26% reduction in perceived pain as indicated by four of the five subjects (22%, 22%, 22%, 34%; Table I). Furthermore, no side effects were associated with UC-II treatment. In essence, treatment with a daily oral dose of 10 mg UC-II was well tolerated and produced a significant reduction in joint pain symptoms.

Discussion

Epitope recognition. Epitopes (antigenic determinants) are structural components of an antigen molecule responsible for its specific interaction with T-cell antibody molecules elicited by the same or related antigen (23). Epitopes of healthy undenatured type II collagen contain the correct composition and structural conformation of galactose-dependent glycoprotein, as evidenced by ELISA analysis (24) (Fig. 2). A novel glycosylated undenatured type II collagen material (UC-II) was developed to preserve biological activity. The presence of glycosylated "active" epitopes in the UC-II collagen matrix is confirmed by a validated ELISA test and distinguishes this form from hydrolyzed, denatured agalactosylated collagen (25). Oral intake of 10 mg of this form of UC-II presents active epitopes, consisting of conformationally correct three-dimensional glycosylated structures, to Peyer's patches in the GALT (22, 26). Following ingestion, UC-II collagen glycoprotein encounters hydrochloric acid and pepsin. Dose- and time-dependent studies show these monomers are still in their triple helical form (Fig. 3) and travel down to the

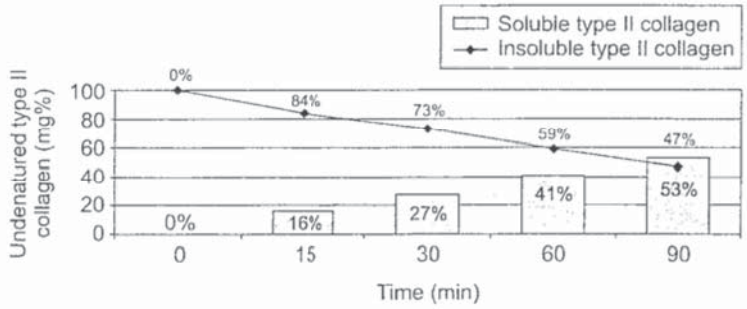

Fig. 3 Time-dose measurements of undenatured type II collagen activity in gastric fluid. Enzyme-linked immunosorbent measurements of undenatured type II collagen epitopes.

Peyer's patches, to which they bind. Pepsin does not break down the triple helical configuration of these monomers due to biochemical limitations, so the active sites always remain intact, which is confirmed by ELISA analysis. Pepsin will not cleave bonds containing the amino acids valine, alanine or glycine (27). The amino acid composition of native type II collagen is heavily distributed with glycine (28, 29). This glycine-rich sequence ensures that pepsin will not cleave the native collagen configuration (27). During digestion, the intact collagen fibril (a combination of collagen monomers, sugars and telopeptides) breaks down into monomeric collagen peptides (smaller glucopeptide units), exposing additional epitopes (30). On the other hand, the telopeptides bound to collagen molecules are susceptible to pepsin and get cleaved in the gut during digestion (31). This allows the collagen triple helix formation to loosen slightly, exposing additional active epitopes of the collagen glycoprotein, resulting in greater binding with and recognition by the Peyer's patches (32). These epitopes positively influence the immunoregulatory response signaling required for the development of tolerance (10, 32).

Properly glycosylated epitopes did not trigger T-cell proliferation, as did modified hybrid epitopes (21). Furthermore, Kim *et al.* (33) demonstrated that a single oral administration of poly(lactic-co-glycolic acid) (PLGA) nanoparticles induced tolerance against collagen II-induced arthritis in mice. Particles of PLGA were evident in the Peyer's patches of animals for 14 days from original feeding (33). Hyporesponsiveness results when epitopes of ingested UC-II collagen interact with the Peyer's patches in the lymphoid tissues of the duodenum, triggering the complex series of immunologic events that, in the case of RA, down-regulate the body's attack on its own type II joint collagen. This research demonstrated that PLGA was well tolerated against collagen II-induced arthritis. These active epitopes meet conformational specifications of the three-dimensional glycoprotein structures required by immune surveillance to signal approval and tolerance. Antigen epitope glycosylation has been shown to play an important role in T-cell recognition and B-cell responsiveness (21, 34, 35). This recognition and approval effectively turns off the up-regulated immune attack by reducing T-cell mediated inflammation, pain and swelling. UC-II has demonstrated its ability to induce tolerance, effectively reducing joint pain and swelling in RA subjects (3-6).

The science of glycobiology is rapidly expanding, uncapping enormous research opportunities and promising therapeutic tools (11). It provides new insights into disease initiation, promotion and progression, especially regarding autoimmune diseases, such as RA (12). A preponderance of the evidence suggests that all autoimmune diseases can be traced back to errors at some juncture of bioidentification, recognition and response signaling. Proper glycosylation is required for glycoconjugation, glycomolecular interconversions, biotransformations, and glycoprotein and glycolipid synthesis (11, 12).

In RA, impaired galactosylation alters the requisite three-dimensional conformations of glycoproteins, including certain immune factors, such as IgG and possibly even type II collagen, producing the loss of self-identity (12). Alterations in glycosylation and of galactosyl structures are hallmark characteristics of RA. This loss of self-identification alters recognition and response signaling during immune surveillance, inciting attack on the body's own joint collagen (13, 18).

Other autoimmune disorders have also been associated with faulty glycosylation (12, 17). This implies that certain autoimmune diseases may result when naturally occurring biomolecules are identified as foreign pathogenic antigens, due to their altered composition and structural conformation. As a result, appropriate immunological alarms are generated and aggressive defense tactics are employed against the host's own tissues (15).

Recently, safe and effective alternatives to traditional models of disease management have been used in RA (36). Oral administration of autoantigens has been shown to suppress a variety of experimentally induced autoimmune diseases, including antigen-induced RA (3-6, 33). As our understanding of glycobiochemistry increases, explanations regarding the reasons for these benefits emerge. Previous studies have shown that small doses of orally administered undenatured type II collagen effectively deactivate killer T-cell attack on type II joint collagen in humans (3, 22). Our pilot study exhibited the efficacy of UC-II (10 mg/day) in effectively reducing joint pain and swelling in human subjects without any adverse effects. UC-II contains conformationally correct "active" epitopes required to interact with Peyer's patches in the GALT and terminate antigenic signaling of a pathogenic nature, characteristic of RA (10). This approach provides new insights into the etiology of autoimmune inflammatory diseases and their amelioration with safe and effective treatments.

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