THE EFFECTS OF ROOSTER COMB EXTRACT ON OSTEOARTHRITIS PAIN IN DOGS

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ABSTRACT

Background: Osteoarthritis (OA) is a slow, progressive degenerative disease which affects 20% of the canine population over the age of one year. Currently, there is no treatment to prevent, delay or reverse this disease and NSAIDs are being used as the gold standard therapy. Due to side-effects this therapy is not suitable for all patients and that is why there is a need for alternative therapies. One of these alternative therapies can be the use of Rooster Comb Extract (RCE). This extract results from a production process involving enzymatic hydrolysis of rooster combs, filtration, concentration and precipitation steps. The glycosaminoglycans (GAGs) hyaluronic acid, chondroitin sulphate A and dermatan sulphate are the principle constituents of RCE. It has been claimed that these ingredients can support dogs with OA pain.

Objective: The aim of this study was to demonstrate the clinical effect, registered by a survey, of a novel nutraceutical for dogs suffering from osteoarthritis. This nutraceutical consisted out of RCE, glucosamine, chondroitin, methyl-sulfonyl-methane (MSM), a proprietary protein mixture and is completed with vitamins and minerals.

Study design: Randomized, blinded, placebo-controlled clinical trial.

Methods: By using the Helsinki Chronic Pain Index the effect of this novel nutraceutical is measured in a period of 6 weeks in 14 dogs suffering from OA. The results were compared to 14 placebo-controlled dogs suffering from OA.

Results: The dogs showed an improved in their HCPI score after the supplementation of the novel nutraceutical. The mean of the supplement group improved from 19.8 (± 7.00) to 13.8 (± 6.50), in the placebo group the mean went from 16.2 (± 4.92) to 15.6 (± 7.00). The results of the repeated measurement ANOVA over time had a p-value of 0.061 (p-value>0.05). The same type ANOVA showed between the groups a p-value of 0.255 (p-value>0.05) and overall a p-value of 0.121 (p-value>0.05).

Conclusion: In this study there was not a significant result found, but according to the owners, the dogs did improve clinically. For a following study a bigger sample size, the use of subobjective and objective parameters are strongly recommended to further investigate the effects of RCE on OA pain in dogs.

INTRODUCTION

Osteoarthritis (OA) is a slow, progressive degenerative disease which is affecting 20% of the canine population over the age of one year (Johnston et al., 2008)(Bhathal et al., 2017). OA is a disorder of the articular joints that is characterized by degeneration of the articular cartilage, inadequate cartilage biosynthesis, joint trauma, instability and inflammatory mechanisms (Bhathal et al., 2017). OA can affect any joint, including smaller joints like the vertebral facets, metacarpophalangeal and

metatarsophalangeal joints (Nelson & Guillermo Couto, 2013). There have been different types of OA described, including obesity-related OA, mechanical-induced OA and aging-relating OA (Conaghan, 2013). The disease presents itself with lameness, but also symptoms such as pain, stiffness and disability can be seen (D'Altilio et al., 2007). The affected animals are often reluctant to do normal activities such as climbing the stairs. The lameness can decrease after a few minutes of activity as the dog is "warming out" their symptoms (Bennet & May, 1994). Dogs of all sizes can be affected by the disease as they age, although large-breed dogs may develop more severe clinical signs (Rychel, 2010). OA often has a negative impact on the affected dog's quality of life.

PATHOPHYSIOLOGY OF OA

Articular cartilage has a complex structure, it is designed to absorb shock and decrease friction. It is composed of chondrocytes embedded in a matrix, which is synthesized by the chondrocytes themselves (Clark, 1991). This matrix consists mainly out of water, collagen and proteoglycans (Martel-Pelletier et al., 2016). These proteoglycans are formed by hydrophilic glycosaminoglycans (GAGs) and form complexes with hyaluronic acid (HA). These complexes act as osmotic traps that hold water between the collagen strands (Nelson & Guillermo Couto, 2013). To withstand normal loading forces, the proteoglycan and water aggregates act as a shock absorber.

Articular cartilage should be seen as a dynamic tissue, with constantly synthesizing products that repair aged or damaged cartilage (Clark, 1991). Likewise, osteoarthritis should not be seen as a static process involving excessive wear and tear on cartilage but rather as an active process that inhibits the normal cartilage regeneration. One of the earliest OA changes in the cartilage is the swelling of the matrix, which is associated with an increased water content and the loss of GAGs (Goldring & Goldring, 2016). OA ultimately results in an abnormal cartilage structure and the loss of cartilage proteoglycans. The proteoglycans of OA are more easily extracted from cartilage due to an abnormal biochemical structure (Nelson & Guillermo Couto, 2013). The proteoglycan and HA content decreases as cartilage degeneration progresses (Clark, 1991). During OA, a wide array of inflammatory cytokines is produced and in turn they stimulate chondrocytes to produce higher levels of metalloproteinases (MMP's) and lower levels of cartilage matrix molecules compared to healthy chondrocytes (Rhouma et al., 2013). In the end, the imbalance between catabolic and anabolic processes is responsible for the progressive matrix degeneration (Tetlow et al., 2001). In figure 1 the difference between a normal joint and a joint suffering from OA is explained and shown.

Although OA is considered as noninflammatory, based on the cytology of the synovial fluid, inflammatory mediators are involved in the disease. These mediators play a role in the clinical manifestations and progression of OA (Nelson & Guillermo Couto, 2013). It has been shown that OA chondrocytes overexpress interleukin-1 and tumor necrosis factor receptors. Besides this, the expression of transforming growth factor BETA-RII receptor is decreased (Attur et al., 2002)(Wang et al., 2003)(Boumediene et al., 1998).

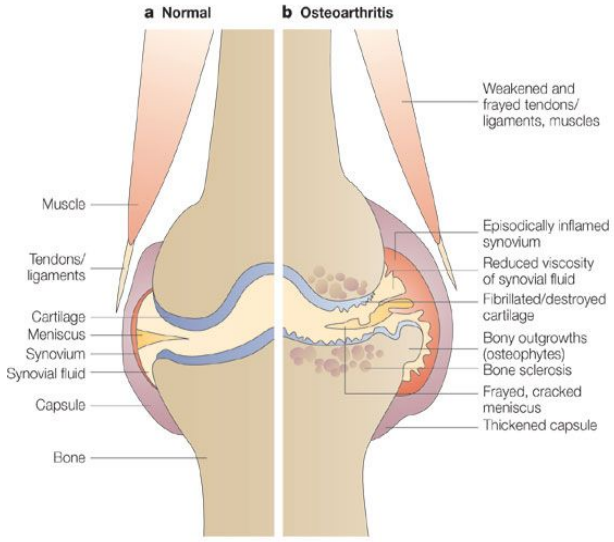

FIGURE 1: DIFFERENCES BETWEEN AN NORMAL AND AN OA JOINT(13)

TREATMENT OPTIONS OF OA

Currently, there is no treatment yet which has been able to prevent, delay or reverse this disease(1). The treatment being used for OA nowadays is symptomatic, nonspecific and involves non-pharmacological and pharmacological options.

Non-pharmacological treatment consists of lifestyle management, such as weight loss, physical therapy and exercise (Rannou & Poiraudeau, 2010). Additional weight puts undue stress on joints that are already affected and obesity contributes to a chronic, systemic inflammation state (Rychel, 2010)(Budsberg & Bartges, 2006). To maintain the mobility and muscle strength low-impact exercises, such as swimming and leash walking, are recommended, whereas high-impact exercise, like jumping, should be discouraged (Nelson & Guillermo Couto, 2013).

The pharmacological treatment is based on anti-inflammatory drugs, chondroprotective drugs and analgesics to relieve pain and improve the function of the joints (de Sousa et al., 2017). Non-steroidal Anti-Inflammatory Drugs (NSAIDs) are the current gold-standard therapy (Nelson & Guillermo Couto, 2013). NSAIDs inhibit cyclo-oxygenase (COX) and therefore reduce the concentration of pro-inflammatory prostaglandins (PGE) (Comblain et al., 2016). Due to the side effects of NSIADs on gastrointestinal ulceration, the contraindication in the presence of renal insufficiency and accelerated cartilage degeneration, this therapy is not suitable for all patients (Bhathal et al., 2017)(Mehler et al., 2016). Steroid injections are usually reserved for severe OA and for dogs that have become refractory for other treatments (Yves Henrotin et al., 2005). Surgical intervention can be done to help to stabilize the joint, correct a deformity and relieve discomfort (Nelson & Guillermo Couto, 2013).

A number of alternative therapies have been evaluated with varying degrees of success (Yves Henrotin et al., 2005). These therapies can be recommended when adverse side-effects of drugs limit treatment options or if pet owners prefer natural or alternative therapies (Vaughan-Scott & Taylor, 1997). Dietary supplementation with nutraceuticals is an example of one of these alternative therapies.

NUTRACEUTICALS IN OA

Nutraceuticals may be defined as a food (or a part of a food) that provides medical or health benefits including the prevention and/or treatment of a (chronic) disease (Kalra, 2003). Thus, these products can be implemented as a treatment option in addition to having nutritional value (Nasri et al., 2014). There are a lot of different types of nutraceuticals used to treat OA in adult dogs. In general, the most commonly used products are:

1) glucosamine sulphate and chondroitin sulphate (Yves Henrotin et al., 2005)(Rhouma et al., 2013)
2) vitamins (especially E and C, sometimes combined with selenium) (Jewell et al., 2000)(Yazar et al., 2005)
3) two different types of collagen (Comblain et al., 2016)
4) polyunsaturated fatty acids (Akhtar & Haqqi, 2012).

These nutraceuticals offer anti-inflammatory and chondroprotective effects. However, the claimed efficacy is not always supported by meticulous scientific studies (Ameye & Chee, 2006).

Glucosamine, an amino sugar, and chondroitin, a sulfated glycosaminoglycan, are commonly recommended by veterinarians for treating OA in dogs (Bhathal et al., 2017). These agents may work as chondroprotective agents as they are precursors for GAGs, which are a major component in joint cartilage (Chan et al., 2005). They could improve cartilage biosynthetic activity, inhibit intraarticular degradative enzymes and decrease synovial inflammation (Nelson & Guillermo Couto, 2013). Glucosamine has a mild anti-inflammatory effect due to its ability to scavenge free radicals. Besides that, it regulates the synthesis of collagen and proteoglycans in cartilage. It

appears that exogenously administered glucosamine is able to be utilized by chondrocytes (*Plumb's Veterinary Drugs*, 2020). Chondroitin can inhibit destructive enzymes in joint fluid and cartilage and it can stimulate the production of GAGs and proteoglycans (*Plumb's Veterinary Drugs*, 2020). There is in-vitro data that supports these effects (Dodge & Jimenez, 2003). The claimed effect found in the in-vitro studies is not always supported by good results in the in-vivo studies. Due to the fact that these clinical trials have been using different products, different dosages and different dosing regimens it is hard to draw meaningful conclusions about the use of these agents (Bhathal et al., 2017). As seen in Plumb's Veterinary Textbook, the recommended daily dosage is 50 mg/kg for glucosamine and 15mg/kg for chondroitin and (*Plumb's Veterinary Drugs*, 2020).

Vitamins are used in OA supplements because of their anti-inflammatory effect. Examples of vitamins used in OA supplementation are vitamin E (VE) and vitamin C (VC). Besides the anti-inflammatory effects of VE, the consumption of VE has been associated with a reduced oxidization of low-density lipoproteins. VE plays a major role in the regulation of arachidonate release from membrane phospholipids and it is subsequent in the metabolism of inflammatory mediators (Rhouma et al., 2013). To get an improvement of the wellbeing in OA dogs, the food must contain 440 IE VE/kg dry matter (Jewell et al., 2000). VC is a powerful antioxidant and it can regenerate other antioxidants and can capture free radicals (intra- and extracellular). Furthermore, VC protects against the protein inactivation reaction, which is mediated by free radicals and this reaction will release a high dose of neutrophils (Dodge & Jimenez, 2003).

There have been different types of collagen supplementation described in the treatment of OA. Firstly, the supplementation of hydrolyzed collagen increases the amount of specific cartilage amino acids, like proline. A higher concentration of proline could help the regeneration of damaged cartilage (García-Coronado et al., 2019). The second type of collagen that is used in OA supplements is undenaturated type II collagen (UC-II). The main characteristic of UC-II is the amino acid composition, which provide higher levels of glycine and proline compared to hydrolyzed collagen. Proline and glycine are essential for the stability and regeneration of cartilage (Walrand et al., 2008). By increasing the synthesis of these macromolecules, UC-II induces cartilage regeneration. With this bystander suppression, UC-II helps in improving activity and it is shown that UC-II can reduce pain, lameness and stiffness in dogs suffering from OA (Comblain et al., 2016). The recommended daily dosage of UC-II is 10 mg (Deparle et al., 2005)(Gupta et al., 2019).

The supplementation of polyunsaturated fatty acids (PUFAs) is based on the fact that PUFA's are incorporated into the phospholipids of the cell membrane. One of these PUFAs is omega-3. The primary omega-3 (n-3) fatty acids are docosahexaenoic acid (DHA), alpha-linolenic acid (ALA) and eicosapentaenoic acid (EPA) (Akhtar & Haqqi, 2012). EPA and DHA are potent anti-inflammatory substances. The supplementation of n-3 fatty acids results in increased EPA content in membrane phospholipids. Another PUFA is omega-6 (n-6) acid. One of these n-6 acids, arachidonic acids (ARA), has metabolites which are inflammatory mediators. The use of EPA and/or DHA could decrease the amount of ARA available as a substrate for inflammatory production. This will lead to the net result that there will be a reduction, systemically and within the joint, in the anti-inflammatory environment (Johnston et al., 2008)(Harris & Shearer, 2014).

The promising theories behind these nutraceuticals are not always supported by sufficient scientific research. The use of EPA+DHA and UC-II is confirmed by several review articles (Bhathal et al., 2017)(Deparle et al., 2005)(Vandeweerd et al., 2012). Due to the contradictions presented in the research of nutraceuticals, there is a need for more data regarding in vivo animal studies with dietary supplementation (Y. Henrotin et al., 2011).

The aim of this randomized, blinded, placebo-controlled study was to demonstrate the clinical effect, registered by a survey, of a 6-week period of supplementation with a novel nutraceutical in 16 dogs with OA signs, in comparison with a 6-week period of supplementation with a placebo in 16 dogs with OA signs.

PRODUCT X

The nutraceutical being evaluated in this study is currently available for human use. The product consists of glucosamine, chondroitin, a proprietary protein mixture, methyl-sulfonyl-methane (MSM), Rooster Comb Extract (RCE) and is completed with some vitamins (C and D3) and a mineral. The ingredients are used together because of the ability to exert a synergistic effect. These ingredients can counteract OA pathogenesis by exerting the antioxidant and inflammatory activities (D'Adamo et al., 2020)(Bottegoni et al., 2014).

Rooster Comb Extract (RCE) is a novel ingredient in the treatment of OA in dogs. The effects of orally administered RCE on OA pain in dogs have not been studied before. RCE is obtained from the combs of *Gallus gallus* roosters. The extract results from a production process involving enzymatic hydrolysis of rooster combs, filtration, concentration and precipitation steps. The GAGs HA, chondroitin sulphate A and dermatan sulphate are the principle constituents of RCE. As mentioned before, GAGs are a major component of joint cartilage. HA has been used in the treatment of OA as an intraarticular injection. HA improves synovial viscosity and decrease inflammation (Nelson & Guillermo Couto, 2013). After oral administration, the percentage of RCE entering the systemic circulation is 5-20%, which is in line with other GAGs (Balogh et al., 2008). RCE stimulates the internal formation of HA, resulting in a multiplied excretion in the synovial fluid. Furthermore, RCE lubricates the joints and therefore decreases joint discomfort and pain. Besides, RCE can downregulate the production of MMP's and the proinflammatory cytokine expression (Jhun et al., 2015).

The proprietary protein mixture consists of Boswellia extracts, ginger and devil's claw. Extracts of the Boswellia tree are expected to have anti-inflammatory properties. In several controlled studies it has been shown to improve clinical signs and pain in humans (Kulkarni et al., 1991)(Kimmatkar et al., 2003). In a study among dogs an improvement in clinical signs, lameness and pain, was found (Reichling et al., 2004).

MSM has been suggested as an agent for pain management, reduction of inflammation and as an antioxidant (Butawan et al., 2017)(Parcell, 2002). There are no randomized controlled clinical trials that are evaluating the effects of MSM. However, in a study conducted by Kim et al., MSM reduced visual analog scale scores, decreased pain and improved physical function compared to the placebo group (Kim et al., 2006).

Because of the contradictory results of studies the efficacy of VC is difficult to determine (Budsberg & Bartges, 2006).

The list of ingredients is summarized in table 1. By using different weight groups, the dosage/day was formed and is listed in table 2.

INGREDIENT OF 1 PILL	
GLUCOSAMINE	500 mg
CHONDROITIN	250 mg
PROPRIETARY PROTEIN MIXTURE	75 mg
MSM	50 mg
VITAMIN C	50 mg
RCE	15 mg
MANGANGLUCONATE	1 mg
CHOLECALCIFEROL	2 µg

TABLE 1: INGREDIENTS OF THE TESTED PRODUCT

DOSAGE	WEIGHT GROUP
1 PILL/DAY	0-20 kg
2 PILLS/DAY	20-40 g
3 PILLS/DAY	>40 kg

TABLE 2: THE USED DOSAGES IN THIS STUDY

MATERIALS AND METHODS

ANIMALS

Owners of 32 dogs were willing to participate in this study. The owners were approached by using an online advertisement on several social media platforms. To be included in this study the dogs had to have signs of OA in one or more joints, had to be on a commercially available dry dog food and the owners needed to be willing to cooperate throughout the study. Dogs that were using NSAIDs, other painkillers and/or supplements within two weeks prior to the start of the study were excluded from the study group. Due to ethical considerations and if needed, the use of NSAIDs was allowed for a couple of days during the study period. If the dog needed a longer period of treatment with NSIADs (or any other painkiller) the dog will be excluded from the study group.

QUESTIONNAIRE

The Helsinki Chronic Pain Index (HCPI), as seen in figure 2, was used for this research (Hielm-Björkman et al., 2009). The questionnaire was translated to Dutch. The same researcher had telephone and email contact with the owners. The HCPI was conducted in a survey program called Qualtrics, which was available at the workspace of Utrecht University.

STUDY PROTOCOL

Before the start of this study an introduction interview and the first Helsinki Chronic Pain Index (HCPI) were completed by the owners (Hielm-Björkman et al., 2009). The interview included questions about the dogs OA and general information about the dog, such as breed, age and weight. The dogs were then randomly given either supplement A or supplement B for 6 weeks. During this period the owner had to refill the HCPI twice. During the study (week 3) and at the end of this 6-week trial a new HCPI was provided. When the last HCPI was completed by the owner a feedback form was distributed and filled in.

The placebo pills consisted mostly out of microcrystalline cellulose. The pills were completed with potato starch, sodium croscarmellose, silicon dioxide and magnesium stereate. The owners were not aware which pill their dog received and were therefore blinded during this study. The researcher was not blinded, this was done to be able to provide information about this research when needed. This was not the case during this research.

STATISTICAL ANALYSIS

First, the data was downloaded into Microsoft Excel (2016) for descriptive analytics. All the details asked in the interview / questionnaire, gender (male/female), neutered (yes/no), weight, age and other general questions about the OA in the dogs were compared. Besides this, the amount and kind of food were analyzed. The results of the 3 HCPI's were analyzed with RStudio 1.3.1073. Summary statistics were used to summarize the results, the Shapiro-Wilkinson and an assumption test were used to test the normality and the

Question asked	0 Points	1 Point	2 Points	3 Points	4 Points
Rate your dog's mood	Very alert	Alert	Neither alert nor indifferent	Indifferent	Very indifferent
Rate your dog's willingness to participate in play	Very willing	Willing	Reluctantly	Very reluctantly	Does not play at all
Rate your dog's vocalization (audible complaining)	Never	Hardly ever	Sometimes	Often	Very often
Rate your dog's willingness to walk	Very willing	Willingly	Reluctantly	Very reluctantly	Does not walk at all
Rate your dog's willingness to trot	Very willing	Willingly	Reluctantly	Very reluctantly	Does not trot at all
Rate your dog's willingness to gallop	Very willing	Willingly	Reluctantly	Very reluctantly	Does not gallop at all
Rate your dog's willingness to jump (e.g., into car, onto sofa)	Very willing	Willingly	Reluctantly	Very reluctantly	Does not jump at all
Rate your dog's ease in lying down	With great ease	Easily	Neither easily nor difficultly	With difficulty	With great difficulty
Rate your dog's ease in rising from a lying position	With great ease	Easily	Neither easily nor difficultly	With difficulty	With great difficulty
Rate your dog's ease of movement after long rest	With great ease	Easily	Neither easily nor difficultly	With difficulty	Very often/always difficulty
Rate your dog's ease of movement after major activity or heavy exercise	With great ease	Easily	Neither easily nor difficultly	With difficulty	Very often/always difficulty

FIGURE 2: THE QUESTION'S ASKED IN THE HELSINKI CHRONIC PAIN INDEX (HCPI) (45)

repeated measurement ANOVA tested the interaction between the two groups and the parameter time during this study. A p-value of <0.05 was considered significant.

RESULTS

Of the 32 dogs that started this study only 28 completed it, 4 dogs therefore did not complete the study due to underlying illnesses. Two of these dogs died (they had been euthanized by their vet because of underlying illness) and two dogs were administered NSAID's for a longer period (> more than 2 weeks continuously). Fourteen dogs were administered supplement A and fourteen dogs received supplement B. Most of the dogs accepted the supplements well. Sixteen different breeds participated in this study. The average age was 9.5 ± 3.7 years. The oldest dog who participated in this study was 15 years old and the youngest dog was 3 years old. 19 dogs were female, and 9 dogs were male. Not all the dogs were neutered, 5 dogs were intact (1 female/4 males). At time of the intake, the clinical signs of OA were at least present for one year, with a mean of 1.9 years. 11 out of the 28 owners did not visited the vet for the OA symptoms their dogs present with. From the 28 dogs, 18 did use supplements or a special diet before. All of these characteristics are shown in table 3. During the intake the kind and the amount of food were asked. The composition of the (dry) foods is shown in table 4. As it is mentioned in the introduction, the addition of polyunsaturated fatty acids and glucosamine/chondroitin could improve the wellbeing of dogs suffering from OA. During this study, one dog received NSAID's because of a subluxation of a toe due to a traumatic event. The administration of these NSAID's was not for a long period (> more than 2 weeks continuously) so this dog was not excluded from the study. None of the other included dogs were in need of any medication during this study.

CHARACTERISTIC	NO.	%
GENDER		
FEMALE	19	67.9%
MALE	9	32.1%
NEUTERED		
YES	23	82.1%
NO	5	17.9%
BREED		
PURE-BRED	21	75.0%
CROSS-BRED	7	25.0%
AGE (YEARS)		
0-5 YEARS	4	14.3%
5-10 YEARS	9	32.1%
>10 YEARS	15	53.6%
WEIGHT GROUPS		
0-20 KG	12	42.9%
20-40 KG	9	32.1%
>40 KG	7	25.0%
VISITED THE VET FOR OA SYMPTOMS		
YES	19	67.9%
NO	9	32.1%
START OA SYMPTOMS IN YEARS		
0-1 YEAR	5	17.9%
1-2 YEARS	11	39.3%
2-3 YEARS	6	21.4%
>3 YEARS	6	21.4%
USED OA SUPPLEMENTS BEFORE		
YES	18	64.3%
NO	10	35.7%

TABLE 3: BASELINE CHARACTERISTICS OF THE RESEARCH GROUP

Dog number	Total N-6	Total N-3	N-6: N-3	Glucosamine + chondroitin
1	na	na	na	-
2	na	na	na	-
3	na	na	na	-
4	4	1.1	4:1.1	yes
5	na	na	na	
6	na	na	na	-
7	3.17	0,75	4.2:1	yes
8	3.85	1.08	3.6:1	-
9	na	na	Na	-
10	na	na	na	-
11	na	na	na	-
12	3.54	na	-	-
13	3.21	0.9	3.6:1	-
14	na	na	na	-
15	4	1.1	4:1.1	yes
16	4	1.1	4:1.1	yes
17	3.85	1.08	3.6:1	-
18	3.21	0.9	3.6:1	-
19	1.70	0.14	12.1:1	-
20	na	na	na	-
21	2.6	0.9	2.9:1	yes
22	6.64	0.09	73:1	-
23	na	na	na	-
24	na	na	na	-
25	na	na	na	-
26	na	na	na	-
27	na	2.2	1:1	-
28	na	na	na	-

TABLE 4: POLYUNSATURATED ACID AND GLUCOSAMINE+CHONDROITIN COMPOSITION OF THE MAINTENANCE DOG FOODS.

NA; NOT AVAILABLE

The mean in the first HCPI in group A was 16.2 ± 4.92 and for group B 19.8 ± 7.01. During the second survey the mean changed in group A to 14.1 ± 6.49 and in group B to 17.3 ± 7.02. The last questionnaire gave in group A a mean of 15.6 ± 7.02 and in group B 13.8 ± 6.45. These summary statistics are shown in table 5 and a boxplot of the findings are shown in figure 4. By using a Shapiro-Wilkinson test the normal distribution is tested. The results are shown in figure 5. The assumption test was used to determine if there were any outliners.

	M(SD)A	M(SD)B
T0	16.2(4.9)	19.8(7.0)
T1	14.1(6.5)	17.3(7.0)
T2	15.6(7.0)	13.8(6.5)

TABLE 5: THE MEAN(M) AND STANDARD DEVATION (SD) OF GROUP A AND B

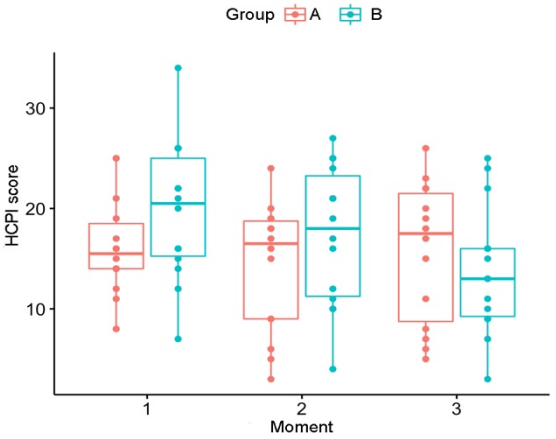

FIGURE 4: BOXPLOT OF THE HELSINKI CHRONIC PAIN INDEX SCORES OF GROUP A AND B DURING THIS STUDY. RED: GROUP A – BLUE: GROUP B

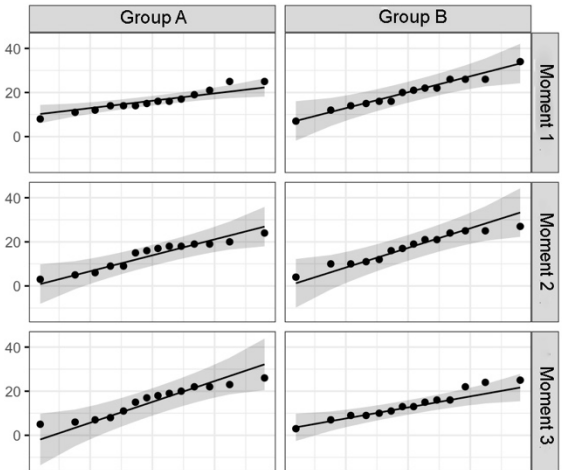

FIGURE 5: THE RESULTS OF THE SHAPIRO-WILKINSON TEST

The repeated measurement ANOVA results are shown in table 6. As seen in table 6, there was no statistically significant difference between the groups. The p-value over time is 0.061 (F=3.60), which is bigger than 0.05. This is also the case for the comparison between group A and B (F=1.31, p=0.225) and the comparison between the groups during the different scores over time (F=2.46, p=0.121).

	F	P
TIME	3.60	0.061
GROUP A/B	1.31	0.255
TIME/GROUPS	2.46	0.121

TABLE 6: THE RESULTS OF THE REPEATED MEASUREMENT ANOVA

DISCUSSION

The aim of this study was to investigate the effect of a 6-week period of a novel nutraceutical on the owner's perception of the behavior and locomotion of their dog with OA signs, in comparison with a 6-week period on a placebo supplementation. This 6-week period was used because in previous studies it was shown that the effects of the ingredients should be measured after 2-6 weeks of supplementation. Although, it is unlikely that a complete other effect was founded in a longer period of supplementation, it is known that the effect of glucosamine and chondroitin with a larger dose or a longer period of supplementation could possibly have given an even better result (*Plumb's Veterinary Drugs*, 2020). For a following study, a longer period of supplementation or a crossover design could be considered. However, a longer period of supplementation or a crossover design could also lead to a decrease of compliance of the owners and can also have given more dropouts.

As seen in the results, the dogs in group B (supplement-group) showed promising results with a great improvement in clinical signs according to their owners. The owners of group B were convinced by the working of the supplement and the effect of product X was the lowering of the mean with 6.0. The results are

in line with a previous study, where a positive effect of RCE in humans with knee OA was found (Oe et al., 2016). In the results, the improvement of group A (placebo) is shown with a lowering of the mean with 0.6 points. Besides the possibility that the improvement of group A is caused by the placebo-effect, it is also possible that this improvement is originating from the better care effect (Dobenecker et al., 2002). However, the owners were blinded to correct interobserver differences.

As it is seen in table 4, an analysis of the commercially dry dog food was done. All of the dogs where on this diet before the start of study, resulting in no changes within the components of food and feeding pattern during this study. There were some dog foods with the addition of glucosamine and chondroitin. These dogs did not show a better result compared to the other dogs. It is known that dog foods nowadays are very likely to be high in n-6 and low in n-3. The optimal n-6/n-3 ratio is 6:1, but none of the foods have this ratio (Biagi et al., 2004). Due to the lack of available data from the dog food analysis, it is hard to draw conclusions of the effect of the dog foods.

A minimum of bias has been present in this study because it was randomized, blinded, and placebo controlled. Despite of this, there are a few shortcomings in this study. First of all, there is a lack of subobjective and objective parameters. The effects on OA-associated pain in dogs may be subjective because of the interpretation by the owner, the placebo effect and the better care effect. In a future study, the available information (history, pain scale) could be completed with the following suggested recommendations. To begin, the severity of OA was not taken into account in this study. The dogs were randomly divided between the two groups by a computer program. As table 5 is showing, group B got a higher mean in their first HCPI. This means that the dogs in group B showed severe OA signs, according to their owners. Nevertheless, it has been showed in this study that even dogs with a high HCPI can improve with the use of this nutraceutical. Secondly, the monitoring by a veterinarian or even an orthopedic surgeon of the dogs during the study could have given valuable information. But these examinations are also an extra obligation for the owner with the potential result of more dropping outs. Thirdly, the use of radiographic imaging can be used to evaluate the effect of this nutraceutical on joint capsular distention, narrowed joint spaces and soft tissue thickening (Bhathal et al., 2017). Anyhow, because of the short period of the study no meaningful changes were expected in radiographic images (Roush et al., 2010). Also, the use of force plate gait analysis can be essential as it gives a good insight of the pain represent in the dog during the study. But this analysis has its limitations as well, as only the force of the limbs is measured. The Liverpool Osteoarthritis in Dogs Clinical Metrology Instrument (LOAD) showed a correlation with force plait gat analysis, this might be a good option for gathering data with additional information from the dog owners (Walton et al., 2013).

Besides the lack of subobjective and objective parameters in this study, there are also some disadvantages of the use of the HCPI. It is known that owners undergo a learning curve and look at different things at different times when using this questionnaire (Hielm-Björkman et al., 2009). The HCPI is validated by a 4-week interval. In this study a 3-week interval was chosen. This slightly shorter interval was chosen because of the length of the study. In reality, the pain status of an OA dog might change from day to day, depending on (extra) activity. This means that any interval can be too long. Besides this, it is also possible that the changings in the scores of the HCPI are correlated to the undulating nature of OA or differences in weather (Hielm-Björkman et al., 2009). Moreover, it is almost impossible to find a chronic pain index that would fit different types of dogs in all kinds of environments. In this study, the dogs have a variety of purposes, from pets to working dogs. So, it is possible that not all questions are suitable for the dog and their specific environment. This could lead to a misinterpretation during filling in the HCPI. Last

but not least, it is known that owners undergo a learning curve and look at different things at different times. Nevertheless, the HCPI is determined to be good and valid as the questionnaire is sensitive enough to measure the difference in mood and mobility as observed by their owners. Even in a small group of dogs it can detect improvement, the strength of the index is that it consists out of 11 questions that all belong to chronic pain in dogs and it can be used as a tool for chronic pain evaluation in clinical research (Hielm-Björkman, 2007).

The sample size of this study was not determined by a power analysis because of the lack of available data in previous studies. Clearly, the aim was to have a sample size large enough to have a good chance of detecting any clinically important treatment differences as statistically significant. As it is shown in table 6, the p-value over time is tended to reach significant difference. It is possible that, when a greater population was tested, a significant difference was shown. The information found in this study can be used to calculate the right power for a following study. The means and standard deviations found in this study can be used to calculate the effect size (Petrie & Watson, 2013).

In conclusion, there is a need for more research to evaluate the effect of RCE in dogs with OA pain. In a following study the patients should be client-owned dogs with naturally occurring OA. The randomization would be based on the disease severity and this can be determined by using radiographic imaging. The use of a standardized, semi-objective veterinary assessment would give additional information. The researcher, owners and clinicians need to be blinded. Last but not least, the right sample size, will give this future study a higher power.

CONCLUSION

A 6-week period on a novel nutraceutical with glucosamine, chondroitin, RCE and MSM changes the owner's perception of some aspects of behavior, locomotion and the HCPI of their dogs, which have OA signs in comparison with a 6-week period on a placebo supplementation. Despite of the improvement the owners found, the results were not significant and further research of the effects of RCE on OA associated pain in dogs should be done.

ACKNOWLEDGMENTS

I would like to thank R.J. Corbee for his support and contribution to this study, Wouter Smink for his help with the statistical analysis. Last but not least I would like to thank Nutramin B.V. and the owners for their cooperation.

REFERENCES

Akhtar, N., & Haqqi, T. M. (2012). Current nutraceuticals in the management of osteoarthritis: A review. *Therapeutic Advances in Musculoskeletal Disease*, *4*(3), 181–207. https://doi.org/10.1177/1759720X11436238

Ameye, L. G., & Chee, W. S. S. (2006). Osteoarthritis and nutrition. From nutraceuticals to functional foods: A systematic review of the scientific evidence. *Arthritis Research & Therapy*, *8*(4), R127. https://doi.org/10.1186/ar2016

Attur, M. G., Dave, M., Akamatsu, M., Katoh, M., & Amin, A. R. (2002). Osteoarthritis or osteoarthrosis: The definition of inflammation becomes a semantic issue in the genomic era of molecular medicine. *Osteoarthritis and Cartilage*, *10*(1), 1–4. https://doi.org/10.1053/joca.2001.0488

Balogh, L., Polyak, A., Mathe, D., Kiraly, R., Thuroczy, J., Terez, M., Janoki, G., Ting, Y., Bucci, L. R., & Schauss, A. G. (2008). Absorption, Uptake and Tissue Affinity of High-Molecular-Weight Hyaluronan after Oral Administration in Rats and Dogs. *Journal of Agricultural and Food Chemistry*, *56*(22), 10582–10593. https://doi.org/10.1021/jf8017029

Bennet, D., & May, C. (1994). *Textbook of veterinary internal medicine* (Fourth edition). W.B. Saunders and Co.

Bhathal, A., Spryszak, M., Louizos, C., & Frankel, G. (2017). Glucosamine and chondroitin use in canines for osteoarthritis: A review. *Open Veterinary Journal*, *7*(1), 36–49. https://doi.org/10.4314/ovj.v7i1.6

Biagi, G., Mordenti, A., & Cocchi, M. (2004). The role of dietary omega-3 and omega-6 essential fatty acids in the nutrition of dogs and cats: A review. *Progress in Nutrition*, *6*, 0–0.

Bottegoni, C., Muzzarelli, R. A. A., Giovannini, F., Busilacchi, A., & Gigante, A. (2014). Oral chondroprotection with nutraceuticals made of chondroitin sulphate plus glucosamine sulphate in osteoarthritis. *Carbohydrate Polymers*, *109*, 126–138. https://doi.org/10.1016/j.carbpol.2014.03.033

Boumediene, K., Conrozier, T., Mathieu, P., Richard, M., Marcelli, C., Vignon, E., & Pujol, J. P. (1998). Decrease of cartilage transforming growth factor-beta receptor II expression in the rabbit experimental osteoarthritis—Potential role in cartilage breakdown. *Osteoarthritis and Cartilage*, *6*(2), 146–149. https://doi.org/10.1053/joca.1997.0104

Budsberg, S. C., & Bartges, J. W. (2006). Nutrition and Osteoarthritis in Dogs: Does It Help? *Veterinary Clinics of North America: Small Animal Practice*, *36*(6), 1307–1323. https://doi.org/10.1016/j.cvsm.2006.08.007

Butawan, M., Benjamin, R. L., & Bloomer, R. J. (2017). Methylsulfonylmethane: Applications and Safety of a Novel Dietary Supplement. *Nutrients*, *9*(3), 290. https://doi.org/10.3390/nu9030290

Chan, P.-S., Caron, J. P., & Orth, M. W. (2005). Effect of glucosamine and chondroitin sulfate on regulation of gene expression of proteolytic enzymes and their inhibitors in interleukin-1–challenged bovine articular cartilage explants. *American Journal of Veterinary Research*, *66*(11), 1870–1876. https://doi.org/10.2460/ajvr.2005.66.1870

Clark, D. M. (Oklahoma S. U. (1991). The biochemistry of degenerative joint disease and its treatment.

The Compendium on Continuing Education for the Practicing Veterinarian (USA). http://agris.fao.org/agris-search/search.do?recordID=US9139004

Comblain, F., Serisier, S., Barthelemy, N., Balligand, M., & Henrotin, Y. (2016). Review of dietary supplements for the management of osteoarthritis in dogs in studies from 2004 to 2014. *Journal of Veterinary Pharmacology and Therapeutics*, *39*(1), 1–15. https://doi.org/10.1111/jvp.12251

Conaghan, P. G. (2013). Parallel evolution of OA phenotypes and therapies. *Nature Reviews Rheumatology*, *9*(2), 68–70. https://doi.org/10.1038/nrrheum.2012.225

D'Adamo, S., Cetrullo, S., Panichi, V., Mariani, E., Flamigni, F., & Borzì, R. M. (2020). Nutraceutical Activity in Osteoarthritis Biology: A Focus on the Nutrigenomic Role. *Cells*, *9*(5). https://doi.org/10.3390/cells9051232

D'Altilio, M., Peal, A., Alvey, M., Simms, C., Curtsinger, A., Gupta, R. C., Canerdy, T. D., Goad, J. T., Bagchi, M., & Bagchi, D. (2007). Therapeutic Efficacy and Safety of Undenatured Type II Collagen Singly or in Combination with Glucosamine and Chondroitin in Arthritic Dogs. *Toxicology Mechanisms and Methods*, *17*(4), 189–196. https://doi.org/10.1080/15376510600910469

de Sousa, E. B., dos Santos Junior, G. C., Duarte, M. E. L., Moura Neto, V., & Aguiar, D. P. (2017). Metabolomics as a promising tool for early osteoarthritis diagnosis. *Brazilian Journal of Medical and Biological Research*, *50*(11). https://doi.org/10.1590/1414-431x20176485

Deparle, L. A., Gupta, R. C., Canerdy, T. D., Goad, J. T., D'Altilio, M., Bagchi, M., & Bagchi, D. (2005). Efficacy and safety of glycosylated undenatured type-II collagen (UC-II) in therapy of arthritic dogs. *Journal of Veterinary Pharmacology and Therapeutics*, *28*(4), 385–390. https://doi.org/10.1111/j.1365-2885.2005.00668.x

Dobenecker, B., Beetz, Y., & Kienzle, E. (2002). A Placebo-Controlled Double-Blind Study on the Effect of Nutraceuticals (Chondroitin Sulfate and Mussel Extract) in Dogs with Joint Diseases as Perceived by Their Owners. *The Journal of Nutrition*, *132*(6), 1690S-1691S. https://doi.org/10.1093/jn/132.6.1690S

Dodge, G. R., & Jimenez, S. A. (2003). Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. *Osteoarthritis and Cartilage*, *11*(6), 424–432. https://doi.org/10.1016/S1063-4584(03)00052-9

García-Coronado, J. M., Martínez-Olvera, L., Elizondo-Omaña, R. E., Acosta-Olivo, C. A., Vilchez-Cavazos, F., Simental-Mendía, L. E., & Simental-Mendía, M. (2019). Effect of collagen supplementation on osteoarthritis symptoms: A meta-analysis of randomized placebo-controlled trials. *International Orthopaedics*, *43*(3), 531–538. https://doi.org/10.1007/s00264-018-4211-5

Goldring, S. R., & Goldring, M. B. (2016). Changes in the osteochondral unit during osteoarthritis: Structure, function and cartilage–bone crosstalk. *Nature Reviews Rheumatology*, *12*(11), 632–644. https://doi.org/10.1038/nrrheum.2016.148

Gupta, R. C., Lall, R., Srivastava, A., & Sinha, A. (2019). Hyaluronic Acid: Molecular Mechanisms and Therapeutic Trajectory. *Frontiers in Veterinary Science*, *6*, 192. https://doi.org/10.3389/fvets.2019.00192

Harris, W. S., & Shearer, G. C. (2014). Omega-6 fatty acids and cardiovascular disease friend, not foe? *Circulation*, *130*(18), 1562–1564. Scopus. https://doi.org/10.1161/CIRCULATIONAHA.114.012534

Henrotin, Y., Lambert, C., Couchourel, D., Ripoll, C., & Chiotelli, E. (2011). Nutraceuticals: Do they

represent a new era in the management of osteoarthritis? - a narrative review from the lessons taken with five products. *Osteoarthritis and Cartilage*, *19*(1), 1–21. https://doi.org/10.1016/j.joca.2010.10.017

Henrotin, Yves, Sanchez, C., & Balligand, M. (2005). Pharmaceutical and nutraceutical management of canine osteoarthritis: Present and future perspectives. *Veterinary Journal (London, England: 1997)*, *170*(1), 113–123. https://doi.org/10.1016/j.tvjl.2004.08.014

Hielm-Björkman, A. (2007). *Assessment of chronic pain and evaluation of three complementary therapies (gold implants, green lipped mussel, and a homeopathic combination preparation) for canine osteoarthritis, using randomized, controlled, double-blind study designs*. Dept. of Equine and Small Animal Medicine, Univ. of Helsinki.

Hielm-Björkman, A., Rita, H., & Tulamo, R. (2009). Psychometric testing of the Helsinki chronic pain index by completion of a questionnaire in Finnish by owners of dogs with chronic signs of pain caused by osteoarthritis. *American Journal of Veterinary Research*, *70*, 727–734. https://doi.org/10.2460/ajvr.70.6.727

Jewell, D. E., Toll, P. W., Wedekind, K. J., & Zicker, S. C. (2000). Effect of increasing dietary antioxidants on concentrations of vitamin E and total alkenals in serum of dogs and cats. *Veterinary Therapeutics: Research in Applied Veterinary Medicine*, *1*(4), 264–272.

Jhun, J., Lee, S. H., Na, H. S., Seo, H.-B., Kim, E.-K., Moon, S.-J., Jeong, J.-H., Lee, D. H., Kim, S. J., & Cho, M.-L. (2015). The chicken combs extract alleviates pain and cartilage degradation in rat model osteoarthritis. *Tissue Engineering and Regenerative Medicine*, *12*(5), 352–361. https://doi.org/10.1007/s13770-015-0028-0

Johnston, S. A., McLaughlin, R. M., & Budsberg, S. C. (2008). Nonsurgical management of osteoarthritis in dogs. *The Veterinary Clinics of North America. Small Animal Practice*, *38*(6), 1449–1470, viii. https://doi.org/10.1016/j.cvsm.2008.08.001

Kalra, E. K. (2003). Nutraceutical-definition and introduction. *AAPS PharmSci*, *5*(3), 27–28. https://doi.org/10.1208/ps050325

Kim, L. S., Axelrod, L. J., Howard, P., Buratovich, N., & Waters, R. F. (2006). Efficacy of methylsulfonylmethane (MSM) in osteoarthritis pain of the knee: A pilot clinical trial. *Osteoarthritis and Cartilage*, *14*(3), 286–294. https://doi.org/10.1016/j.joca.2005.10.003

Kimmatkar, N., Thawani, V., Hingorani, L., & Khiyani, R. (2003). Efficacy and tolerability of Boswellia serrata extract in treatment of osteoarthritis of knee – A randomized double blind placebo controlled trial. *Phytomedicine*, *10*(1), 3–7. https://doi.org/10.1078/094471103321648593

Kulkarni, R. R., Patki, P. S., Jog, V. P., Gandage, S. G., & Patwardhan, B. (1991). Treatment of osteoarthritis with a herbomineral formulation: A double-blind, placebo-controlled, cross-over study. *Journal of Ethnopharmacology*, *33*(1), 91–95. https://doi.org/10.1016/0378-8741(91)90167-C

Martel-Pelletier, J., Barr, A. J., Cicuttini, F. M., Conaghan, P. G., Cooper, C., Goldring, M. B., Goldring, S. R., Jones, G., Teichtahl, A. J., & Pelletier, J.-P. (2016). Osteoarthritis. *Nature Reviews Disease Primers*, *2*(1), 1–18. https://doi.org/10.1038/nrdp.2016.72

Mehler, S. J., May, L. R., King, C., Harris, W. S., & Shah, Z. (2016). A prospective, randomized, double blind, placebo-controlled evaluation of the effects of eicosapentaenoic acid and docosahexaenoic acid on the clinical signs and erythrocyte membrane polyunsaturated fatty acid concentrations in dogs with

osteoarthritis. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *109*, 1–7. https://doi.org/10.1016/j.plefa.2016.03.015

Nasri, H., Baradaran, A., Shirzad, H., & Rafieian-Kopaei, M. (2014). New Concepts in Nutraceuticals as Alternative for Pharmaceuticals. *International Journal of Preventive Medicine*, *5*(12), 1487–1499.

Nelson, R. W., & Guillermo Couto, C. (2013). *Small Animal Internal Medicine—EBooks from Elsevier UK* (Fith edition). https://elsevierelibrary-co-uk.proxy.library.uu.nl/product/small-animal-internal-medicine60119

Oe, M., Tashiro, T., Yoshida, H., Nishiyama, H., Masuda, Y., Maruyama, K., Koikeda, T., Maruya, R., & Fukui, N. (2016). Oral hyaluronan relieves knee pain: A review. *Nutrition Journal*, *15*. https://doi.org/10.1186/s12937-016-0128-2

Parcell, S. (2002). Sulfur in human nutrition and applications in medicine. *Alternative Medicine Review: A Journal of Clinical Therapeutic*, *7*(1), 22–44.

Petrie, A., & Watson, P. (2013). *Statistics for Veterinary and Animal Science*. John Wiley & Sons, Incorporated. http://ebookcentral.proquest.com/lib/uunl/detail.action?docID=1178710

Plumb's Veterinary Drugs. (2020, April 9). https://www-plumbsveterinarydrugs-com.proxy.library.uu.nl/#!/monograph/kJWIDAfFGr/

Rannou, F., & Poiraudeau, S. (2010). Non-pharmacological approaches for the treatment of osteoarthritis. *Best Practice & Research Clinical Rheumatology*, *24*(1), 93–106. https://doi.org/10.1016/j.berh.2009.08.013

Reichling, J., Schmökel, H., Fitzi, J., Bucher, S., & Saller, R. (2004). Dietary support with Boswellia resin in canine inflammatory joint and spinal disease. *Schweizer Archiv Für Tierheilkunde*, *146*(2), 71–79. https://doi.org/10.1024/0036-7281.146.2.71

Rhouma, M., de Oliveira El Warrak, A., Troncy, E., Beaudry, F., & Chorfi, Y. (2013). Anti-inflammatory response of dietary vitamin E and its effects on pain and joint structures during early stages of surgically induced osteoarthritis in dogs. *Canadian Journal of Veterinary Research = Revue Canadienne De Recherche Veterinaire*, *77*(3), 191–198.

Roush, J. K., Cross, A. R., Renberg, W. C., Dodd, C. E., Sixby, K. A., Fritsch, D. A., Allen, T. A., Jewell, D. E., Richardson, D. C., Leventhal, P. S., & Hahn, K. A. (2010). Evaluation of the effects of dietary supplementation with fish oil omega-3 fatty acids on weight bearing in dogs with osteoarthritis. *Journal of the American Veterinary Medical Association*, *236*(1), 67–73. https://doi.org/10.2460/javma.236.1.67

Rychel, J. K. (2010). Diagnosis and treatment of osteoarthritis. *Topics in Companion Animal Medicine*, *25*(1), 20–25. https://doi.org/10.1053/j.tcam.2009.10.005

Tetlow, L. C., Adlam, D. J., & Woolley, D. E. (2001). Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: Associations with degenerative changes. *Arthritis and Rheumatism*, *44*(3), 585–594. https://doi.org/10.1002/1529-0131(200103)44:3<585::AID-ANR107>3.0.CO;2-C

Vandeweerd, J.-M., Coisnon, C., Clegg, P., Cambier, C., Pierson, A., Hontoir, F., Saegerman, C., Gustin, P., & Buczinski, S. (2012). Systematic review of efficacy of nutraceuticals to alleviate clinical signs of osteoarthritis. *Journal of Veterinary Internal Medicine*, *26*(3), 448–456. https://doi.org/10.1111/j.1939-1676.2012.00901.x

Vaughan-Scott, T., & Taylor, J. H. (1997). The pathophysiology and medical management of canine osteoarthritis. *Journal of the South African Veterinary Association*, *68*(1), 21–25. https://doi.org/10.4102/jsava.v68i1.861

Walrand, S., Chiotelli, E., Noirt, F., Mwewa, S., & Lassel, T. (2008). Consumption of a functional fermented milk containing collagen hydrolysate improves the concentration of collagen-specific amino acids in plasma. *Journal of Agricultural and Food Chemistry*, *56*(17), 7790–7795. https://doi.org/10.1021/jf800691f

Walton, M. B., Cowderoy, E., Lascelles, D., & Innes, J. F. (2013). Evaluation of Construct and Criterion Validity for the 'Liverpool Osteoarthritis in Dogs' (LOAD) Clinical Metrology Instrument and Comparison to Two Other Instruments. *PLOS ONE*, *8*(3), e58125. https://doi.org/10.1371/journal.pone.0058125

Wang, J., Verdonk, P., Elewaut, D., Veys, E. M., & Verbruggen, G. (2003). Homeostasis of the extracellular matrix of normal and osteoarthritic human articular cartilage chondrocytes in vitro. *Osteoarthritis and Cartilage*, *11*(11), 801–809. https://doi.org/10.1016/S1063-4584(03)00168-7

Yazar, M., Sarban, S., Kocyigit, A., & Isikan, U. E. (2005). Synovial fluid and plasma selenium, copper, zinc, and iron concentrations in patients with rheumatoid arthritis and osteoarthritis. *Biological Trace Element Research*, *106*(2), 123–132. https://doi.org/10.1385/BTER:106:2:123