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(54) Title: JOINT CARE COMPOSITION

(57) Abstract: The present invention relates to a composition comprising curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline for use in preventing or treating osteoarthritis. It also relates to a method of preventing or treating osteoarthritis in mammals, the method comprising administering to said mammal a composition which comprises curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline.

JOINT CARE COMPOSITION

The present invention relates to a composition comprising curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline for use in preventing or treating osteoarthritis. It also relates to a method of preventing or treating osteoarthritis in mammals, the method comprising administering to said mammal a composition which comprises curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline.

Cartilage deterioration can be caused by several reasons such as repeated exercise, instability of the joint, etc., which may result in inflammation of the joints. While a greater portion of humans with arthritis have rheumatoid arthritis, most of the arthritis occurring in companion animals is osteoarthritis.

Nowadays, no cure exists for osteoarthritis, and the pharmacological treatment is limited to alleviating symptoms. The most popular are non-steroidal anti-inflammatory drugs, but these are associated with adverse effects. A safer treatment is desirable.

The first aspect of this invention relates to a composition comprising curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline for use in preventing or treating osteoarthritis. Treating osteoarthritis includes ameliorating osteoarthritis symptoms.

The present invention relates, for all aspects, to any mammal, including a human. In particular, the present invention relates to a companion animal such as a dog, a cat or an equine animal (e.g. a horse) or any other such animal that suffers or is prone to suffer from osteoarthritis.

The composition of the present invention comprises curcuminoid. Curcuminoid is curcumin or a derivative of curcumin. The chemical structures of curcuminoids differ in their functional groups.

Curcuminoid includes curcumin, demethoxycurcumin, *bis*-methoxycurcumin and/or

tetrahydrocurcumin.

Curcuminoids are natural phenols that are present, in particular, in the Indian spice turmeric. Turmeric is derived from the roots of the plant *Curcuma longa*. Curcuminoids have also been found in roots of other species in the plant family Zingiberaceae of the *Curcuma* genus. Curcuminoids have a distinctly earthy, bitter, peppery flavour and a mustardy smell.

In particular, turmeric contains 60-80% curcumin, 15-30% demethoxycurcumin and 2-6% *bis*-demethoxycurcumin.

The curcuminoid in the composition of the invention can be of any format, including a powder or lipid extract.

In some embodiments, curcuminoid can be mixed with phospholipids and/or cellulose, starch or derivatives thereof to form complexes. This may assist in stability and/or to further increase solubility and bioavailability of the curcuminoid.

The curcuminoid can be mixed with essential oils, piperine or bromelain. The curcuminoid can be mixed with phosphatidycholine, for example lecithin.

Preferably, the curcuminoid of the present invention is curcumin, which is the most active curcuminoid. Curcumin according to the present invention includes demethoxycurcumin, *bis*-demethoxycurcumin and/or tetrahydrocurcumin.

The composition of the invention comprises curcuminoid and green tea polyphenol.

Tea (*Camellia sinensis*), in particular green tea, has a high content of flavonoids, including polyphenols, in particular catechins. Catechins in tea include epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), catechin, and gallocatechin (GC).

Preferably, the green tea polyphenols include catechin. Preferably, the catechin includes EGCG. Green tea extract usually contains at least about 25% polyphenols, about 12.5% of catechins and about 9.3% of EGCG.

Epigallocatechin gallate (EGCG) is the ester of epigallocatechin and gallic acid. EGCG is the most abundant catechin in tea and is a potent antioxidant. It is particularly found in green tea. EGCG is a major polyphenol of green tea and exhibits anti-oxidant, anti-tumour and anti-mutagenic activities.

The composition of the invention comprises curcumin and a combination of glycine, proline and hydroxyproline.

A combination of glycine, proline and hydroxyproline represents 50% of the total amino acid content of hydrolyzed collagen. Preferably, a combination of glycine, proline and hydroxyproline is hydrolyzed collagen. The amino acid composition of hydrolyzed collagen is as set in the table below;

Table 1

Amino acids	**Percentage**
Proline/Hydroxyproline	25%
Glycine	20%
Glutamic acid	11%
Arginine	8%
Alanine	8%
Other essential amino acids	16%
Other non-essential amino acids	12%

Hydrolyzed collagen is obtained by the enzymatic hydrolysis of collagenous tissues found in the bones, skin, and connective tissue of animals such as cattle, fish, horses, pigs, and rabbits. Hydrolyzed collagen is well digested and is preferentially accumulated in cartilage.

A preferred composition includes curcuminoid, green tea polyphenol and a combination of glycine, proline and hydroxyproline. Preferably, this composition includes curcumin, green tea polyphenol and hydrolyzed collagen.

The invention is preferably a foodstuff. It can be any foodstuff, such as dry, semi moist or wet food product. In particular, the foodstuff may be a pet food product.

The pet foodstuff is preferably a commercial pet food product. Such a product is preferably sold as a product for feeding to a pet animal, in particular a pet cat or a pet dog.

A typical pet foodstuff contains about 20-30% crude protein and about 10-20% fat, the remainder being carbohydrate, including dietary fibre and ash. A typical wet or moist product contains (on a dry matter basis) about 40% fat, 50% protein and the remainder being fibre and ash. The foodstuff of the invention may be a dry product (with approximately 5 to approximately 15% moisture), a semi-moist product (with approximately 15 to approximately 70% moisture) or a wet product (with approximately 70 to approximately 90% moisture).

The remaining components of the foodstuff are not essential to the invention and typical standard products can be included. The combined ingredients of the foodstuff according to the invention can provide all of the recommended vitamins and minerals for the particular animal in question (a complete and balanced food).

The foodstuff according to the present invention encompasses any product which a pet consumes in its diet. Thus, the invention covers standard food products including liquids, as well as pet food snacks (for example, snack bars, pet chew, crunchy treat, cereal bars, snacks, biscuits and sweet products) and supplements.

The foodstuff can be provided as a food supplement. The food supplement can be a powder, sauce, topping, biscuit, kibble, pocket or tablet that can be administered with or without an additional foodstuff. Where the food supplement is administered with an additional foodstuff, the food supplement can be administered sequentially simultaneously or separately. The food supplement may be mixed with the foodstuff, sprinkled over the foodstuff or served separately. Alternatively, the food supplement can be added to a liquid provided for drinking such as water or milk.

The foodstuff is preferably a cooked product. It may incorporate meat or animal derived material (such as beef, chicken, turkey, lamb, fish, blood plasma, marrow bone etc. or one or more thereof). The product alternatively may be meat free (preferably including a meat substitute such as soya, maize gluten or a soya product) in order to provide a protein source. The foodstuff may contain additional protein sources such as soya protein concentrate, milk proteins, gluten etc. The foodstuff may also contain a starch source such as one or more grains (e.g. wheat, corn, rice, oats, barley etc.), or may be starch free.

The foodstuff of the invention is preferably produced as a dry product containing from approximately 5% to approximately 15% moisture. The preferred dry food is more preferably presented as a small biscuit – like kibbles.

The table below details the amount of the composition according the present invention and the amount of the composition for the dogs to take according to the present invention:

Table 2

	Diet 1	Diet 2	Diet 3	Energy need (kcal/kg^0,75)		
	3515 kcal	2500 kcal	5600 kcal	80	95	145
	%DM	%DM	%DM	mg/400 kcal	mg/400 kcal	mg/400 kcal
Curcuma (turmeric) extract	0,171	0,07	0,32	210	175	102
Curcuminoids	0,034	0,013	0,065	43	35	22
Curcumin	0,026	0,01	0,05	33	27	15
Green tea extract	0,341	0,155	0,63	414	349	225
Green tea poly	0,085	0,035	0,16	106	87	51
Green tea EGCG	0,032	0,01	0,06	39	32	15
Collagen hydrolyzed	1,706	0,7	3,2	2108	1747	1016
Glycine	1,158	0,53	2,15	1416	1185	767
Proline	1,358	0,63	2,5	1651	1390	911
Hydroxyproline	0,177	0,08	0,33	217	182	116
Total gly+pro+hydroxypro	2,693	1,2	5	3295	2757	1736

The composition in the first aspect of the invention may comprise curcuminoid at an amount ranging from about 0.005 to 1.1% by weight of curcuminoid on an “as is” weight percent of the food. The amount of curcuminoid can be any amount from 0.005 to 1.1% (as is). The amount of curcuminoid can be any amount from 0.1 to 1% (as is). The amount of curcuminoid can be any amount from 0.1 to 0.6% (as is). The amount of curcuminoid can be any amount from 0.3 to 0.6% (as is).

The composition in the first aspect of the invention may comprise curcuminoid at an amount ranging from about 0.005 to 0.15% by weight of curcuminoid on an “as is” weight percent of the food. The amount of curcuminoid can be any amount from 0.005 to 0.15% (as is) (7 to 99 mg/400 kcal).

When the diet is dry the “as is” weight is the same as the “dry matter weight”.

Preferably, the amount of curcuminoid in the composition ranges from about 0.01 to 0.07% (as is) (14 to 46 mg/400 kcal). Most preferably, the amount of curcuminoid is 0.035% (as is) (36 mg/400 kcal).

In some embodiments, the curcuminoid in the composition is curcumin at an amount ranging from about 0.005 to 0.15% by weight of curcumin on an “as is” weight percent of the food. The amount of curcumin can be any amount from 0.005 to 0.15% (as is) (7 to 99 mg/400 kcal). Preferably, the amount of curcumin ranges from about 0.01 to 0.05% (as is) (14 to 32 mg/400 kcal). Most preferably, the amount of curcumin is 0.026% (as is) (27 mg/400 kcal).

The composition in the first aspect of the invention may comprise green tea polyphenol in an amount ranging from about 0.01 to 1.1 % by weight of green tea polyphenol on an “as is” weight percent of the food. The amount of green tea polyphenol can be any amount from 0.01 to 1.1% (as is). The amount of green tea polyphenol can be any amount from 0.1 to 1% (as is). The amount of green tea polyphenol can be any amount from 0.1 to 0.6% (as is). The amount of green tea polyphenol can be any amount from 0.3 to 0.6% (as is).

The composition in the first aspect of the invention may comprise green tea polyphenol in an amount ranging from about 0.01 to 0.3 % by weight of green tea polyphenol on an “as is” weight percent of the food. The amount of green tea polyphenol can be any amount from 0.01 to 0.3 % (as is) (14 to 197 mg/400 kcal). Preferably, the amount of green tea polyphenol ranges from about 0.03 to 0.17% (as is) (43 to 113 mg/400 kcal). Most preferably, the amount of green tea polyphenol is 0.085% (as is) (87 mg/400 kcal).

In some embodiments, the green tea polyphenol is EGCG at an amount ranging from about 0.005 to 0.2% by weight of EGCG on an “as is” weight percent of the food (7 to 131 mg/400 kcal). The amount of EGCG can be any amount from 0.01 to 0.06% (as is) (14 to 39 mg/400 kcal). Most preferably, the amount of EGCG is 0.032% (as is) (33 mg/400 kcal).

The composition in the first aspect of the invention may comprise a combination of glycine, proline and hydroxyproline in an amount ranging from about 0.5 to 10% by weight of combined glycine, proline and hydroxyproline on an “as is” weight percent of the food. The amount of combined glycine, proline and hydroxyproline can be any amount from 0.5 to 10% (as is) (720 to 6591 mg/400 kcal). Preferably, the amount of combined glycine, proline and hydroxyproline ranges from about 1.2 to 5% (as is) (1736 to 3295 mg/400 kcal). Most preferably, the amount of combined glycine, proline and hydroxyproline is 2.7% (as is) (2780 mg/400 kcal).

In some embodiment, the combination of glycine, proline and hydroxyproline is hydrolyzed collagen in an amount ranging from about 0.5 to 5% by weight of combined glycine, proline and hydroxyproline on an “as is” weight percent of the food. The amount of hydrolyzed collagen can be any amount from 0.5 to 5% (as is) (720 to 3295 mg/400 kcal). Preferably, the amount of hydrolyzed collagen ranges from about 0.7 to 3.2% (as is) (1016 to 2138 mg/400 kcal). Most preferably, the amount of hydrolyzed collagen is 1.7% (as is) (1750 mg/400 kcal).

In other embodiments, the composition may comprise curcumin in an amount of about 27 mg/400 kcal (35 mg/400 kcal of curcuminoids) with about 87 mg/400 kcal of green tea polyphenol and with about 2757 mg/400 kcal of combined glycine, proline and hydroxyproline, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen. Preferably, wherein the combination of glycine, proline and

hydroxyproline is hydrolyzed collagen and is present in the composition at an amount of about 1747mg/400kcal.

In other embodiments, the composition may comprise curcumin in an amount of about 33 mg/400 kcal (43mg/400 kcal of curcuminoids) with about 106 mg/400 kcal of green tea polyphenol and with about 3295 mg/400 kcal of combined glycine, proline and hydroxyproline, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen. Preferably, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen and is present in the composition at an amount of about 2108mg/400kcal.

In other embodiments, the composition may comprise curcumin in an amount of about 15 mg/400 kcal (22mg/400 kcal of curcuminoids) with about 51mg/400 kcal of green tea polyphenol and with about 1736 mg/400 kcal of combined glycine, proline and hydroxyproline, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen. Preferably, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen and is present in the composition at an amount of about 1016mg/400kcal.

These values apply to a composition for feeding to a mammal, in particular a companion animal.

The second aspect of the invention relates to a method of preventing or treating osteoarthritis in mammals.

Osteoarthritis (OA) is a degenerative and inflammatory condition that affects the joints in mammals. It is also known as degenerative arthritis or degenerative joint disease. Osteoarthritis is a group of abnormalities involving degradation of joints, including articular cartilage and sub-chondral bone.

Osteoarthritis is the consequence of an imbalance of catabolism and anabolism, wherein catabolism is increased; anabolism is decreased causing the inflammation of chondrocytes. Chondrocytes are the only cells found in healthy cartilage. They produce and maintain the cartilaginous matrix, which consists mainly of collagen and proteoglycans. The composition of the invention has demonstrated to provide, *inter alia*, a decrease in inflammation, a decrease in catabolism and an increase in anabolism in *in*

vitro inflammation-induced chondrocytes and in *in vitro* healthy chondrocytes. Thus, the composition of the invention prevents and/or treats osteoarthritis in animals.

The present invention relates, for all aspects, to any mammal, including a human. In particular the present invention relates to a companion animal such as a dog, a cat or an equine animal (e.g. a horse) or any other such animal that suffers or is prone to suffer from osteoarthritis.

In particular, it is a desire in the area of pet foodstuff and companion animal health to provide foodstuff including supplements suitable to support the health of the companion animals. In particular, it is desire to provide diets suitable to promote or maintain the health of already healthy companion animals.

In particular, the second aspect of the invention provides a method for preventing and treating osteoarthritis in mammals, including ameliorating the symptoms of osteoarthritis, in particular companion animals. The method comprises administering to said animal a composition which comprises curcumin with green tea polyphenol or with a combination of glycine, proline and hydroxyproline. The animal may be in need thereof. Since a significant number of dogs suffer from osteoarthritis in their lifetime, all dogs can be considered as in need of prevention.

In particular embodiments, the method comprises administering to said animal a composition comprising curcumin, green tea polyphenol and a combination of glycine, proline and hydroxyproline. Most preferably, the combination of glycine, proline and hydroxyproline is hydrolysed collagen.

Further, the method is preferably administered to an animal, in particular a companion animal, that suffers from osteoarthritis and is in need of ameliorating the symptoms of osteoarthritis or in need of preventing further symptoms of osteoarthritis or in need of treatment of osteoarthritis. This may be to, for example a young pet animal, such as a puppy, or an older companion animal. Where the composition is a foodstuff, the foodstuff may be administered in a dietary regime in accordance with the usual dietary regime of the companion animal. The foodstuff may comprise 100% of the diet of the companion animal or a lesser proportion, depending on the level of prevention or treatment required. The foodstuff allows the composition to be administered with ease thus avoiding a need to supplement the companion animal's food. In addition, the foodstuff can be administered

by the animal's owner thus avoiding constant veterinary supervision. The foodstuff may be available at any outlet selling pet food products or may be available from a veterinarian. The foodstuff may be as described above according to the first aspect of the invention.

As used herein, the term "administration" also includes feeding or any other method of oral administration. Other means of administration may include tablets, capsules, injection, suppositories or any other suitable means.

Preferred features for the second aspect of the invention apply as for the first aspect *mutatis mutandis*.

The present description includes a method for preparing the composition of the first aspect of the invention.

The foodstuff can be made according to any method known in the art such as in Waltham Book of Dog and Cat Nutrition, Ed. ATB Edney, Chapter by A. Rainbird, entitled "A Balanced Diet" in pages 57 to 74 Pergamon Press Oxford.

For example, a process for the manufacture of a foodstuff as defined herein comprises mixing together ingredients with the composition comprising curcuminoid with green tea polyphenol or with a combination of glycine, proline and hydroxyproline and forming a foodstuff, in particular a pet foodstuff. Heating/cooking may be applied to any one or more of the ingredients prior to, during or following the mixing.

The composition can be sprayed onto the foodstuff, mixed in with the foodstuff or incorporated into the foodstuff in a matrix. Methods of inclusion of the composition are known in the art.

The importance of the present invention is the beneficial properties of curcuminoid with either green tea polyphenol or with a combination of glycine, proline and hydroxyproline (optionally as hydrolyzed collagen). In particular, an effect which is more than the cumulative effect is seen.

A further benefit is seen with the triple combination of ingredients of: curcuminoid, green tea polyphenol and glycine, proline and hydroxyproline (optionally as hydrolyzed collagen).

The combination of the compounds of the composition of the present invention can provide a synergistic effect in terms of one or more of decreasing inflammation, decreasing catabolism and increasing anabolism.

The invention will now be further described by way of reference to the following Examples, which are provided for the purpose of illustration only and are not to be construed as being limiting on the invention.

Example 1: Individual screening of compounds

Experiments were carried out to assess the effect of several compounds on primary culture of bovine chondrocytes, in which inflammatory and catabolic processes are induced by interleukin-1beta to mimic the effect of arthritic chondrocytes.

The table below details the biomarkers that were measured throughout the experiments to show the effect of the compounds of the three metabolic pathways on chondrocytes.

Table 3: Biomarkers tested

Biomarkers produced by chondrocytes	Inflammation	NO
		PGE2
Genes expressed by chondrocytes		IL-6
		COX2
		iNOS
	Catabolism	MMP3
		ADAMTS4
		ADAMTS5
	Anabolism	COL2
		AGG

Primary culture of bovine chondrocytes in monolayer

Normal bovine articular cartilage was obtained from the metacarpal-phalangeal joint of 1 to 2 year old steers shortly after death. Full-depth articular cartilage was excised and immersed in Dulbecco's Modified Eagle Medium (DMEM) (with phenol red and 4.5 g/L glucose) supplemented with N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

(HEPES) 10 mM, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (all from Lonza, Verviers, Belgium). After three washings, chondrocytes were released from cartilage by sequential enzymatic digestions with 0.5 mg/ml hyaluronidase type IV S (Sigma-Aldrich, Bornem, Belgium) for 30 min at 37 °C, 1 mg/ml pronase E (Merck, Leuven, Belgium) for 1 h at 37 °C and 0.5 mg/ml clostridial collagenase IA (Sigma-Aldrich, Bornem, Belgium) for 16 to 20 h at 37 °C. The enzymatically isolated cells were then filtered through a nylon mesh (70 µm), washed three times, counted and filled to the density of 0.25 x 106 cells/ml of DMEM (with phenol red and 4.5 g/L glucose) supplemented with 10 % foetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine (all from Lonza, Verviers, Belgium) and 20 µg/ml proline (Sigma-Aldrich, Bornem, Belgium). Cells were seeded in a 6-well plate at 0.5 X 106 cells/well by adding 2 ml of the previously described culture medium/well and cultured in monolayer for 5 days. Chondrocytes were then cultured in monolayer until confluence (for about 2 days) in DMEM (phenol red-free and containing only 1 g/L glucose) (Lonza, Verviers, Belgium) supplemented with 1 % fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine and 20 µg/ml proline. Only primary cultures were used to ensure the stability of chondrocyte phenotype.

When cells achieved confluence, the culture medium was removed and replaced by fresh culture medium (DMEM phenol red-free and containing only 1 g/L glucose supplemented with 1 % fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine and 20 µg/ml proline) containing some nutraceuticals (12.5 µg/ml of each of them) and in the absence or in the presence of recombinant porcine IL-1β (10^{-11} M) (RD System, Abingdon, UK).

The anti-inflammatory power of the compounds (firstly individually and then in combination) was tested by measuring the chondrocyte viability and the production of PGE2 and NO.

The compounds were added in the culture medium either before inflammation (prevention effect measurement), either simultaneously of the inflammation (treatment effect measurement).

List of compounds screened:

1) Fish oil: 18%EPA+10%DHA (DSM)
2) EPA 99% (Sigma):
3) DHA 99% (Sigma):

4) Aloe Vera (Naturex)

5) Nettle leaf extract (Naturex)

6) Resvida: 99% Resvératrol (DSM)

7) Green tea extract: 25% polyphenols of which 12.5 % are catéchines and 8% is EGCG: (Naturex)

8) Pine bark extract: Pycnogenol: 65-75% procyanidines (Biolandes)

9) Premix of vitamins including vitamin D3

10) GLM (AromaNZ)

11) collagen hydrolysate (Fortigel de Gelita [3,3kDa] : hydrolyzed pork collagen)

12) ASU (Sochim)

13) Curcuma powder : 85% curcuminoids (Naturex)

The results showed the 3 compounds to use were curcuma extract, hydrolysate collagen and green tea extract, which showed significant effects on different parameters. After that, these 3 compounds were tested in response-dose.

Example 2: Dose responses

The methodology of example 1 was followed. Four different concentrations were tested to cover the range of concentrations corresponding to 10^{-5} M, depending on the molecular weight: 0.5 µg/ml, 2.5 µg/ml, 12.5 µg/ml and 62.5 µg/ml.

The results showed that the concentration which gave the best effects without giving toxic effects was 12.5µg/ml for each compound. This is why the concentration of 12.5µg/ml was used for testing compounds in combination with each other.

Example 3: Testing particular combinations of the compounds and the synergistic effects

The method of example 1 was followed.

Supplementation with compounds

When cells achieved confluence, the culture medium was removed and replaced by fresh culture medium (DMEM phenol red-free and containing only 1 g/L glucose supplemented with 1 % fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine and 20 µg/ml proline) containing some compounds (12.5 µg/ml of each of

them) and in the absence or in the presence of recombinant porcine IL-1β (10^{-11} M) (RD System, Abingdon, UK).

The three compounds were tested namely, curcuma extract (Naturex, Avignon, France), hydrolysate collagen (Gelita, Eberbach, Germany) and green tea extract (Naturex, Avignon, France). Curcuma extract was prepared as a 12.5 mg/ml solution in tetrahydrofuran (Merck, Leuven, Belgium) and then further diluted 1000 times in cell culture medium. Hydrolysate collagen and green tea extract were dissolved in water at the concentration of 12.5 mg/ml, filtered through a sterile mesh (0.20 µm) and then further diluted 1000 times in cell culture medium. The compounds were tested alone at the final concentration of 12.5 µg/ml or in combination (12.5 µg/ml curcuma extract + 12,5 µg/ml hydrolysate collagen; 12.5 µg/ml curcuma extract + 12.5 µg/ml green tea extract; 12.5 µg/ml curcuma extract + 12.5 µg/ml hydrolysate collagen + 12.5 µg/ml green tea extract) in the absence or in the presence of recombinant porcine IL-1β (10^{-11} M). The effects of the compounds were compared to controls: DMEM alone or DMEM + IL-1β.

Culture stop

After 24 h in these conditions, conditioned culture medium of three wells of each condition was collected and stored at -20°C. The cells of these corresponding wells were scrapped, an RNA extraction was made using RNeasy mini kit (Qiagen, Venlo, Netherlands), a reverse transcriptase polymerase chain reaction was realised and then a quantitative real time polymerase chain reaction was realised, using the LightCycler 480 (Roche, Vilvoorde, Belgium) to analyse gene expression.

After 48 h in these conditions, conditioned culture medium of the remaining wells (3 of each condition) was collected (lactate dehydrogenase release assay) and stored at -20°C until analysis (nitrite and prostaglandin E2 assays). Cells were scrapped and homogenized in 500 µl of Tris-HCl buffer by ultrasonic dissociation for 20 s at 4 °C, to measure DNA content.

Lactate dehydrogenase release assay

Cell viability was estimated by quantifying the release of lactate dehydrogenase (LDH) in the culture supernatant. A 100 µl sample of the supernatant or dilutions of standard solution (LDH from rabbit muscle) was mixed with 50 µl of Tris buffer (10 mM Tris-HCl (pH 8.5), 0.1 % bovine serum albumin) containing 800 mM lactate. Then, 50 µl of colorimetric

reagent, 1.6 mg/ml iodonitrotetrazolium chloride (Sigma-Aldrich, Bornem, Belgium), 4 mg/ml nicotinamide adenine dinucleotide (Roche Diagnostics, Brussels, Belgium), and 0.4 mg/ml phenazine methosulfate (Sigma-Aldrich, Bornem, Belgium) were added, and the absorbance at 492 nm was read after 10 min of incubation at room temperature.

DNA assay

Chondrocytes were homogenized in 500 µl of Tris-HCl buffer by ultrasonic dissociation for 15 s at 4 °C. DNA content was measured in the cell extracts using the fluorimetric method of Hoechst.

Nitrite assay

Nitric oxide (NO) production was determined by quantifying its derived product, nitrite, in the culture supernatant using a spectrophotometric method based upon the Griess reaction. Briefly, 100 µl of the supernatant or sodium nitrite ($NaNO_2$) standard dilutions were mixed with 100 µl of Griess reagent (0.5 % sulphanilamide, 0.05 % naphtyl ethylene diamine dihydrochloride, 2.5 % H_3PO_4). The absorption was measured at 540 nm. The production of NO was expressed per microgram of DNA.

PGE2 assay

Prostaglandin E2 (PGE2) production was measured in the culture supernatant using the DetectX PGE2 High Sensitivity Immunoassay kit (Arbor Assays, Michigan, USA). Briefly, 100 µl of the supernatant or PGE2 standard dilutions were pipetted into a clear microtiter plate coated with an antibody to capture mouse IgG. A PGE2-peroxidase conjugate (25 µl) is added to the standards and supernatants in the wells. The binding reaction is initiated by the addition of 25 µl of a monoclonal antibody to PGE2. After an overnight incubation at 4°C, the plate is washed and 100 µl of substrate is added. The substrate reacts with the bound PGE2-peroxidase conjugate. After a short incubation, the reaction is stopped and the intensity of the generated colour is detected at 450 nm wavelength. The production of PGE2 was expressed per microgram of DNA.

Quantitative real-time reverse transcriptase polymerase chain reaction (RT PCR)

RNA from cells from 3 wells of each condition was isolated using RNeasy mini kit (Qiagen, Venlo, Netherlands). Then, RNA was reverse transcribed. Quantitative real time

Polymerase Chain Reaction (PCR) was performed by using the SYBR Premix Ex Taq (Tli RNaseH Plus) (Westburg, Leusden, Netherlands). The PCR template source was either first-strand cDNA or purified DNA standard. Primer sequences used to amplify the desired cDNA were as follows: bovine HPRT forward and reverse primers: 5'-AGTTTGGAAATACCTGGCG-3' and 5'-AGTCTTTAGGCTCGTAGTGC-3'; bovine interleukin (IL)-6 forward and reverse primers: 5'- TGGTGATGACTTCTGCTTTCC-3' and 5'- TGCCAGTGTCTCCTTGC-3'; bovine cyclooxygenase (COX)2 forward and reverse primers: 5'-GTCTGATGATGTATGCCACC-3' and 5'-ACGTAGTCTTCAATCACAATCT-3'; bovine induced nitric oxide synthase (iNOS) forward and reverse primers: 5'-GGCAAGCACCACATTGAGA-3' and 5'- TGCGGCTGGATTTCGGA-3'; bovine aggrecans (AGG) forward and reverse primers: 5'-TGCCTTTGACGTGAGC-3' and 5'-GCATTGTTGTTGACAAACT-3'; bovine type II collagen (COL2) forward and reverse primers: 5'-CTGCGTCTACCCCAAC-3' and 5'-GGGTGCAATGTCAATGAT-3'; bovine metalloproteinase (MMP)-3 forward and reverse primers: 5'-TCTATGAAGGAGAAGCTGACATAAT-3' and 5'-TTCATGGGCAGCAACAAG-3'; bovine A Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTS) 4 forward and reverse primers: 5'- CTTTCAATGTCCCACAGGC-3' and 5'-CAGGAACGGAAGCGGGTA-3'; bovine ADAMTS 5 forward and reverse primers: 5'-GACACCCTGGGAATGGCA-3' and 5'- CACAGAACTTGGAATCGTCA-3'.
Amplification was performed with a spectrofluorometric thermal cycler (LightCycler 480, Roche Diagnostics, Vilvoorde, Belgium). To standardize mRNA levels, we amplified HPRT, a housekeeping gene, as an internal control. Gene expression was normalized by calculating the ratio between the number of cDNA copies of IL-6, COX2, iNOS, AGG, COL2, MMP-3, ADAMTS4, ADAMTS5, and that of HPRT.

Results were expressed as the mean percentage of increase compared to the control. Statistical significance was assessed using the t-test. Differences were considered statistically significant at $p < 0.05$. Table below details the results provided when combining the compounds and the synergistic effects observed.

Table 4

			Control	Curcumin (=C)	Hydrolyzed collagen (=O)	Green tea (=T)	CO	CT	COT
Chondrocyte produced biomarkers	Inflammation	NO	0	0	0	0	0	0	0
		NO+IL1B	100	-96*	23**	-14*	-100***	-100*	-100***
		PGE2	0	0	0	0	0	0	0
		PGE2+ IL1B	100	-99*	1**	363**	-100*	-100***	-100***
Genes expressed by chondrocytes	Inflammation	IL-6	100	-82*	307**	252**	-43*	-38*	-65*
		IL-6+ IL1B	100	-84*	-1*	-8*	-89*	-100*	-99*
		COX2	100	49**	54**	86**	378**	84**	27**
		COX2+IL1B	100	-51*	-24*	-26*	-58*	-87*	-92*
		iNOS	100	-76*	123**	207**	-65***	-27***	-25***
		iNOS+IL1B	100	-86*	-13*	15**	-91*	-96*	-97*
	Catabolism	MMP3	100	40**	109**	161**	91*	-10***	-15***
		MMP3+IL1B	100	-58*	-20*	-22*	-85*	-99*	-99*
		ADAMTS4	100	-21*	12**	9**	16**	-14*	-58***
		ADAMTS4 + IL1B	100	-55*	-23*	-28*	-68*	-83*	-84*
		ADAMTS5	100	-16*	2**	32**	-31***	-28***	-41***
		ADAMTS5+ IL1B	100	-47*	-13*	22**	-52*	-76***	-71***
	Anabolism	COL2	100	-77**	13*	18*	-84**	-62**	-74**
		COL2+IL1B	100	-57**	-2**	55*	-67**	29***	67***
		AGG	100	-77**	54*	33*	-78**	-30**	-53**
		AGG+ IL1B	100	-77**	-4**	123*	-82**	186***	337***

(* Beneficial effect; ** Negative effect; *** More beneficial than expected)

Discussion

The results of the combinations were better than the additive effect of each compound. An explanation is that because compounds act on different metabolic ways which are related, when there is inflammation, catabolism increases and anabolism decreases. Thus, our non-limiting hypothesis is that curcumin inhibits inflammation induced by IL-1 β (and also induced by collagen and green tea). Once the inflammation is inhibited, catabolism decreases and collagen and green tea polyphenols can have their positive effect on anabolism. Given arthrosis is a vicious circle (inflammation induces catabolism which induces inflammation, etc), when catabolism decreases (and anabolism increases), there is a decrease of inflammation and we recover a virtuous circle.

Moreover, in general, in healthy cells there is always a balance between catabolism and anabolism. We saw that the combinations could have positive effects on the metabolism

of healthy cells (with no induction of inflammation by IL-1β). It is very interesting because, in case of arthrosis or before arthrosis, cells which are still in good health can be protected by our combinations.

CLAIMS

1. A composition comprising curcuminoid with green tea polyphenol and with a combination of glycine, proline and hydroxyproline for use in the prevention and/or treatment of osteoarthritis.

2. A composition for use as claimed in claim 1, wherein the prevention and/or treatment is for a human or a pet companion such as a cat, a dog or a horse.

3. A composition for use as claimed in any one of claims 1 to 2, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen.

4. A composition for use as claimed in any one of claims 1 to 3, wherein the curcuminoid is curcumin.

5. A composition for use as claimed in any one of claims 1 to 4, wherein the composition is in the form of a foodstuff.

6. A composition for use as claimed in any one of claims 1 to 5, wherein the curcuminoid is present at an amount of about 0.005% to 1.1% on a dry matter basis.

7. A composition for use as claimed in any of claims 1 to 6, wherein the green tea polyphenol is present at an amount of about 0.01% to 1.1% on a dry matter basis.

8. A composition for use as claimed in any of claims 1 to 7, wherein the combination of glycine, proline and hydroxyproline is present at an amount of about 0.5% to 10% on dry matter basis.

9. A composition for use as claimed in any one of claims 1 to 8, wherein the curcuminoid is curcumin and is present at an amount of about 0.005% to 0.15% on a dry matter basis.

10. A method of preventing or treating osteoarthritis in a mammal, comprising the step of administering to said mammal a composition comprising curcuminoid with green tea polyphenol and with a combination of glycine, proline and hydroxyproline.

11. The method of any one of claim 10, wherein the mammal is a human or pet companion such as a cat, a dog or a horse.

12. The method of any one of claims 10 to 11, wherein the combination of glycine, proline and hydroxyproline is hydrolyzed collagen.

13. The method of any one of claims 10 to 12, wherein curcuminoid is curcumin.

14. The method of any one of claims 10 to 13, wherein the composition is in the form of a foodstuff.

15. The method of any one of claims 10 to 14, wherein the curcuminoid is present at an amount of about 0.005% to 1.1% on a dry matter basis.

16. The method of any one of claims 10 to 15, wherein the green tea polyphenol is present at an amount of about 0.01 to 1.1% on a dry matter basis.

17. The method of any one of claims 10 to 16, wherein the combination of glycine, proline and hydroxyproline is present at an amount of about 0.5 to 10% on a dry matter basis.

18. The method of any one of claims 10 to 17, wherein the curcuminoid is curcumin and is present at an amount of 0.005% to 0.15% on a dry matter basis.

19. A composition comprising curcuminoid, green tea polyphenol and a combination of glycine, proline and hydroxyproline.

20. A composition as claimed in claim 19, wherein the combination of glycine, proline and hydroxyproline is hydrolysed collagen.

21. A composition as claimed in claim 19 or claim 20, wherein the curcuminoid is curcumin.

22. A composition as claimed in any one lf claims 19 to 21, wherein the composition is in the form of a foodstuff.

23. A composition as claimed in any one of claims 19 to 22, wherein the curcuminoid is present at an amount of about 0.005% to 1.1% on a dry matter basis.

24. A composition as claimed in any one of claims 19 to 23, wherein the green tea polyphenol is present at an amount of about 0.01% to 1.1% on a dry matter basis.

25. A composition as claimed in any one of claims 19 to 24, wherein the combination of glycine, proline and hydroxyproline is present at an amount of about 0.5% to 10% on dry matter basis.

26. A composition as claimed in any one of claims 19 to 25, wherein the curcuminoid is curcumin and is present at an amount of about 0.005% to 0.15% on a dry matter basis.

27. A process for the preparation of a composition as claimed in any one of claims 19 to 26 comprising mixing together the ingredients and optionally presenting the mixture in a form suitable for composition and administration.

28. A process as claimed in claim 27, wherein the composition is added to the mixture in the form of a spray, coating or in a starch pocket matrix.

29. A composition as claimed in any one of claims 19 to 26, for use in medicine.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2014/059850

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☒ subsequently to this Authority for the purpose of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/059850

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K36/82 A61K38/00 A61K45/06 A61K31/05 A61K31/353
A61P19/02
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2010/106191 A1 (BIOXTRACT S A [BE]; PRIEM FABIAN [BE]; JACQUEMOND-COLLET INGRID [BE]) 23 September 2010 (2010-09-23) page 18 - page 19; example 1 page 26 - page 28; example 6 claims 1, 16, 20, 21 -----	1-29
Y	KR 2012 0033633 A (UNIV ULSAN FOUND FOR IND COOP [KR]) 9 April 2012 (2012-04-09) claims 1, 6-8 paragraph [0018] - paragraph [0022] -----	1-29
Y	WO 00/74662 A2 (UNIV SHEFFIELD [GB]; BUTTLE DAVID [GB]; ADCOCKS CLAIR [GB]; COLLIN PET) 14 December 2000 (2000-12-14) page 8 claims 1, 2, 6 ----- -/--	1-29

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 2014	14/08/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Uryga-Polowy, V

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	US 2006/172012 A1 (FINLEY JOHN W [US] ET AL) 3 August 2006 (2006-08-03) page 7; table 7 claims 1-4, 36 paragraph [0099] example 1	1-29
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Y	BENITO-RUIZ P ET AL: "A randomized controlled trial on the efficacy and safety of a food ingredient, collagen hydrolysate, for improving joint comfort", INTERNATIONAL JOURNAL OF FOOD SCIENCES AND NUTRITION, CARFAX PUBLISHING LTD, GB, vol. 60, no. Suppl. 2, 1 January 2009 (2009-01-01), pages 99-113, XP009139049, ISSN: 0963-7486, DOI: 10.1080/09637480802498820 abstract page 111	1-29

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2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TOMÃ AA TRÄ ET AL: "Efficacy and tolerance of enzymatic hydrolysed collagen (EHC) vs. glucosamine sulphate (GS) in the treatment of knee osteoarthritis (KOA)", INTERNATIONAL ORTHOPAEDICS, SPRINGER, BERLIN, DE, vol. 35, no. 3, 19 April 2010 (2010-04-19) , pages 341-348, XP019887381, ISSN: 1432-5195, DOI: 10.1007/S00264-010-1010-Z abstract page 341, column 2, paragraph 2 page 345 page 348 -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/059850

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		WO 2006083666 A1	10-08-2006

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A61P 19/02(2006.01)

权利要求书2页 说明书12页
序列表4页

(54)发明名称

关节护理组合物

(57)摘要

本发明涉及一种用于预防或治疗骨关节炎用途的组合物，所述组合物包含姜黄色素与绿茶多酚，或与甘氨酸、脯氨酸和羟脯氨酸的组合。本发明还涉及一种在哺乳动物中预防或治疗骨关节炎的方法，所述方法包括对所述哺乳动物给药一种组合物，所述组合物包含姜黄色素与绿茶多酚，或它们与甘氨酸、脯氨酸和羟脯氨酸的组合。

CN 105431159 A

1. 一种用于预防和 / 或治疗骨关节炎用途的组合物，所述组合物包含姜黄色素与绿茶多酚，以及与甘氨酸、脯氨酸和羟脯氨酸的组合。

2. 如权利要求 1 所述用途的组合物，其中，所述预防和 / 或治疗是用于人类或者如猫、犬或马的宠物伴侣。

3. 如权利要求 1-2 中任一项所述用途的组合物，其中，所述甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原。

4. 如权利要求 1-3 中任一项所述用途的组合物，其中，所述姜黄色素是姜黄素。

5. 如权利要求 1-4 中任一项所述用途的组合物，其中，所述组合物是以食料的形式。

6. 如权利要求 1-5 中任一项所述用途的组合物，其中，所述姜黄色素以干物质为基础以约 0.005％至 1.1％的量存在。

7. 如权利要求 1-6 中任一项所述用途的组合物，其中，所述绿茶多酚以干物质为基础以约 0.01％至 1.1％的量存在。

8. 如权利要求 1-7 中任一项所述用途的组合物，其中，所述甘氨酸、脯氨酸和羟脯氨酸的组合以干物质为基础以约 0.5％至 10％的量存在。

9. 如权利要求 1-8 中任一项所述用途的组合物，其中，所述姜黄色素是姜黄素，并以干物质为基础以约 0.005％至 0.15％的量存在。

10. 一种预防或治疗哺乳动物中的骨关节炎的方法，所述方法包括对所述哺乳动物给药一种组合物的步骤，所述组合物包含姜黄色素与绿茶多酚以及与甘氨酸、脯氨酸和羟脯氨酸的组合。

11. 如权利要求 10 所述的方法，其中所述哺乳动物是人类或者如猫、犬或马的宠物伴侣。

12. 如权利要求 10-11 中任一项所述的方法，其中所述甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原。

13. 如权利要求 10-12 中任一项所述的方法，其中所述姜黄色素是姜黄素。

14. 如权利要求 10-13 中任一项所述的方法，其中所述组合物是以食料的形式。

15. 如权利要求 10-14 中任一项所述的方法，其中，所述姜黄色素以干物质为基础以约 0.005％至 1.1％的量存在。

16. 如权利要求 10-15 中任一项所述的方法，其中，所述绿茶多酚以干物质为基础以约 0.01％至 1.1％的量存在。

17. 如权利要求 10-16 中任一项所述的方法，其中，所述甘氨酸、脯氨酸和羟脯氨酸的组合以干物质为基础以约 0.5％至 10％的量存在。

18. 如权利要求 10-17 中任一项所述的方法，其中，所述姜黄色素是姜黄素，并以干物质为基础以 0.005％至 0.15％的量存在。

19. 一种组合物，所述组合物包含姜黄色素、绿茶多酚以及甘氨酸、脯氨酸和羟脯氨酸的组合。

20. 如权利要求 19 所述的组合物，其中，所述甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原。

21. 如权利要求 19 或 20 所述的组合物，其中，所述姜黄色素是姜黄素。

22. 如权利要求 19-21 中任一项所述组合物，其中，所述组合物是以食料的形式。

23. 如权利要求 19-22 中任一项所述的组合物，其中，所述姜黄色素以干物质为基础以约 0.005％至 1.1％的量存在。

24. 如权利要求 19-23 中任一项所述的组合物，其中，所述绿茶多酚以干物质为基础以约 0.01％至 1.1％的量存在。

25. 如权利要求 19-24 中任一项所述的组合物，其中，所述甘氨酸、脯氨酸和羟脯氨酸的组合以干物质为基础以约 0.5％至 10％的量存在。

26. 如权利要求 19-25 中任一项所述的组合物，其中，所述姜黄色素是姜黄素，并以干物质为基础以约 0.005％至 0.15％的量存在。

27. 一种制备权利要求 19-26 中任一项所述的组合物的方法，所述方法包括将配料混合在一起，并任选地以适合组合物和给药的形式呈现混合物。

28. 如权利要求 27 所述的方法，其中，将所述组合物以喷雾、涂层或淀粉囊基质的形式添加至混合物中。

29. 权利要求 19-26 中任一项所述的组合物用在药物中的用途。

关节护理组合物

[0001] 本发明涉及一种用于预防或治疗骨关节炎用途的组合物，其包含姜黄色素(curcuminoid)与绿茶多酚，或与甘氨酸、脯氨酸和羟脯氨酸的组合。本发明还涉及一种在哺乳动物中预防或治疗骨关节炎的方法，所述方法包括对所述哺乳动物给药一种组合物，所述组合物包含姜黄色素与绿茶多酚，或与甘氨酸、脯氨酸和羟脯氨酸的组合。

[0002] 软骨退化可由几种原因引起，如反复运动、关节的不稳定性等，其可能导致关节的炎症。虽然较大部分患有关节炎的人类具有类风湿性关节炎，但是发生在伴侣动物中的大部分关节炎是骨关节炎。

[0003] 现今，对骨关节炎的治愈并不存在，并且药物治疗限于减轻症状。最常用的是非甾体抗炎药，但是这些药物与不良反应相关。较安全的治疗是所期望的。

[0004] 本发明的第一方面涉及一种用于预防或治疗骨关节炎用途的组合物，其包含姜黄色素与绿茶多酚，或与甘氨酸、脯氨酸和羟脯氨酸的组合。治疗骨关节炎包括减轻骨关节炎的症状。

[0005] 本发明对于所有方面涉及任何哺乳动物，包括人类。具体而言，本发明涉及患有或易于患有骨关节炎的伴侣动物，如犬、猫或马科动物（例如马）或任何其它这种动物。

[0006] 本发明的组合物包含姜黄色素。姜黄色素(curcuminoid)是姜黄素(curcumin)或姜黄素的衍生物。多种姜黄色素的化学结构在它们官能团上存在不同。

[0007] 姜黄色素包括姜黄素、去甲氧基姜黄素(demethoxycurcumin)、双去甲氧基姜黄素(bis-methoxycurcumin)和/或四氢姜黄素(tetrahydrocurcumin)。

[0008] 姜黄色素是天然酚类，其特别存在于印度香料姜黄(turmeric)中。姜黄来源于植物姜黄(Curcuma longa)的根。姜黄色素还存在于姜科(Zingiberaceae)姜黄属(Curcuma genus)植物的其它种的根中。姜黄色 素明显具有土味、苦味、辛辣味和芥末的气味。

[0009] 具体而言，姜黄含有60-80％姜黄素、15-30％去甲氧基姜黄素和2-6％双去甲氧基姜黄素。

[0010] 本发明的组合物中的姜黄色素可以是任何形式的，包括粉末或脂质提取物。

[0011] 在一些实施方案中，姜黄色素可以与磷脂和/或纤维素、淀粉或其衍生物混合，以形成复合物。这可以有助于稳定性和/或进一步增加姜黄色素的溶解性和生物利用度。

[0012] 姜黄色素可以与精油(essential oils)、胡椒碱或菠萝蛋白酶混合。姜黄色素可以与磷脂酰胆碱例如卵磷脂混合。

[0013] 优选地，本发明的姜黄色素是姜黄素，其是最具活性的姜黄色素。根据本发明的姜黄素包括去甲氧基姜黄素、双去甲氧基姜黄素和/或四氢姜黄素。

[0014] 本发明的组合物包含姜黄色素和绿茶多酚。

[0015] 茶（茶树(Camellia sinensis)），特别是绿茶，具有高含量的类黄酮，包括多酚，特别是儿茶精类(catechins)。茶中的儿茶精类包括表没食子酸儿茶素-3-没食子酸酯(epigallocatechin-3-gallate,EGCG)、表儿茶素(epicatechin,EC)、表儿茶素-3-没食子酸酯(epicatechin-3-gallate,ECG)、表没食子酸儿茶素(epigallocatechin,EGC)、儿茶素和没食子儿茶素(gallocatechin,GC)。

[0016] 优选地，绿茶多酚包括儿茶素。优选地，儿茶素包括EGCG。绿茶提取物通常含有至少约25％多酚、约12.5％儿茶精类和约9.3％EGCG。

[0017] 表没食子酸儿茶素没食子酸酯(epigallocatechin gallate，EGCG)是表没食子酸儿茶素和没食子酸的酯。EGCG是茶中最丰富的儿茶素并且是强抗氧化剂。其特别存在于绿茶中。EGCG是主要的绿茶多酚并显示抗氧化、抗肿瘤和抗诱变活性。

[0018] 本发明的组合物包含姜黄素以及甘氨酸、脯氨酸和羟脯氨酸的组合。

[0019] 甘氨酸、脯氨酸和羟脯氨酸的组合占水解胶原的总氨基酸含量的50％。优选地，甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原。水解胶原的氨基 酸组成列于下表中：

[0020] 表1

[0021]

氨基酸	百分比
脯氨酸/羟脯氨酸	25％
甘氨酸	20％
谷氨酸	11％
精氨酸	8％
丙氨酸	8％
其它必需氨基酸	16％
其它非必需氨基酸	12％

[0022] 水解胶原是通过存在于动物如牛、鱼、马、猪和兔的骨、皮(skin)和结蹄组织中的胶原组织的酶法水解获得。水解胶原是好消化的，并优先积累在软骨中。

[0023] 优选的组合物包括姜黄色素、绿茶多酚以及甘氨酸、脯氨酸和羟脯氨酸的组合。优选地，该组合物包括姜黄素、绿茶多酚和水解胶原。

[0024] 本发明优选地是食料。其可以是任何食料，如干食品、半潮湿食品或湿食品。特别是，所述食料可以是宠物食品。

[0025] 宠物食料优选地是商品化的宠物食品。这种产品优选作为用于喂给宠物动物特别是宠物猫或宠物犬的产品而售卖。

[0026] 典型的宠物食料含有约20-30％粗蛋白和约10-20％脂肪，余量为碳水化合物，包括膳食纤维和灰分。典型的湿或潮湿产品包含(以干物质为基础)：约40％脂肪、50％蛋白质和余量的纤维和灰分。本发明的食料可以是干产品(具有约5％至约15％水分)、半潮湿产品(具有约15％至约70％水分)或湿产品(具有约70％至约90％水分)。

[0027] 食料的剩余组分对本发明不是必需的，并且典型的标准产品可以被包括在内。根据本发明的食料的组合成分可提供针对所讨论的特定动物的所 有建议的维生素和矿物质(完全且均衡的食物)。

[0028] 根据本发明的食料包括宠物在其饮食中消耗的任何产品。因此，本发明涵盖了标准食品，包括液体，以及宠物食物点心(snacks)(例如，点心棒(snack bar)、宠物咀嚼物、松脆零食、谷物棒(cereal bar)、点心(snacks)、饼干和甜味产品)和补充剂。

[0029] 食料可以作为食物补充剂提供。食物补充剂可以是可与其它食料或不与其它食料一同给予的粉末、调味汁、顶料(topping)、饼干、粗粒物(kibble)、囊(pocket)或小块(tablet)。当食物补充剂与其它食料一同给予时，食物补充剂可以顺序地同时或分开给予。食物补充剂可以与食料混合、撒在食料上或分开食用。或者，可将食物补充剂加入为饮用而提供的液体如水或奶中。

[0030] 食料优选地是熟食 (cooked food)。其可以包括肉或动物来源的材料 (如牛肉、鸡肉、火鸡肉、羔羊肉、鱼肉、血浆、髓性骨 (marrow bone) 等,或它们的一种或多种)。该产品或者可以是不含肉的 (优选地包括肉替代物,如大豆、玉米麸质或大豆制品),以便提供蛋白质源。食料可以含有其它蛋白质源,如大豆蛋白浓缩物、奶蛋白、麸质等。食料还可以含有淀粉源,如一种或多种谷物 (例如小麦、玉米、大米、燕麦、大麦等),或者可以是无淀粉的。

[0031] 本发明的食料优选地生产成含有约 5％至约 15％水分的干产品。优选的干食物更优选地以小饼干 - 样粗粒物出现。

[0032] 下表具体描述了根据本发明的组合物的量和根据本发明的为犬食用的组合物的量 :

[0033] 表 2

[0034]

	食物 1	食物 2	食物 3	能量需求（kcal/kg^0.75）		
	3515 千卡（kcal）	2500 千卡（kcal）	5600 千卡（kcal）	80	95	145
	%DM	%DM	%DM	mg/400 kcal	mg/400 kcal	mg/400 kcal
姜黄（curcuma）（姜黄(turmeric)）提取物	0.171	0.07	0.32	210	175	102
姜黄色素	0.034	0.013	0.065	43	35	22
姜黄素	0.026	0.01	0.05	33	27	15
绿茶提取物	0.341	0.155	0.63	414	349	225
绿茶多酚（green tea poly）	0.085	0.035	0.16	106	87	51
绿茶 EGCG	0.032	0.01	0.06	39	32	15
水解胶原	1.706	0.7	3.2	2108	1747	1016
甘氨酸	1.158	0.53	2.15	1416	1185	767
脯氨酸	1.358	0.63	2.5	1651	1390	911
羟脯氨酸	0.177	0.08	0.33	217	182	116
甘氨酸+脯氨酸+羟脯氨酸的总量	2.693	1.2	5	3295	2757	1736

[0035] 在本发明的第一方面中的组合物可以包含姜黄色素，按食物的“现状（as is）”重量百分比以姜黄色素的重量计，其量的范围为约 0.005％至 1.1％。姜黄色素的量可以是 0.005％至 1.1％（现状）的任意量。姜黄色素的量可以是 0.1％至 1％（现状）的任意量。姜黄色素的量可以是 0.1％至 0.6％（现状）的任意量。姜黄色素的量可以是 0.3％至 0.6％（现状）的任意量。

[0036] 在本发明的第一方面中的组合物可以包含姜黄色素，按食物的“现状”重量百分比以姜黄色素的重量计，其量的范围为约 0.005％至 0.15％。姜黄 色素的量可以是 0.005％至 0.15％（现状）(7 至 99mg/400kcal) 的任意量。

[0037] 当食物是干的时，“现状”重量与“干物质重量”相同。

[0038] 优选地，姜黄色素在组合物中的量的范围是约 0.01％至 0.07％（现状）(14 至 46mg/400kcal)。更优选地，姜黄色素的量是 0.035％（现状）(36mg/400kcal)。

[0039] 在一些实施方案中，组合物中的姜黄色素是姜黄素，姜黄素的量按食物的“现状”重量百分比以姜黄素的重量计范围为约 0.005％至 0.15％。姜黄素的量可以是 0.005％至 0.15％（现状）(7 至 99mg/400kcal) 的任意量。优选地，姜黄素的量的范围是约 0.01％至 0.05％（现状）(14 至 32mg/400kcal)。最优选地，姜黄素的量是 0.026％（现状）(27mg/400kcal)。

[0040] 在本发明的第一方面中的组合物可以包含绿茶多酚，按食物的“现状”重量百分比以绿茶多酚的重量计，其量的范围为约 0.01％至 1.1％。绿茶多酚的量可以是 0.01％至 1.1％（现状）的任意量。绿茶多酚的量可以是 0.1％至 1％（现状）的任意量。绿茶多酚的量可以是 0.1％至 0.6％（现状）的任意量。绿茶多酚的量可以是 0.3％至 0.6％（现状）的任意量。

[0041] 在本发明的第一方面中的组合物可以包含绿茶多酚，按食物的“现状”重量百分比以绿茶多酚的重量计，其量的范围为约 0.01％至 0.3％。绿茶多酚的量可以是 0.01％至 0.3％（现状）(14 至 197mg/400kcal) 的任意量。优选地，绿茶多酚的量的范围是约 0.03％至 0.17％（现状）(43 至 113mg/400kcal)。最优选地，绿茶多酚的量是 0.085％（现状）(87mg/400kcal)。

[0042] 在一些实施方案中，绿茶多酚是 EGCG，EGCG 的量按食物的“现状”重量百分比以 EGCG 的重量计范围为约 0.005％至 0.2％。EGCG 的量可以是 0.01％至 0.06％（现状）(14 至 39mg/400kcal) 的任意量。最优选地，EGCG 的量是 0.032％（现状）(33mg/400kcal)。

[0043] 在本发明的第一方面中的组合物可以包含甘氨酸、脯氨酸和羟脯氨酸的组合，按食物的“现状”重量百分比以组合的甘氨酸、脯氨酸和羟脯氨酸的重量计，其量的范围为约 0.5％至 10％。组合的甘氨酸、脯氨酸和羟脯氨酸的量可以是 0.5％至 10％（现状）(720 至 6591mg/400kcal) 的任意量。优选地，组合的甘氨酸、脯氨酸和羟脯氨酸的量的范围是约 1.2％至 5％（现 状）(1736 至 3295mg/400kcal)。最优选地，组合的甘氨酸、脯氨酸和羟脯氨酸的量是 2.7％（现状）(2780mg/400kcal)。

[0044] 在一些实施方案中，甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原，按食物的“现状”重量百分比以组合的甘氨酸、脯氨酸和羟脯氨酸的重量计，其量的范围是约 0.5％至 5％。水解胶原的量可以是 0.5％至 5％（现状）(720 至 3295mg/400kcal) 的任意量。优选地，水解胶原的量的范围是约 0.7％至 3.2％（现状）(1016 至 2138mg/400kcal)。最优选地，水解胶原的量是 1.7％（现状）(1750mg/400kcal)。

[0045] 在其它实施方案中，组合物可包含约 27mg/400kcal(35mg/400kcal 的姜黄色素）的姜黄素和约 87mg/400kcal 的绿茶多酚以及约 2757mg/400kcal 的组合的甘氨酸、脯氨酸和羟脯氨酸，其中，甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原。优选地，其中甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原，且在组合物中存在的量为约 1747mg/400kcal。

[0046] 在其它实施方案中，组合物可包含约 33mg/400kcal 的姜黄素 (43mg/400kcal 的姜黄色素）和约 106mg/400kcal 的绿茶多酚以及约 3295mg/400kcal 的组合的甘氨酸、脯氨酸和羟脯氨酸，其中，甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原。优选地，其中甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原，且在组合物中存在的量为约 2108mg/400kcal。

[0047] 在其它实施方案中，组合物可包含约 15mg/400kcal 的姜黄素 (22mg/400kcal 的姜黄色素）和约 51mg/400kcal 的绿茶多酚以及约 1736mg/400kcal 的组合的甘氨酸、脯氨酸

和羟脯氨酸，其中，甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原。优选地，其中甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原，且在组合物中存在的量为约 1016mg/400kcal。

[0048] 这些值适用于喂给哺乳动物、特别是伴侣动物的组合物。

[0049] 本发明的第二方面涉及一种预防或治疗哺乳动物中的骨关节炎的方法。

[0050] 骨关节炎 (OA) 是影响哺乳动物中的关节的退行性且炎性的病症。其也被称为退行性关节炎或退行性关节疾病。骨关节炎是一类涉及关节包括关节软骨和软骨下骨的退化的异常。

[0051] 骨关节炎是分解代谢和合成代谢的不平衡的结果，其中，分解代谢增 加；合成代谢降低，导致软骨细胞的炎症。软骨细胞是健康软骨中仅存的细胞。它们产生并维持软骨基质，软骨基质主要由胶原和蛋白聚糖组成。本发明的组合物已证明在体外炎症诱导的软骨细胞和体内健康软骨细胞中提供了尤其是减少的炎症、降低的分解代谢和增加的合成代谢。因此本发明的组合物预防和 / 或治疗动物中的骨关节炎。

[0052] 本发明对于所有方面涉及任何哺乳动物，包括人类。具体而言，本发明涉及患有或易于患有骨关节炎的伴侣动物，如犬、猫或马科动物 (例如马) 或任何其它这种动物。

[0053] 具体而言，在宠物食料和伴侣动物健康领域中所期望的是提供包括适合支持伴侣动物的健康的补充剂。具体而言，期望的是提供适合促进或维持已经健康的伴侣动物的健康的饮食。

[0054] 具体而言，本发明的第二方面提供一种用于预防和治疗哺乳动物、特别是伴侣动物中的骨关节炎的方法，包括减轻骨关节炎的症状。所述方法包括给予所述动物一种组合物，所述组合物包含姜黄素与绿茶多酚，或与甘氨酸、脯氨酸和羟脯氨酸的组合。所述动物可能是需要该组合物的。因为显著数量的犬在其一生中患有骨关节炎，因此所有犬可被认为需要预防。

[0055] 在具体实施方案中，所述方法包括给予所述动物一种组合物，所述组合物包括姜黄素、绿茶多酚以及甘氨酸、脯氨酸和羟脯氨酸的组合。更优选地，所述甘氨酸、脯氨酸和羟脯氨酸的组合是水解胶原。

[0056] 并且，所述方法优选地给药至患有骨关节炎和需要减轻骨关节炎的症状或需要进一步预防骨关节炎的症状或需要治疗骨关节炎的动物，特别是伴侣动物。这可以施用于例如幼年宠物动物，如幼犬，或大龄伴侣动物。在组合物是食料的情况下，食料可按照伴侣动物平时的饮食方案 (dietary regime) 而被给予至饮食方案中。根据所需的预防或治疗的水平，食料可包括 100％的伴侣动物的饮食，或少于该比例。食料允许容易地给药组合物，由此避免补充伴侣动物的食物的需要。此外，食料可通过动物的主人给予，由此避免持续的兽医监督。食料可以从售卖宠物食品的任何零售商店 (outlet) 获得，或者从兽医处获得。食料可以是根据本发明的第一方面的如上所述的食料。

[0057] 本文使用的“给药”还包括喂食或任何其它的口服给药方法。给药的其它方式还包括片剂、胶囊、注射、栓剂或任何其它适合的方式。

[0058] 本发明的第二方面的优选特征在进行必要的变化的情况下适用于第一方面。

[0059] 本发明包括用于制备本发明的第一方面的组合物的方法。

[0060] 食料可通过本领域中的例如帕加马牛津出版社出版的由 ATB 埃德尼编辑、A·瑞博德分章节的犬与猫的营养丛书中第 57-74 页中的标题为“平衡饮食”的文章 (Waltham Book

of Dog and Cat Nutrition,Ed.ATB Edney,Chapter by A.Rainbird,entitled"A Balanced Diet"on pages 57 to 74,Pergamon Press Oxford) 中的任何已知的方法制成。

[0061] 例如,本文所限定的用于制造食料的方法包括将配料与组合物混合在一起,所述组合物包含姜黄色素与绿茶多酚或与甘氨酸、脯氨酸和羟脯氨酸的组合物,并形成食料,特别是宠物食料。在混合之前、之中或之后,可将加热 / 烹调应用于任意一种或多种配料。

[0062] 组合物可被喷洒在食料上,与食料混合或掺入基质中的食料中。将组合物包含在内的方法是本领域中已知的。

[0063] 本发明的重要性在于姜黄色素与绿茶多酚或与甘氨酸、脯氨酸和羟脯氨酸的组合(任选地作为水解胶原)的有益性质。特别是,可观察到比累加效应更大的效果。

[0064] 对于姜黄色素、绿茶多酚以及甘氨酸、脯氨酸和羟脯氨酸(任选地作为水解胶原)的三重成分组合,观察到了进一步的益处。

[0065] 本发明的组合物的化合物的组合在降低炎症、降低分解代谢和提高合成代谢中的一个或多个方面上可提供协同效应。

[0066] 现通过参考以下实施例进一步描述本发明,这些实施例仅出于说明的目的提供,不应视为限制本发明。

[0067] 实施例 1:化合物的单独筛选

[0068] 进行实验以评价几种化合物对牛软骨细胞的原代培养 (primary culture) 的作用,其中,通过白细胞介素 -1β 诱导炎症和分解代谢过程,以模拟关节炎软骨细胞的作用。

[0069] 下表具体描述了贯穿整个实验中测定的生物标记,以示出化合物对软骨细胞上的三种代谢途径的作用。

[0070] 表 3 :检测的生物标记

[0071]

由软骨细胞产生的生物标记	炎症	NO
		PGE2
由软骨细胞表达的基因		IL-6
		COX2
		iNOS
	分解代谢	MMP3
		ADAMTS4
		ADAMTS5
	合成代谢	COL2
		AGG

[0072] 单层牛软骨细胞的原代培养

[0073] 从 1 岁至 2 岁的死后不久的公牛的掌指关节 (metacarpal-phalangeal joint) 获得正常的牛关节软骨。全厚 (full-depth) 关节软骨被切除并浸入补充有 N-(2- 羟乙基)哌嗪 -N'-(2- 乙磺酸)(HEPES)10mM、青霉素 (100U/ml) 和链霉素 (0.1mg/ml)(均来自龙沙

(Lonza)韦尔维耶,比利时)的达尔伯克氏改良伊格尔培养基(Dulbecco's Modified Eagle Medium,DMEM)(含酚红和4.5g/L葡萄糖)中。在三次洗涤后,通过在37℃下用0.5mg/ml透明质酸酶IV S型(西格玛-奥德里奇(Sigma-Aldrich),博尔内姆,比利时)进行30min,在37℃下用1mg/ml链霉蛋白酶E(默克,鲁汶(Leuven),比利时)进行1h和在37℃下用0.5mg/ml梭菌胶原酶(clostridial collagenase)IA(西格玛-奥德里奇,博尔内姆,比利时)进行16h至20h的连续的酶消化从软骨中释放出软骨细胞。然后将酶解分离的细胞通过尼龙网(70μm)过滤,洗涤三次,计数并填充至DMEM(含酚红和4.5g/L葡萄糖)的$0.25x10^6$个细胞/ml的密度,所述DMEM补充有10%胎牛血清、10mM HEPES、100U/ml青霉素、0.1mg/ml链霉素、2mM谷氨酰胺(均来自龙沙,韦尔 维耶,比利时)和20μg/ml脯氨酸(西格玛-奥德里奇,博尔内姆,比利时)。通过加入2ml的前述培养基/孔,将细胞以$0.5X10^6$个细胞/孔接种于6孔板中并以单层培养5天。然后,在补充有1%胎牛血清、10mM HEPES、100U/ml青霉素、0.1mg/ml链霉素、2mM谷氨酰胺和20μg/ml脯氨酸的DMEM(无酚红并仅含有1g/L葡萄糖)(龙沙,韦尔维耶,比利时),以单层培养软骨细胞直到汇合(confluence)(进行约2天)。仅使用原代培养物以确保软骨细胞表型的稳定性。

[0074] 当细胞实现汇合时,去除培养基并由新鲜培养基(补充有1%胎牛血清、10mM HEPES、100U/ml青霉素、0.1mg/ml链霉素、2mM谷氨酰胺和20μg/ml脯氨酸的无酚红且仅含有1g/L葡萄糖的DMEM)替换,所述新鲜培养基含有一些营养物质(nutraceuticals)(其中的每一种为12.5μg/ml)并且无或有重组猪IL-1β(10^{-11}M)(RD系统,阿宾顿,UK)。

[0075] 通过测量软骨细胞的生存力(viability)和PGE2与NO的产生来测试化合物的抗炎效力(首先单独测试,然后组合测试)。

[0076] 将化合物或者在炎症前(预防效果测定)或者在炎症同时(治疗效果测定)加入培养基中。

[0077] 筛选的化合物的列表:

[0078] 1)鱼油:18% EPA+10% DHA(DSM)

[0079] 2)EPA 99%(西格玛(Sigma))

[0080] 3)DHA 99%(西格玛)

[0081] 4)芦荟(纳图瑞克斯(Naturex))

[0082] 5)荨麻叶提取物(纳图瑞克斯)

[0083] 6)高纯度反式白藜芦醇(Resvida):99%白藜芦醇(Resvératrol)(DSM)

[0084] 7)绿茶提取物:25%多酚,其中12.5%是儿茶素(catéchines),8%是EGCG(纳图瑞克斯)

[0085] 8)松树皮提取物:碧萝芷(Pycnogenol):65%-75%原花青素(procyanidines)(碧欧蓝德(Biolandes))

[0086] 9)维生素预混料,包括维生素D3

[0087] 10)GLM(阿若马NZ(AromaNZ))

[0088] 11)胶原水解物(富迪格嘉利达(Fortigel de Gelita)[3.3kDa]:水解猪肉胶原)

[0089] 12)ASU(索齐姆(Sochim))

[0090] 13)姜黄粉:85%姜黄色素(纳图瑞克斯)

[0091] 结果显示,使用的3种化合物为姜黄提取物、水解胶原和绿茶提取物,其显示出针

对不同参数的显著效果。之后，对这 3 种化合物的反应 - 剂量进行测试。

[0092] 实施例 2:剂量反应

[0093] 按照实施例 1 的方法进行。根据分子重量，测试 4 个不同浓度 :0.5μg/ml、2.5μg/ml、12.5μg/ml 和 62.5μg/ml，以覆盖对应于 10^{-5}M 的浓度范围。

[0094] 结果显示，对于每个化合物，给出最佳效果且没有给出毒性作用的浓度是 12.5μg/ml。这是 12.5μg/ml 的浓度相互组合地被用于测试化合物的原因。

[0095] 实施例 3:测试化合物的特定组合和协同效应

[0096] 进行实施例 1 的方法。

[0097] 化合物的补充

[0098] 当细胞实现汇合时，去除培养基并由新鲜培养基（补充有 1％胎牛血清、10mM HEPES、100U/ml 青霉素、0.1mg/ml 链霉素、2mM 谷氨酰胺和 20μg/ml 脯氨酸的无酚红且仅含有 1g/L 葡萄糖的 DMEM）替换，所述新鲜培养基含有一些化合物（其中的每一种为 12.5μg/ml）并且无或有重组猪 IL-1β（10^{-11}M）(RD 系统，阿宾顿，UK)。测试了三种化合物，即姜黄提取物（纳图瑞克斯，阿维尼翁，法国）、水解胶原（嘉利达，埃贝尔巴赫，德国）和绿茶提取物（纳图瑞克斯，阿维尼翁，法国）。姜黄提取物制备成 12.5mg/ml 在四氢呋喃（默克，鲁汶，比利时）中的溶液，然后在细胞培养基中进一步稀释 1000 倍。将水解胶原和绿茶提取物以 12.5mg/ml 的浓度溶解于水中，通过无菌网 (0.20μm) 过滤，然后在细胞培养基中进一步稀释 1000 倍。在无或有重组猪 IL-1β（10^{-11}M）时，在 12.5μg/ml 的终浓度下化合物得到单独测试，或者组合测试 (12.5μg/ml 姜黄提取物 +12.5μg/ml 水解胶原 ;12.5μg/ml 姜黄提取物 +12.5μg/ml 绿茶提取物 ;12.5μg/ml 姜黄提取物 +12.5μg/ml 水解胶原 +12.5μg/ml 绿茶提取物）。将化合物的效果与对照（仅 DMEM 或 DMEM+IL-1β）进行对比。

[0099] 培养停止

[0100] 在这些条件下培养 24h 后，收集每种条件的三个孔的条件培养基，并在 -20℃下储存。解体这些对应的孔的细胞，使用 RNeasy 迷你试剂盒 (RNeasy mini kit)（凯杰 (Qiagen)，芬洛，荷兰）进行 RNA 提取，使用 LightCycler 480（罗氏 (Roche)，韦尔维耶，比利时）实施逆转录酶聚合酶链反应 (a reverse transcriptase polymerase chain reaction)，然后实施定量实时聚合酶链反应 (a quantitative real time polymerase chain reaction)，以分析基因表达。

[0101] 在这些条件下培养 48h 后，收集剩余孔（每种条件三个孔）的条件培养基（乳酸脱氢酶释放试验），并在 -20℃下储存，直到进行分析（亚硝酸盐和前列腺素 E2 试验）。解体细胞，并通过在 4℃下超声解离 20s 在 500μl Tris-HCl 缓冲液中均质化，以测定 DNA 含量。

[0102] 乳酸脱氢酶释放试验

[0103] 通过在培养上清液中定量乳酸脱氢酶 (LDH) 的释放来估计细胞生存力。将 100μl 上清液样品或标准溶液（来自兔肌肉的 LDH）的稀释液与含有 800nM 乳酸盐的 50μl Tris 缓冲液 (10mM Tris-HCl(pH 8.5)、0.1％牛血清白蛋白）混合。然后加入 50μl 比色试剂、1.6mg/ml 碘硝基氯化四氮唑蓝 (iodonitrotetrazolium chloride)（西格玛 - 奥德里奇，博尔内姆，比利时）、4mg/ml 烟酰胺腺嘌呤二核苷酸（罗氏诊断 (Roche Diagnostics)，布鲁塞尔，比利时）和 0.4mg/ml 吩嗪硫酸甲酯（西格玛 - 奥德里奇，博尔内姆，比利时），在室温下

孵育 10min 后读取 492nm 下的吸光度。

[0104] DNA试验

[0105] 通过在 4℃下超声解离 15s 在 500μl Tris-HCl 缓冲液中使软骨细胞均化。使用赫斯特 (Hoechst) 荧光法在细胞提取液中测定 DNA 含量。

[0106] 亚硝酸盐试验

[0107] 基于格里斯反应 (Griess reaction) 使用分光光度法在培养上清液中,通过定量一氧化氮的衍生产物亚硝酸盐来测定一氧化氮 (NO) 的产生量。 简言之,将 100μl 上清液或亚硝酸钠 ($NaNO_2$) 标准稀释液与 100μl 格里斯试剂 (Griess reagent)(0.5％磺胺、0.05％萘基乙二胺二盐酸盐、2.5％ H_3PO_4) 混合。测定在 540nm 处的吸收。NO 的产生量由每毫克 DNA 表示。

[0108] PGE2试验

[0109] 使用 DetectX PGE2 高灵敏度免疫试剂盒 (DetectX PGE2High Sensitivity Immunoassay kit)(阿伯试验 (Arbor Assays),密歇根州,美国) 在培养上清液中测定前列腺素 E2(PGE2) 的产生量。简言之,将 100μl 上清液或 PGE2 标准稀释液用移液器滴入包被有捕获小鼠 IgG 的抗体的清洁的微量滴定板中。将 PGE2- 过氧化物酶缀合物 (25μl) 加入孔中的标准液和上清液中。通过加入 25μl 抗 PGE2 单克隆抗体来起始结合反应。在 4℃下孵育过夜后,洗涤板,并加入 100μl 底物。底物与结合的 PGE2- 过氧化物酶缀合物反应。在短暂的孵育后,停止反应,在 450nm 波长下检测产生的颜色的强度。PGE2 的产生量由每毫克 DNA 表示。

[0110] 定量实时逆转录酶聚合酶链反应 (Quantitative real-time reverse transcriptase polymerase chain reaction,RT PCR)

[0111] 使用 RNeasy 迷你试剂盒 (凯杰,芬洛,荷兰) 分离来自每种条件的 3 个孔的细胞的 RNA。然后,逆转录 RNA。通过使用 SYBR Premix Ex Taq(带有预先混合 SYBR 染料的 DNA 聚合酶 Ex Taq)(Tli RNaseH Plus)(维斯特博格 (Westburg),勒斯登,荷兰) 进行定量实时聚合酶链反应 (Polymerase Chain Reaction,PCR)。PCT 模板源为第一链 cDNA 或纯化的 DNA 标准链。用于扩增所需 cDNA 的引物序列如下:牛 HPRT 正向和反向引物:5’-AGTTTGGAAATACCTGGCG-3’ 和 5’-AGTCTTTAGGCTCGTAGTGC-3’;牛白细胞介素 (IL)-6 正向和反向引物:5’-TGGTGATGACTTCTGCTTTCC-3’ 和 5’-TGCCAGTGTCTCCTTGC-3’;牛环氧酶 (COX)2 正向和反向引物:5’-GTCTGATGATGTATGCCACC-3’ 和 5’-ACGTAGTCTTCAATCACAATCT-3’;牛诱导型一氧化氮合成酶 (iNOS) 正向和反向引物:5’-GGCAAGCACCACATTGAGA-3’和 5’-TGCGGCTGGATTTCGGA-3’;牛聚集蛋白聚糖 (aggrecans)(AGG) 正向和反向引物:5’-TGCCTTTGACGTGAGC-3’和 5’-GCATTGTTGTTGACAAACT-3’;牛 II 型胶原 (COL2) 正向和反向引物:5’-CTGCGTCTACCCCAAC-3’ 和 5’-GGGTGCAATGTCAATGAT-3’;牛金属蛋白酶 (MMP)-3 正向和反向引物:5’-TCTATGAAGGAGAAGCTGACATAAT-3’ 和 5’-TTCATGGGCAGCAACAAG-3’;牛的带有血小板反应蛋白模体的解整合素及金属蛋白酶 (A Disintegrin and Metalloproteinase with Thrombospondin Motifs,ADAMTS)4 正向和反向引物:5’-CTTTCAATGTCCCACAGGC-3’ 和 5’-CAGGAACGGAAGCGGG TA-3’;牛 ADAMTS 5 正向和反向引物:5’-GACACCCTGGGAATGGCA-3’ 和 5’-CACAGAACTTGGAATCGTCA-3’。

[0112] 用分光荧光热循环仪 (spectrofluorometric thermal cycler)

(LightCycler480,罗氏诊断,韦尔维耶,比利时)进行扩增。为了标准化 mRNA 水平,扩增作为内部对照的管家基因 HPRT。通过计算 IL-6、COX2、iNOS、AGG、COL2、MMP-3、ADAMTS4、ADAMTS5 和 HPRT 的 cDNA 拷贝数量之间的比例标准化基因表达。

[0113] 结果表示为与对照相比的平均增加百分比。使用 t- 测试评价统计显著性。$p<0.05$ 时的偏差被认为是统计上显著的。下表详细记载了在结合化合物与观察到的协同作用时所提供的结果。

[0114] 表 4

[0115]

			对照	姜黄素 (=C)	水解胶原 (=O)	绿茶 (=T)	CO	CT	COT
软骨细胞产生的生物标记		NO	0	0	0	0	0	0	0
		NO+IL1B	100	-96*	23**	-14*	-100***	-100*	-100***
		PGE2	0	0	0	0	0	0	0
		PGE2+ IL1B	100	-99*	1**	363**	-100*	-100***	-100***
由软骨细胞表达的基因	炎症	IL-6	100	-82*	307**	252**	-43*	-38*	-65*
		IL-6+ IL1B	100	-84*	-1*	-8*	-89*	-100*	-99*
		COX2	100	49**	54**	86**	378**	84**	27**
		COX2+IL1B	100	-51*	-24*	-26*	-58*	-87*	-92*
		iNOS	100	-76*	123**	207**	-65***	-27***	-25***
		iNOS+IL1B	100	-86*	-13*	15**	-91*	-96*	-97*
	分解代谢	MMP3	100	40**	109**	161**	91*	-10***	-15***
		MMP3+IL1B	100	-58*	-20*	-22*	-85*	-99*	-99*
		ADAMTS4	100	-21*	12**	9**	16**	-14*	-58***
		ADAMTS4 + IL1B	100	-55*	-23*	-28*	-68*	-83*	-84*
		ADAMTS5	100	-16*	2**	32**	-31***	-28***	-41***
		ADAMTS5+ IL1B	100	-47*	-13*	22**	-52*	-76***	-71***
	合成代谢	COL2	100	-77**	13*	18*	-84**	-62**	-74**
		COL2+IL1B	100	-57**	-2**	55*	-67**	29***	67***
		AGG	100	-77**	54*	33*	-78**	-30**	-53**
		AGG+ IL1B	100	-77**	-4**	123*	-82**	186***	337***

[0116] (* 有益作用 ;** 负作用 ;*** 比预期的更有益)

[0117] 讨论

[0118] 组合的结果比每个化合物的叠加效果要好。一种解释是因为化合物对相关的不同代谢途径起作用,当有炎症时,分解代谢增加而合成代谢降低。因此,我们的非限定性假设是,姜黄素抑制由 IL-1β 诱导的炎症(并且也由胶原和绿茶诱导)。一旦炎症被抑制,分解代谢降低,并且胶原和绿茶多酚可对合成代谢具有其正向作用。由于关节炎是恶性循环(炎症诱导分解代谢,分解代谢诱导炎症等),当分解代谢降低(且合成代谢增加)时,会有炎症的降低,并弥补恶性循环。

[0119] 然而,通常,在健康细胞中,分解代谢和合成代谢之间总有平衡。可见, 该组合可

对健康细胞的代谢具有正向作用（没有通过 IL-1β 诱导炎症）。非常有趣的是，在关节炎或关节炎之前的情况中，仍处于良好健康的细胞可被这些组合所保护。

[0001]

序列表

<110> 马斯公司
马斯宠物护理英国公司

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