Original Research

Collagen hydrolysate inhibits zymosan-induced inflammation

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Abstract

During the past years, evidence accumulated showing that glycine comprises anti-inflammatory activities. These effects occur, at least in part, via the activation of glycine-gated chloride channels (GlyR). Glycine is one of the major structural units of collagen, making up about 30% of the amino acids. This study aims to investigate the anti-inflammatory potential of collagen hydrolysate (CH) using the zymosan-induced ear-skin inflammation mouse model. After oral intake of 12.5, 25 or 50 mg CH the plasma levels of glycine increased in a concentration-dependent manner. CH was able to counteract zymosan-induced ear-skin inflammation locally (ear swelling) as well as systemically (IL-6 production by lipopolysaccharide (LPS)-stimulated whole blood cells). The LPS-stimulated IL-6 production in whole blood correlated positively with the ear swelling response. This correlation was abolished by strychnine (a glycine receptor antagonist), indicating the involvement of GlyR. Collectively, these data show that CH is able to modulate inflammatory responses both locally as well as systemically. This effect might be constituted by inhibiting pro-inflammatory cytokine production via GlyR.

Keywords: Collagen hydrolysate, GlyR, Glycine, inflammation

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Introduction

The simple nonessential amino acid glycine is found in many different proteins. It acts as an inhibitory neurotransmitter in the central nervous system via glycine-gated chloride channels (GlyR). The existence of GlyR has been demonstrated on a wide variety of cells including different cell types involved in immune responses, such as macrophages, monocytes, neutrophils and T lymphocytes.[1–3] In the past years, evidence accumulated showing that glycine causes an anti-inflammatory response, at least in part via the activation of GlyR.[3] Activation of GlyR in immune cells results in an influx of chloride ions, membrane hyperpolarisation and inhibition of voltage-operated calcium channels and cell activation. Glycine largely prevents endotoxin-induced TNF-α production by Kupffer cells and alveolar macrophages, and it reduces IL-1β and TNF-α expression while simultaneously stimulating IL-10 expression in lipopolysaccharide (LPS)-activated monocytes.[1,4,5] Moreover, glycine interferes with TNF-α-mediated NF-κB activation in human coronary arterial endothelial cells and differentiated 3T3-L1 adipocytes.[6,7]

The *in vitro* data of the effects of glycine are reinforced by *in vivo* results derived from different animal models including: the inhibition of pro-inflammatory cytokine expression in surgical manipulation-induced inflammation in the intestine, a decrease in pro-inflammatory cytokine expression in the fat tissue of lean and obese mice, the inhibition of peptidoglycan polysaccharide-induced arthritis, and the inhibition of zymosan-induced inflammation.[8–11]

Glycine is one of the major structural units of collagen, making up about 30% of the amino acids. It has been indicated that serum glycine levels increase after intake of collagen hydrolysate (CH).[12] Clinical studies point to a possible beneficial effect of CH in osteoarthritis by a reduction in hip or knee pain, after daily intake of 10 g CH for 60 days to six months.[13–16]

This study aims to answer the question whether CH is able to modulate inflammatory responses. The anti-inflammatory potential of CH was examined using a mouse model for zymosan-induced ear-skin inflammation.

Materials and methods

All experimental procedures using laboratory animals were approved by an independent animal experiments committee (DEC Consult, Bilthoven, the Netherlands).

Prompting zymosan-induced ear-skin inflammation

Male Balb/C mice (Charles River, Maastricht, the Netherlands), aged 14 weeks at the start of the experiment were randomly assigned to groups of six animals and

acclimatized to the animal housing for one-week prior to the start of the experiment. All animals had free access to a standard rodent diet and tap water. Different amounts of CH (0, 12.5, 25 or 50 mg/mouse) were administered daily for three constitutive days (day 1 till 3) by oral gavage. Tap water (vehicle) was applied in the same volume, 200 µL, to the control mice. Inflammation was induced at day 3, 2 h after administration of CH, by injecting 25 µL zymosan (0.5% suspended in PBS) or PBS (sham), intradermally in both ears.[17,18] Ear thickness was measured prior to and at 3 and 6 h after zymosan injection using an engineer's micrometer (Mitutoyo Dinamic, Veenendaal, the Netherlands). After the final ear thickness measurement, mice were bled under terminal anaesthesia (isoflurane/N_2O/O_2) and sacrificed.

In a second group of mice, blood was taken at different time points (1 and 3 h) after CH (50 mg/mouse) intake.

Plasma isolation

Blood was collected in Lithium Heparin MiniCollect tubes (Greiner Bio-One B.V, Alphen a/d Rijn, the Netherlands) and preserved at room temperature awaiting whole blood analysis. After starting the whole blood cell assay, the remaining blood was centrifuged for 5 min at 6000 rpm in an Eppendorf® centrifuge, and the plasma was then collected and stored at −20°C for glycine detection.

Plasma glycine detection

After precipitation of proteins and polypeptides with perchloric acid, the plasma samples were centrifuged. Glycine concentrations were determined in the supernatant by fully automated High Performance Liquid Chromatography (Figure 1, HPLC, Shimadzu, 's-Hertogenbosch, the Netherlands).

Whole blood assay

Heparinized murine blood (50 µL) was incubated for 20 h in the presence of LPS (*E. coli*, B55:055, final concentration 1 µg/mL, Sigma-Aldrich BV, Zwijndrecht, the Netherlands) with and without strychnine (a glycine receptor antagonist, final concentration 10 μM, Sigma-Aldrich BV) in a total volume of 200 µL in culture medium (RPMI-1640 containing 25 mM HEPES and 2 mM L-glutamine, Life-Technologies, Merelbeke, Belgium, enriched with 100 U/mL penicillin/streptomycin 100 µg/mL, Life-Technologies). The plates were subsequently centrifuged and the supernatants were harvested and stored at −80°C until cytokine detection.

Cytokine detection

Cytokine levels were detected using a commercial Multiplex Bead immunoassay (BioRad, Veenendaal, the Netherlands) including IL-6 and TNF-α, according to the manufacturer's protocol. The analysis was performed by using the Bio-Plex system (BioRad). Results were calculated using Bio-Plex Manager Software 3.1 (BioRad).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). All readout conditions were compared to the control using the analysis of variance (one-way ANOVA). The overall significance of differences for all calculations was tested using the *post hoc* Dunnett's test. Differences between LPS stimulated IL-6 production and the LPS stimulated IL-6 production in the presence of strychnine were calculated by a T-test. Correlations were calculated using the Pearson's linear regression model.

Results

Plasma glycine levels

Plasma glycine levels were detected in control conditions and at 1 (50 mg/mouse), 3 (50 mg/mouse) and 8 h (12.5, 25

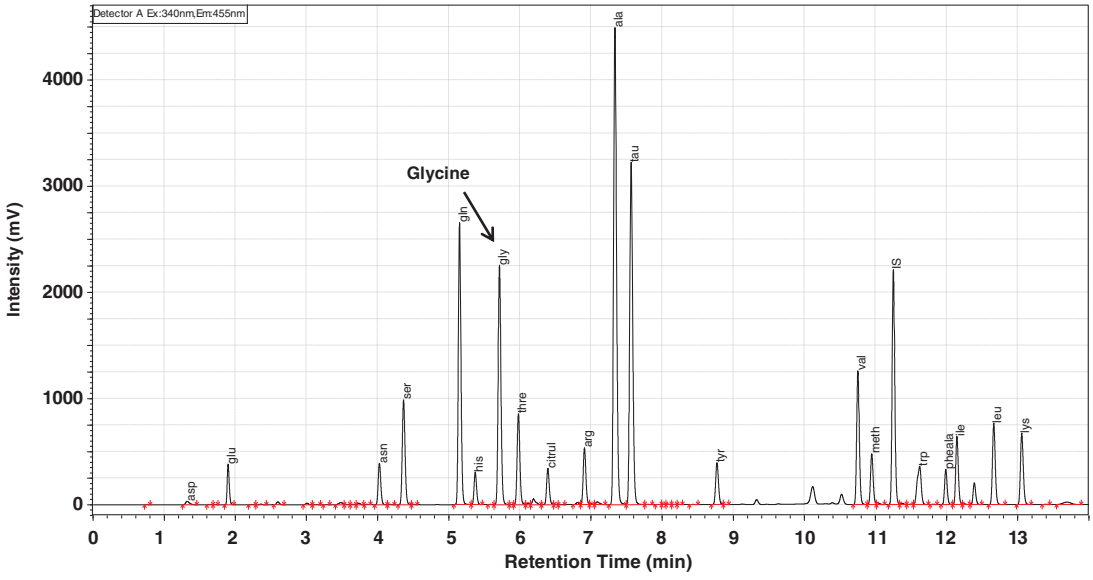

Figure 1 A typical chromatogram of the plasma free amino acid analysis by HPLC. (A color version of this figure is available in the online journal)

and 50 mg/mouse) after CH intake. CH increased the plasma glycine level (detected at 8 h after intake) in a concentration-dependent manner (Figure 2A). Intake of 50 mg of CH more than doubled the glycine plasma levels at 1 and 3 h after intake (Figure 2B).

Ear-skin inflammation

To study the effect of CH on zymosan-induced ear-skin inflammation in mice, CH was administered orally for three days. On the third day, zymosan or PBS were injected into both ears intradermally. Ear thickness was measured before injection and at 3 and 6 h after injection (5 and 8 h after CH intake, respectively). Basal ear thickness was subtracted from the ear thickness at the different time-points after zymosan injection to calculate ear swelling. In the vehicle-supplemented animals, the sham injection (PBS) induced an ear swelling of 79 μm and 112μm after 3 and 6 h respectively. This PBS-induced swelling was subtracted from the zymosan-induced ear swelling to acquire the 'zymosan specific' skin-swelling. The ear swelling of the CH supplemented animals was compared to the control animals (vehicle supplemented). CH inhibited the zymosan induced ear-swelling in a concentration dependent manner at 3 h after injection (Figure 3A). At 6 h post zymosan challenge the effect was no longer significant (Figure 3B).

Cytokine levels in an LPS-induced whole blood assay

Blood was incubated, *ex vivo,* in the presence of LPS with and without strychnine. After 20 h, the supernatant was collected. TNF-α and IL-6 levels were detected by a Multiplex Beat Immunoassay. CH was able to inhibit IL-6 production significantly at 25 mg/mL (Figure 4A). Strychnine counteracted this effect (Figure 4B). Strychnine significantly increased IL-6 levels in all collagen-treated conditions but not in the control (Figure 4C1, C2, C3 and C4). There were no differences detectable in the TNF-α level at the different tested conditions (data not shown).

Possible correlations between LPS induced IL-6 levels in whole blood, plasma glycine levels and ear-swelling at 6 h after zymosan injection were calculated. The IL-6 production and ear-swelling at 6 h after zymosan injection showed a significant correlation (Pearson $r = 0.63$, $P = 0.002$, Figure 5A). No correlation was found between the ear-swelling and IL-6 production in the strychnine supplemented whole blood cell assay (Pearson $r = 0.20$, $P = 0.377$, Figure 5B).

Discussion

Collagen is a natural component of the diet, found in animal products such as meat and fish. Small peptides and amino acids derived from CH are detectable in blood.[10,19] The present study demonstrated that orally administered CH increases plasma glycine levels in mice in a concentration-dependent manner. Anti-inflammatory effectiveness was indicated by counteraction of the zymosan-induced ear swelling at 3 h after zymosan injection (5 h after CH intake). Glycine serum levels, tested for the most effective anti-inflammatory dose (50 mg/day), were more than

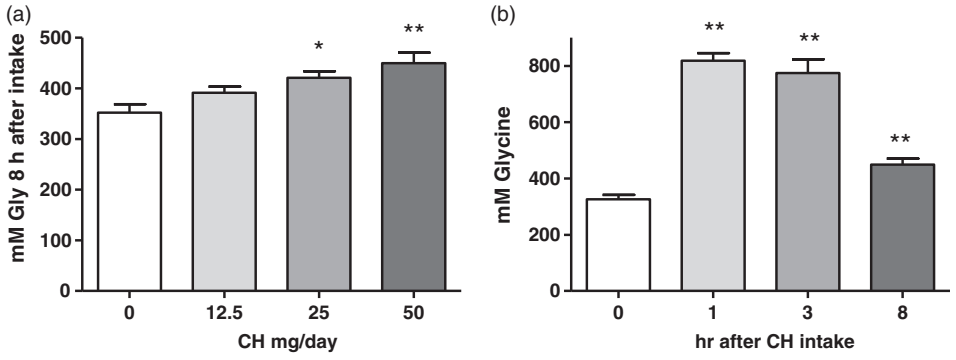

Figure 2 Plasma concentrations of Gly, 8 h after intake of vehicle (0) or different concentrations CH (a). Plasma concentration of Gly before (0) and 1, 3 and 8 h after, intake of 50 mg CH (b). Data are means (mM) ± SEM, $n = 6$. Significant differences from no CH intake (0) are indicated $^{*}P < 0.05$, $^{**}P < 0.01$

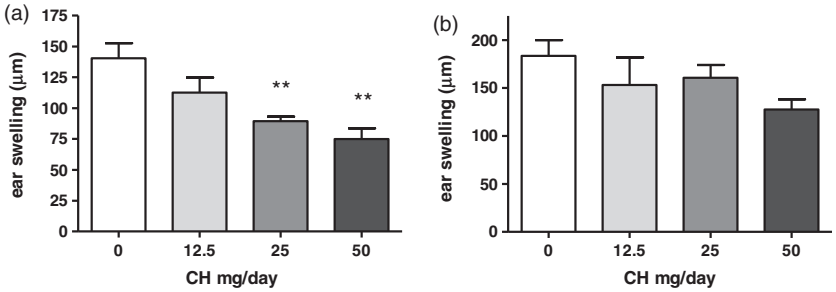

Figure 3 Zymosan-induced ear inflammation (swelling) 3 (a) and 6 (b) hours after zymosan injection. Data are means (μm) ± SEM, $n = 6$. Significant differences from no CH intake (0) are indicated $^{**}P < 0.01$

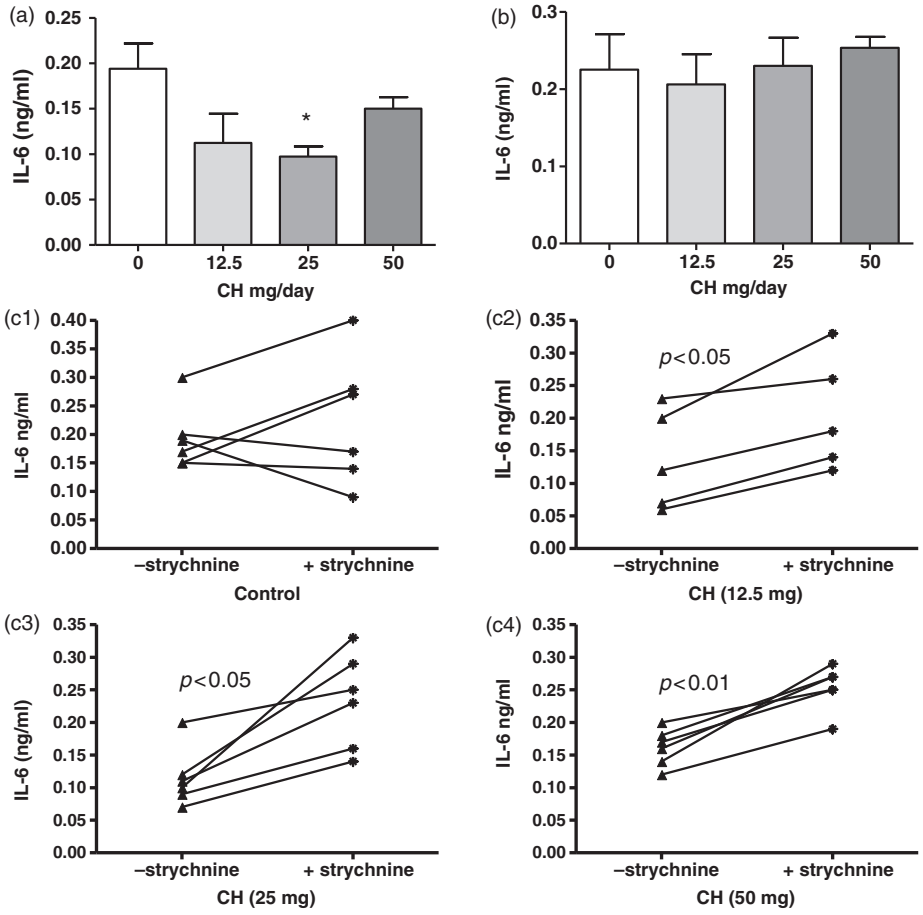

Figure 4 LPS-induced (1 μg/mL) IL-6 production by whole blood cells, without (A) and in the presence of strychnine (10 μM, B). Data are means (ng/mL) ± SEM, $n = 6$. Significant differences from no CH intake (0) are indicated $^*P < 0.05$. Figure C shows LPS-induced IL-6 production by whole blood cells with and without strychnine in control condition (1) and after intake of 12.5 mg (2), 25 mg (3) and 50 mg (4) CH. Significant differences between the control and strychnine condition as detected by T-test are indicated

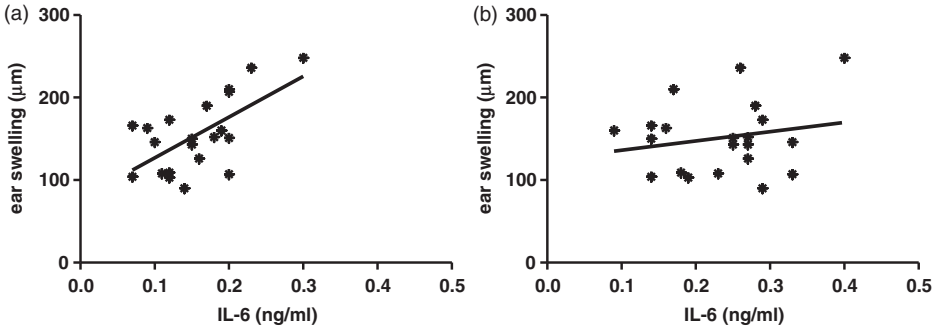

Figure 5 The correlation between ear-swelling 6 h after zymosan injection and LPS induced IL-6 production in whole blood ($n = 21$) in the absence (a) and presence (b) of strychnine

doubled at the time point of induction of the inflammation (2 h after CH intake).

Effectiveness of CH has been tested in different disease models. In the ethanol-induced ulcer model in rats, a single dose of CH resulted in a strong reduction of ulcer formation[19,20] and, in a spontaneous hyperlipidemic mouse model, C57BL/6.KOR-Apoe(shl), CH feeding showed besides a lipid-lowering effect a decrease in plasma IL-6 and TNF-α levels.[21] Moreover, in osteoarthritis (which is associated with significant functional impairment in combination with synovial inflammation) CH is one of the candidate disease modifying osteoarthritis drugs (DMOADs).[22] Inflammatory cells play a role in osteoarthritis and the aforementioned animal models in which the effects of CH are described. The results of this study suggest that modulation of the inflammatory response by CH could contribute to the protective effectiveness as detected in the different studies.

Eight hours after intake of different amounts of CH, blood was collected and stimulated with LPS in the

presence or absence of strychnine. The neurotoxin strychnine acts as an antagonist on the GlyR, neutralizing the anti-inflammatory effects of glycine on immune cells.[5] It was found that the LPS-stimulated IL-6 production was lower after intake of 25 mg CH, demonstrating a systemic effect. The addition of strychnine significantly increased the production of IL-6 in the CH groups but not in the control group. Recent literature indicates that extracellular pH changes influence glycine receptor functionality.[23] Both, the plasma and the cell cultures showed a physiological pH, excluding pH-induced receptor modifications. LPS induced IL-6 production and the detected ear swelling at 6 h after zymosan injection were highly correlated. Although the plasma glycine levels were low at the time of LPS stimulation, this correlation in combination with the strychnine results suggest that the GlyR are involved, pointing to a contribution of glycine in the CH-induced anti-inflammatory effect.

Collectively, these data show that CH is able to modulate inflammatory responses. This effect might be constituted by inhibiting pro-inflammatory cytokine production via the GlyR.

Author contributions: All authors participated in the interpretation of the study data and review of the manuscript; AH and JG designed the experiments; AH, MC and GdV conducted the experiments; AH wrote the manuscript.

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REFERENCES

1. Froh M, Thurman RG, Wheeler MD. Molecular evidence for a glycine-gated chloride channel in macrophages and leukocytes. *Am J Physiol Gastrointest Liver Physiol* 2002;**283**:G856–63
2. Wheeler M, Stachlewitz RF, Yamashina S, Ikejima K, Morrow AL, Thurman RG. Glycine-gated chloride channels in neutrophils attenuate calcium influx and superoxide production. *FASEB J* 2000;**14**:476–84
3. Zhong Z, Wheeler MD, Li X, Froh M, Schemmer P, Yin M, Bunzendaul H, Bradford B, Lemasters JJ. L-glycine: a novel antiinflammatory, immunomodulatory, and cytoprotective agent. *Curr Opin Clin Nutr Metab Care* 2003;**6**:229–40
4. Ikejima K, Iimuro Y, Forman DT, Thurman RG. A diet containing glycine improves survival in endotoxin shock in the rat. *Am J Physiol* 1996;**271**:G97–103
5. Spittler A, Reissner CM, Oehler R, Gornikiewicz A, Gruenberger T, Manhart N, Brodowicz T, Mittlboeck M, Boltz-Nitulescu G, Roth E. Immunomodulatory effects of glycine on LPS-treated monocytes: reduced TNF-alpha production and accelerated IL-10 expression. *FASEB J* 1999;**13**:563–71
6. Blancas-Flores G, Alarcon-Aguilar FJ, Garcia-Macedo R, lmanza-Perez JC, Flores-Saenz JL, Roman-Ramos R, Ventura-Gallegos JL, Kumate J, Zentella-Dehesa A, Cruz M. Glycine suppresses TNF-alpha-induced activation of NF-kappaB in differentiated 3T3-L1 adipocytes. *Eur J Pharmacol* 2012;**689**:270–7
7. Hasegawa S, Ichiyama T, Sonaka I, Ohsaki A, Okada S, Wakiguchi H, Kudo K, Kittaka S, Hara M, Furukawa S. Cysteine, histidine and glycine exhibit anti-inflammatory effects in human coronary arterial endothelial cells. *Clin Exp Immunol* 2012;**167**:269–74
8. Hartog A, Leenders I, van der Kraan PM, Garssen J. Anti-inflammatory effects of orally ingested lactoferrin and glycine in different zymosan-induced inflammation models: evidence for synergistic activity. *Int Immunopharmacol* 2007;**7**:1784–92
9. Li X, Bradford BU, Wheeler MD, Stimpson SA, Pink HM, Brodie TA, Schwab JH, Thurman RG. Dietary glycine prevents peptidoglycan polysaccharide-induced reactive arthritis in the rat: role for glycine-gated chloride channel. *Infect Immun* 2001;**69**:5883–91
10. Stoffels B, Turler A, Schmidt J, Nazir A, Tsukamoto T, Moore BA, Schnurr C, Kalff JC, Bauer AJ. Anti-inflammatory role of glycine in reducing rodent postoperative inflammatory ileus. *Neurogastroenterol Motil* 2011;**23**:76–87,e8
11. Alarcon-Aguilar FJ, Almanza-Perez J, Blancas G, Angeles S, Garcia-Macedo R, Roman R, Cruz M. Glycine regulates the production of pro-inflammatory cytokines in lean and monosodium glutamate-obese mice. *Eur J Pharmacol* 2008;**599**:152–8
12. Walrand S, Chiotelli E, Noirt F, Mwewa S, Lassel T. Consumption of a functional fermented milk containing collagen hydrolysate improves the concentration of collagen-specific amino acids in plasma. *J Agric Food Chem* 2008;**56**:7790–5
13. Benito-Ruiz P, Camacho-Zambrano MM, Carrillo-Arcentales JN, Mestanza-Peralta MA, Vallejo-Flores CA, Vargas-Lopez SV, Villacis-Tamayo RA, Zurita-Gavilanes LA. A randomized controlled trial on the efficacy and safety of a food ingredient, collagen hydrolysate, for improving joint comfort. *Int J Food Sci Nutr* 2009;**60**:99–113
14. Bello AE, Oesser S. Collagen hydrolysate for the treatment of osteoarthritis and other joint disorders: a review of the literature. *Curr Med Res Opin* 2006;**22**:2221–32
15. Carpenter RL, Peel JB, Carpenter MR, Lowndes J, Angelopoulos T, Rippe JM. Effectiveness of a collagen hydrolysate-based supplement on joint pain, range of motion and muscle function in individuals with mild osteoarthritis of the knee: a randomized clinical trial. *Ann Rheum Dis* 2005;**64**:476–476
16. Bruyere O, Zegels B, Leonori L, Rabenda V, Janssen A, Bourges C, Reginster JY. Effect of collagen hydrolysate in articular pain: a 6-month randomized, double-blind, placebo controlled study. *Complement Ther Med* 2012;**20**:124–30
17. Hougee S, Hartog A, Sanders A, Graus YM, Hoijer MA, Garssen J, van den Berg WB, van Beuningen HM, Smit HF. Oral administration of the NADPH-oxidase inhibitor apocynin partially restores diminished cartilage proteoglycan synthesis and reduces inflammation in mice. *Eur J Pharmacol* 2006;**531**:264–9
18. Erdo F, Torok K, Aranyi P, Szekely JI. A new assay for antiphlogistic activity: zymosan-induced mouse ear inflammation. *Agents Actions* 1993;**39**:137–42
19. Castro GA, Sgarbieri VC, Carvalho JE, Tinti SV, Possenti A. Protective effect of collagen derivates on the ulcerative lesions caused by oral administration of ethanol. *J Med Food* 2007;**10**:154–8
20. Castro GA, Carvalho JE, Tinti SV, Possenti A, Sgarbieri VC. Anti-ulcerogenic effect of a whey protein isolate and collagen hydrolysates against ethanol ulcerative lesions on oral administration to rats. *J Med Food* 2010;**13**:83–90
21. Zhang Y, Kouguchi T, Shimizu K, Sato M, Takahata Y, Morimatsu F. Chicken collagen hydrolysate reduces proinflammatory cytokine production in C57BL/6.KOR-ApoEshl mice. *J Nutr Sci Vitaminol (Tokyo)* 2010;**56**:208–10
22. Van Vijven JP, Luijsterburg PA, Verhagen AP, van Osch GJ, Kloppenburg M, Bierma-Zeinstra SM. Symptomatic and chondroprotective treatment with collagen derivatives in osteoarthritis: a systematic review. *Osteoarthr Cartilage* 2012;**20**:809–21
23. Song YP, Schlesinger F, Ragancokova D, Calixto R, Dengler R, Krampfl K. Changes in extracellular pH affect glycine receptor channels expressed in HEK 293 cells. *Eur J Pharmacol* 2010;**636**:59–64

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