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Randomized Clinical Trial to Analyze the Efficacy of an Eggshell Membrane Dietary Supplementation in the Concomitant Treatment of Osteoarthritis in Dogs

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Abstract: Osteoarthritis has become one of the most prevalent conditions that affect joints in dogs. Cartilage degradation and subsequent chronic inflammatory process affect synovial membranes, induce pain and decrease joint functionality. The aim of this research is to study the effects of supplementation with a nutraceutical based on internal egg shell membrane in dogs diagnosed with osteoarthritis in knee or hip joints. During a consumption period of 10 weeks, efficacy was evaluated in a placebo controlled clinical trial. Bioarth functional scale (BAS) was used to estimate joint functionality. Serum levels of C-telopeptides of type II collagen (CTx-II) were also collected as a biomarker of osteoarthritis. Functional limitation and joint mobility parameters showed an improvement by a decrease of BAS scores, although muscle atrophy showed no differences between any of the groups or periods considered in this study. Sterilization seems to have a higher influence in that parameter, indicating sex and condition as confounding variables. Antalgic postures significantly decreased in treatment group therefore indicating an improvement in the quality of life of subjects. Despite the fact that biomarker CTx-II has been used frequently in evaluation of cartilage degradation, no significant differences were observed in this study, although individual analysis revealed a greater reduction in a higher percentage of dogs from the treatment group. Osteoarthritis is a long-term disease, often frustrating for both veterinaries and owners. ESM could become a safe, effective alternative for chronic, long-term management of osteoarthritis in dogs.

Keywords: Knee Pain, Hip Pain, Nutraceutical, Joint Functionality, Bioarth Assessment Scale

1. Introduction

1.1. Background

Osteoarthritis (OA) is a progressive disease that is characterized by cartilage degradation, subchondral bone sclerosis, periarticular bone growing, and a development of a chronic inflammation process, affecting mainly synovial membranes [1, 2]. Clinical signs could include pain or tenderness, decreased range of motion, swelling, stiffness, muscle atrophy, crepitus, and effusion, that could lead to a change in animal's behavior [1, 2]. Affecting most of dogs over 5 years old [3, 4], OA prevalence increases because of a series of risk factors. Unmodifiable factors (e.g., sex, breed, and age) allow to identify individuals that could be more susceptible to suffer from this condition, meanwhile other

modifiable factors such as castration and body condition (overweight and obesity), increase the risk [5]. Other arthropathies are also considered predisposing risk factors. Hypocaloric diets and physical exercise has been successfully applied in weight loss and retention of lean body mass [6, 7]. However, therapeutic goals should be extended further pain control and joint functionality increase to ultimately improve the quality of life.

A variety of medications may be prescribed for pain relief. Non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and opiates are among the most common medication used for pain management. They must be prescribed by a veterinarian and have adverse effects [8], including accelerate cartilage degeneration with long-term use. Fortunately, our understanding about pain mechanisms of OA has led to a growing number of pharmaceutical treatment options over the last decade. Other new medications such as anti-NGF mAbs and piprants, as well as regenerative therapies, offer alternatives to improve welfare and quality of life for dogs with OA. Furthermore, novel curative treatments are opening exciting research areas, such as gene and mRNA therapy [9]. OA is a long-term disease, whose management supposes a challenge for both veterinarians and pet owners who support costly and frustrating treatments. As a consequence, a multimodal approach to OA management seems to become a better alternative to alleviate pain and in terms, slow the progression of this disease.

1.2. Literature Review

Nutraceutical interventions have garnered popularity, experiencing a rapid and substantial economic growth [3], and helping to diminish the use of other traditional therapies with potential minor adverse effects. Although their mechanisms of action have not been well-determined, in some cases, they provide the building blocks to some of the constituents of joint cartilage, decrease the effect of destructive enzymes, as well as also enhance the lubricating effect of the joint fluid and target anti-inflammatory, anti-oxidative and anti-catabolic actions [4]. Therapeutic diets usually incorporate these common substances, including omega-3-based nutraceuticals, collagen-based nutraceuticals, nutraceuticals based on chondroitin–glucosamine, cannabinoid-based nutraceuticals, nutraceuticals based on hydroxycitric acid, nutraceuticals based on calcium fructoborate, curcumoids, *Boswellia serrata* extracts, green-lipped mussel (*Perna canaliculus*), avocado and soybean unsaponifiables, or yeasts among other less commonly used and studied [4].

Among them, collagen has demonstrated an ability to prevent cartilage destruction [10]. Mechanism of action is based on the production of macromolecules and inhibition of catabolic enzymes, therefore increasing the synthesis of collagen type II and reducing inflammation [10]. Oral tolerance phenomenon of this nutraceutical also leads to elevated levels of 17 collagen-derived peptides transiently in the blood, with a particular enrichment in Gly-Pro-Hyp [11],

that involves activation of regulatory T cells (Tregs) [12]. Tregs are probably secreting IL-4, IL-10, and transforming growth factor-β [12], supporting anti-inflammatory activity and secondary processes of cartilage repairment. Moreover, it has been hypothesized that oral supplementation could be a therapeutic measure itself [13]. Exposition of collagen to gut immune system could be a way to regulated later immune response against collagen in an altered joint.

Such nutraceuticals have been supported by various research studies in dogs [14-22]. A recent review on this topic has led to important conclusions [4]. Collagen-based nutraceuticals showed lower efficacy compared with Omega-3 enriched diets, although the same authors pointed as a main cause for this observation to a lesser quality of included studies due to a number of causes such as small sample size (N lower than 10 dogs), non-validated subjective tools without observer guidance, non-adapted statistical methodology, and single assessment times in the follow-up period [4]. Uncertainty about their efficacy is in contrast with the results observed from the comparison of studies included in such review. Collagen-based nutraceuticals alone were scored the third out of nine considered categories of nutraceuticals (45.4% of studies that proven effect), after therapeutic diets based on omega-3 (50% of studies that proven effect), and omega-3 nutraceuticals that was the best classified category of them (70% of studies that proven effect). Authors concluded that collagen based nutraceuticals can not be properly evaluated, and further studies with better quality should be performed to provide conclusive results.

Among collagen formulations, eggshell membrane (ESM) derived nutraceuticals has been previously used in both humans [23-29] and pets [14, 19, 21]. Safety together with its natural origin make this nutraceutical a promising target as possible adjuvant treatment of OA [25]. Therefore, the objective of this study was to determine the efficacy of a food supplement extracted from the internal membrane of the egg shell to control pain in dogs that have been diagnosed with OA in knee or hip joints. During a consumption period of 10 weeks, efficacy was evaluated in a placebo controlled clinical trial. Bioarth functional scale was used to estimate functionality of both knee and hip joints. Such variable of quality of life was combined with serum levels of C-telopeptides of type II collagen (CTx-II) to estimate disease severity.

2. Methodology

A randomized, controlled, triple-blind, unicenter clinical trial was designed, in which individuals were assigned to internal membrane egg shell-based supplement (ESM) or placebo groups (Figure 1). This study was conducted during a period of 10-weeks (April-June, 2021). Study protocol was in compliance with national guidelines for animal research. This study was carried out in cooperation between the Catholic University of Murcia, and Veterinary Clinic Lur Gorri-Barañain and Veterinary Center Lur Gorri-Orkoien that were responsible for recruitment, getting owners' signed

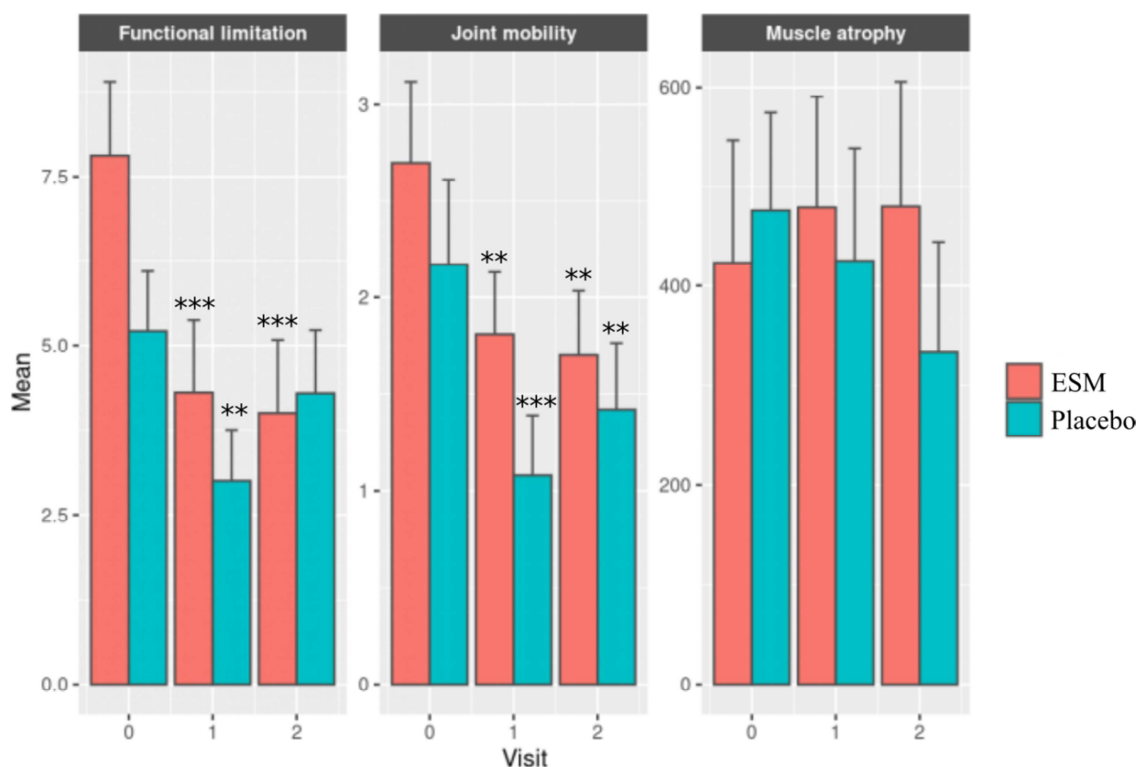consent and performing standard clinical tests.

*Figure 1. BAS results for each of the three parameters: functional limitation, joint mobility, and muscle atrophy [31], for both groups through the three visits programmed in this trial. Significance level is represented by p-values over mean bars following: 0.05 < * < 0.01 < ** < 0.001 < ***.*

To be eligible for enrollment, dogs were required to be more than 1 y.o., owner identified mobility impairment, and diagnosed with OA on hips or knees. Dogs were not allowed to receive glucocorticoids, e.g. oral prednisone, during the course of this study and, other analgesic drugs (e.g. gabapentin, tramadol, polysulfated glycosaminoglycan) were allowed occasionally under prescription. Exclusion criteria included concomitant treatments with omega-3, glucosamine, chondroitin sulphate, collagen o hyaluronic acid infiltrations or consumption of any supplement indicated for joint health, known allergy to eggs, and inability to comply with the protocol during the period of study. Subjects were also excluded when recorded known chronic inflammatory diseases affecting the musculoskeletal system (rheumatoid arthritis, etc.) and other serious illnesses (limiting pneumopathy, presence of arrhythmia). Owners should not change basic management protocol including diet, exercise and daily routines during this trial. Dogs remained with their owners during this trial.

ESM® was obtained from Torolis Explotaciones, S. L. (Navarra, Spain) and dispensed in capsules that were stored at ambient temperature, containing 200 mg of ESM. Control group was administered with a placebo based on encapsulated maltodextrin matching in size to the nutraceutical. The ESM was composed mainly of collagen proteins (I, V and X), glycosaminoglycans (chondroitin sulfate and dermatan sulfate), hexosamines (glucosamine), and hyaluronic acid in significant amounts. [24]. Doses were estimated according to a general dosage of 1 capsule for small-medium size (0-25 Kg of weight)

and 2 capsules for large dogs (>25 Kg). At screening, veterinary recorded breed, age, and sex, performing an individual physical and neurological examination. Dogs were then randomized to either get the nutraceutical or the placebo. Primary outcomes measures were gathered at screening/visit 0 (day 0), visit 1 (week 3), and visit 2 (week 10). On visit 0 and visit 2, serum CTx-II biochemistry profile was performed. Researchers and staticians involved in this study as well as owners, were masked until statistical analyses were finalized. Compliance with the treatment was evaluated during the last visit by owner's interviews and counting the number of capsules still remaining unused.

The primary outcome variable was the functional evaluation of OA that was assessed by a veterinary clinician by using Bioarth Assessment Scale (BAS) [29, 30]. BAS established a scoring system (from 0 to either 2 or 3), depending on the 12 different parameters considered that were grouped in 3 categories: functional limitation, joint mobility, and muscle atrophy [30]. Functional limitation considered changes in posture (antalgic postures), characteristics of lameness, and reluctance to move and play and scored up to 23 points. Range of motion was used to assess joint mobility by determining maximal extension and flexion by using a goniometer centered on the hip (scored up to 7 points). Additionally, any signs of pain due to manipulation during this procedure was also recorded. Two different veterinary clinicians performed this procedure for each group to allow different blinded observer. Muscle atrophy was qualified using a 3 degrees scale scoring

as no atrophy (0 points), mild atrophy (1 point), or severe atrophy (2 points). Only one clinician recorded this observation for all the groups and during all the visits. Lameness severity was then calculated by the sum of all functional parameters together to obtain the degree of arthrosis: higher scores indicated more severity (Additional file 2).

Telopeptides consist on the non-helical sequences at the end of the molecule of collagen. Monitoring of this marker has been demonstrated useful for evaluating OA progression [28]. Serum levels of C-telopeptide of type II collagen (CTx-II) were measured at both initial and ending visits. Blood samples were processed by serum separation, and stored. Upon completion of the trial, samples were packaged and shipped on dry ice to a laboratory, for analysis by using an available Serum Pre-clinical Cartilaps enzyme-linked Immunosorbent assay (ELISA) (Nordic Bioscience, Herlev, Denmark) with modifications proposed by [28].

Data analysis was performed by using the statistical software R version 4.2.2 Patched [31]. Parameters were estimated by using linear regressions models, and analyzed by a researcher who was not involved in the trial.

Data were examined for their distribution, and parametric or non-parametric statistical approaches were used as appropriate. The increases of each variable, both from visit 1 and 2, with respect to visit 0 were calculated. Then, the increases between the ESM group and the placebo group were compared using linear regression models, using the group as an explanatory variable and including in the model the baseline values (visit 0) centered on their mean, in order to control for the regression to the mean effect. Non-parametric distributions were compared using Kruskal-Wallis test. Type I error rate was set at alpha = 0.05.

Measured CTx-II biomarker was compared between screening (day 0) and study completion (week 10) for both nutraceutical and placebo groups.

3. Results

51 dogs diagnosed with knee and hip osteoarthritis,

chronic pain and lack of functionality, completed a 10-week study. Demographic information can be found in Table 1 and list of individual information can be also found in Additional file 1. 27 dogs were included in the ESM group registering a mean age of 10.3 ± 1.2 y.o. (12 females and 14 males). Placebo group was made up of 24 dogs in the ESM supplement group with an average age of 10.7 ± 1.39 y.o. (16 females and 8 males). No statistical differences between groups in terms of age and weight were found at Day 0. Evolution of weight throughout this study also showed no significant differences. Owners did not report any adverse events related to the consumption of the nutraceutical administered, therefore, indicating a good tolerability.

Functional limitation scores showed an improvement, decrease of BAS score for that parameter, for both groups during the first 3 weeks of this trial. However, such improvement was only significant at the end of the trial for ESM group (Figure 1). Placebo showed an increase in the score indicating a worsening for that parameter of the assessment using BAS. Joint mobility showed similar results to those registered for functional limitation. Both groups showed an improvement in the second visit, with a subsequent worsening in the placebo group. Muscle atrophy showed no differences between any of the groups or periods considered in this study. However, sterilization seemed to have a higher influence in the evaluation of this particular parameter. Individuals that were sterilized showed a significant trend towards an increase in atrophy (data not showed here). No other differences were observed in age or sex when establishing such comparisons (confounding). ESM group showed a statistically significant decrease in global BAS score through the period of study (Table 2). Although, there was a significant decrease from the beginning of this study to visit 1 (week 3) in both groups (-2.785 and -2.950 for ESM and placebo groups, respectively), such decrease was only significantly observed in visit 2 (week 10) for ESM group (-2.997 and -0.628 for ESM and placebo groups, respectively). Both groups showed significant differences throughout the trial.

Table 1. *Demographic information of dogs that completed this 10-week study. Age and weight are represented by the 95% confidence interval.*

| | N | male /female | sterilized | age | weight (Kg) | | |
					visit 0	visit 1	visit 2
ESM	27	14 / 12	6	10.3 ± 1.2	18.4 ± 5.3	20.7 ± 6.1	19.4 ± 5.5
Placebo	24	8 / 16	5	10.7 ± 1.4	15.2 ± 4.7	13.4 ± 4.3	13.5 ± 4.1
P-value	-	0.240	-	0.552	0.528	0.339	0.208

Quality of life was also evaluated as changes in posture (antalgic postures), that was directly estimated from functional limitation parameter in BAS (Figure 2). Scoring significantly decreased in ESM group measured throughout the 2 periods and compared to placebo group. No other significant differences were observed for this subcategory.

Table 2. *Mean differences for BAS scores in both ESM and placebo groups between visits: visit 0 and visit 1 (week 3), and visit 1 (week 3) and visit 2 (week 10).*

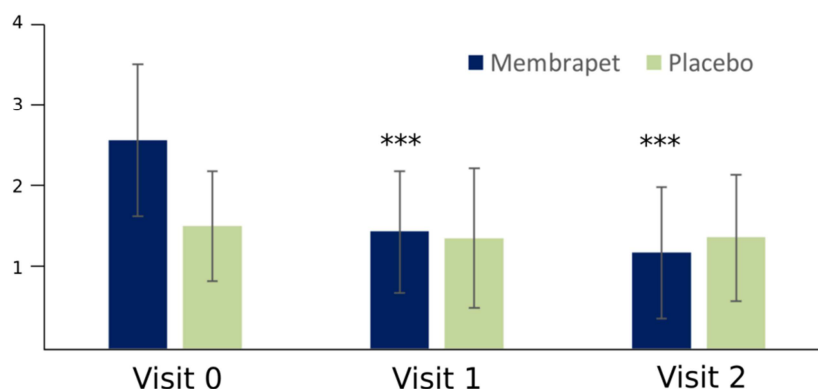|	group	differences	p-value
visit 1 (week3)	Membrapet (N=27)	-2.785	0.004
	Placebo (N=24)	-2.950	0.004
visit 2 (week 10)	Membrapet (N=27)	-2.997	0.002
	Placebo (N=24)	-0.628	0.518

Figure 2. Quality of life evaluated as changes in posture (antalgic postures), as directly extracted from functional limitation parameter in BAS. Both groups through the three visits programmed in this trial are represented. Significance level is represented by p-values over mean bars between visits, following: 0.05 < * < 0.01 < ** < 0.001 < ***.

Biomarker CTx-II showed high variability intragroup, what can be inferred from the confidence interval (Table 3). Despite that fact, both groups showed a significant reduction for this marker of degradation of cartilage. Comparison between groups at both beginning and ending of this trial showed no significant differences (Table 3). In order to establish the role of such reduction in both groups, individual changes were analysed in those individuals that showed a reduction in this biomarker, discriminating from those in whom no reduction was observed. 42.8 % of individuals treated with ESM showed a reduction higher than 50 mg/dL, in comparison with 31.57% of individuals from the placebo group.

Table 3. 95% confidence interval for CTx-II biochemistry profile in both ESM and placebo groups at visit 0, visit 1 (week 3), and visit 2 (week 10).

	N	visit 0	visit 2	P-value
ESM	27	50.8 ± 23.8	17.7 ± 11.0	0.000
Placebo	24	47.7 ± 14.2	18.9 ± 15.4	0.000

4. Discussion

Osteoarthritis is a long-term disease that suppose a challenge for veterinary clinicians and owners, including a number of clinical signs such as pain or tenderness, decreased range of motion, swelling, stiffness, muscle atrophy, crepitus, and effusion, that could led animal's behavior to change [1, 2]. Intervention measures such as physical rehabilitation, weight loss, and therapeutic diets, may increase quality of life and allow to reduce NSAIDs use and dose [3]. In fact, some nutraceuticals have been determined really effective, such as fatty acid omega-3 (alone or as enriched in therapeutic diets), followed by collagen-based nutraceuticals. The most common form is based on internal egg shell membrane [4].

ESM-based nutraceuticals have been demonstrated useful for the treatment of OA. Our results agree with such findings. Functional limitation evaluation based on BAS global scores shows a significant decrease. However, during the first 3 weeks of this study, results were similar to those in the control group. Such observation is not recorded after 10

weeks of treatment, when ESM sustains the same level of improvement meanwhile control group suffers a worsening in clinical signs of OA. This fact could be indicating that nutraceuticals from this family, are long term action compounds. Oral ingestion leads to elevated levels of collagen-derived peptides in the blood [11]. However, mechanisms of functional peptides transference to the joint cartilage remain still unclear. Effects could be limited by the bio-availability of the different transient types of collagen-derived peptides, therefore emphasizing long term effects of this nutraceutical and prolonged treatments to enjoy the benefits of its use.

Functional limitation evaluation by studying the three BAS parameters (functional limitation, joint mobility and muscle atrophy), allows us to understand effects of ESM on quality of life in dogs with OA. Functional limitation and joint mobility show a similar behaviour during this study. In fact, both parameters contribute to a general conclusion of improvement after 10 weeks of treatment. However, both parameters do not perform better than control group after 3 weeks of treatment. This observation could be indicating a mask effect due to other treatments (e.g., NSAIDs) which could be improving clinical signs of OA at short term, that could be only sustained after 10 weeks due to amelioration of joint cartilage quality as a result of nutraceutical consumption [11].

Skeletal muscle wasting is probably playing an important role in the development and progression of OA. Our findings disagree with previous studies that showed an increase in muscular perimeter in the legs [14]. Muscle atrophy is not showing any improvements in our study. Pain can cause a disruption in muscle fibers, that when not fully active, weakness and atrophy are the most likely outcomes. As a consequence, pain control due to this nutraceutical could be limited. Previous studies have already showed that inflammatory mediators such as TNF-α or N-methylhistamine, are not being modified by ESM consumption, although IL-2 over time is experiencing a significant decrease during the consumption of this nutraceutical [19]. Despite that fact, methodology of such study could be also influencing authors' observations. A subjective scale built from questionnaires filled by the owners is used. Owners' perception could be bias

because of expectancy and knowledge about the nutraceutical that could lead to contradictory results when compared to objective measures from blood serum parameters. Such observation will be congruent with our findings, therefore indicating the role of long term effects as a result of ESM chronic use rather than immediate, greater effects. In any case, other confounding variables seem to play a significant role in muscle atrophy as significant results are found in sterilized males. Reduced testosterone is associated with both loss of muscle and increased adiposity [32]. Such effect could be masking beneficial effects from ESM supplementation on this parameter.

Quality of life is evaluated by combining BAS scoring results from changes in posture. Such partial scores significantly decrease during this trial in ESM group. Improvement in antalgic postures could be considered as a marker of wellness since it is indicating both amelioration of joint functionality and decrease in noxious stimuli. Further studies focused on an extended set of markers could provide a better understanding about this particular variable.

Objective marker used in this study do not provide significant insides about ESM mechanism of action. Biomarker CTx-II did not report significant results towards protection effects against degradation of joint cartilage. However, individual analysis of results from subjects that experienced a reduction in that biomarker, showed that CTx-II shows higher reduction (more than 50 mg/dL) in ESM group for a higher percentage of subjects (42.8 % compared to 31.57% from the placebo).

5. Conclusion

Our results are supported by previous evidences of the benefits of ESM supplementation on animal welfare, mainly by improving joints in dogs with osteoarthritis. Beneficial effects suggest that peptide availability provided by this supplement may improve cartilage state. Further studies are needed to establish the basis of the mechanism of action of ESM supplementation in joint health as well as possible synergies among different nutraceuticals to increase overall effectiveness. ESM supplementation shows positive effects to ameliorate osteoarthritis but limited significant findings in reducing pain. Due to an increase in the prevalence of conditions that limited joint functionality and therefore decrease quality of life of pets around the world, ESM could become a safe, effective alternative to practitioners and owners to provide chronic, long-term treatment alternatives.

Acknowledgements

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