

Evaluation of the effect of the administering of an N-acetyl-glucosamine-containing green tea supplement on biomarkers for cartilage metabolism in healthy individuals without symptoms of arthritis: a randomized double-blind placebo-controlled clinical study

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ABSTRACT

Background: To evaluate the chondroprotective action of an N-acetyl-glucosamine (GlcNAc)-containing supplement on the joint health of healthy individuals without symptoms of arthritis, we conducted a randomized double-blind placebo-controlled clinical trial.

Methods: Subjects (n=100, 51.3 ± 1.0 years (mean ± SE)) without symptoms of arthritis were randomly assigned to receive a 1000 mg GlcNAc-containing diet (GlcNAc group) or a placebo diet (placebo group) once a day for 16 weeks, and the effect on the cartilage metabolism was evaluated by analyzing the ratio of type II collagen degradation to synthesis using type II collagen degradation (C2C) and synthesis (PIICP) markers.

Results: The results indicated that the changes in the C2C/PIICP ratios from the baseline were slightly suppressed in the GlcNAc group compared with those in the placebo group at weeks 16 during the intervention and 4 weeks after the intervention. However, there was no significant difference between the two groups. To make the effect of GlcNAc even more clear, the subjects with joint loading and impaired cartilage metabolism were evaluated. Interestingly, the changes in the C2C/PIICP ratios from the baseline were significantly suppressed in the GlcNAc group compared with the placebo group at weeks 16 during the intervention and 4 weeks after the intervention. Moreover, test supplement-related adverse events were not essentially observed during and after the intervention.

Conclusions: These observations suggest that the oral administration of GlcNAc at a dose of 1000 mg/day exerts a chondroprotective action on the healthy individuals by lowering the C2C/PIICP ratio, which indicates relative reduction of type II collagen degradation and increase of type II collagen synthesis, without apparent adverse effect.

Key words: N-acetyl-glucosamine, biomarker, cartilage metabolism, joint health

BACKGROUND

Osteoarthritis, which develops due to the progressive destruction of articular cartilage, is the most common joint disease and the leading cause of pain and physical disability in elderly people [1-3]. In osteoarthritis, knee joints are especially impaired as they are weight-bearing joints. Importantly, studies with experimental osteoarthritis models have shown that the changes in the metabolic and chemical properties of cartilage matrix can be detected in the early stages of arthritis [4]. Thus, various molecular markers have been developed as indicators of cartilage metabolism in joint disorders [5-8]. Furthermore, such biomarkers are used for evaluating the actions of disease-modifying drugs and chondroprotective supplements on the cartilage, since they specifically reflect the alterations in the cartilage metabolism [9].

Type II collagen is one of the major constituents of cartilage and represents 90-95% of the cartilage collagens [7]. Thus, fragments of type II collagen have been utilized as biomarkers for cartilage metabolism. A C-terminal crosslinking peptide (CTX-II) is cleaved during degradation of type II collagen [10], whereas a neoepitope (C2C) is generated by intrahelical cleavage at the C terminus of the 3/4 piece of degraded type II collagen [11]. Thus, both CTX-II and C2C are used as markers for type II collagen degradation. In contrast, a C-terminal type II procollagen peptide (CPII or PIICP), which is cleaved during the processing of newly synthesized type II procollagen, can be used as a marker for type II collagen synthesis [12].

Nutritional supplements (such as glucosamine, N-acetylglucosamine, chondroitin and collagen) are now used for 'joint health' to treat or prevent cartilage disorders (including osteoarthritis) [13, 14]. Among these substances, glucosamine suppresses the degradation and stimulates the synthesis of glycosaminoglycans (proteoglycans) [15, 16]. Additionally, glucosamine suppresses the expression of collagen-degrading enzymes (matrix metalloproteinases, MMPs) and enhances the synthesis of type II collagen in chondrocytes [17, 18]. As a result, glucosamine exhibits a chondroprotective action on cartilage disorders by retaining not only proteoglycans but also type II collagen in the articular cartilage, and is thereby used to treat osteoarthritis in humans [19-21]. More importantly, we evaluated the effect of glucosamine on the cartilage metabolism in healthy individuals using bicycle racers (an average age of 20 years) with normal cartilage metabolism, by analyzing the levels of CTX-II and CPII [22]. The results indicated that oral administration of glucosamine exhibits a chondroprotective action in these healthy individuals by preventing type II collagen degradation (CTX-II) and maintaining type II collagen synthesis [CPII].

Furthermore, N-acetylglucosamine (GlcNAc), a derivative of glucosamine, is reported to stimulate the hyaluronan synthesis via the upregulation of hyaluronan synthase-2 in chondrocytes [23], while it inhibits the IL-1 β -mediated expression of inducible NO synthase, cyclooxygenase-2 and IL-6 in chondrocytes [24]. These findings suggest that GlcNAc exerts chondroprotective and antiinflammatory actions in cartilage disorders. In fact, intra-articular injection of GlcNAc protects animals from experimental osteoarthritis [25, 26]. Furthermore, the administration of GlcNAc-containing beverage enhances the type II collagen synthesis, and relieves the symptoms of knee osteoarthritis in humans [27]. Moreover, we have recently evaluated the action of GlcNAc on the cartilage metabolism in healthy individuals without symptoms of arthritis, and demonstrated that the oral administration of GlcNAc at doses of 500 mg and 1000 mg/day similarly exhibits a chondroprotective action by lowering the C2C/PIICP

ratio, which indicates relative reduction of type II collagen degradation and increase of type II collagen synthesis. However, the effect of GlcNAc-containing diet was not significantly different from that of a placebo diet [28]. Thus, in the present study, to make the effect of a GlcNAc-containing diet more clear, the number of enrolled healthy subjects was increased and the effect of oral administration of GlcNAc-containing diet (a dose of 1000 mg/day GlcNAc) on the cartilage metabolism was evaluated by analyzing the ratio of type II collagen degradation to synthesis using type II collagen degradation (C2C) and synthesis (PIICP) markers. The results demonstrated that the GlcNAc-containing diet significantly suppresses the C2C/PIICP ratios compared with the placebo diet in the subject with joint loading and impaired cartilage metabolism among the enrolled healthy participants.

METHODS

Study design

A prospective randomized double-blind placebo-controlled, parallel-group comparative study was designed to assess the actions of a GlcNAc-containing diet and a placebo diet on the cartilage metabolism (type II collagen synthesis and degradation) in healthy individuals without symptoms of joint disorders. Additionally, the safety of the test supplement was evaluated. The study was performed from October 2014 to August 2015 and involved three clinical service organization centers in Japan. The study protocol with the title of “Evaluation of N-acetyl-glucosamine-containing diet on various cartilage markers” (protocol number: 25905) was approved by the local ethics committee and was conducted in accordance with the principles of the amended Declaration of Helsinki and ‘Ethical Guidelines for Epidemiological Research’ (established by the Japanese Government in 2008). Written informed consent was obtained from all participants prior to their enrollment in the study. The whole design of the study consisted of a 4-week run-in (screening) period, a 16-week intervention period, and a 4-week follow-up period without intervention. Subjects were screened at a baseline visit by a physical examination, a knee radiograph according to a standardized method, a symptom questionnaire and routine laboratory tests. Additionally, medical examinations and laboratory tests were performed at weeks 4, 8, 12 and 16 during the intervention, and 4 weeks after the intervention for the enrolled subjects. Furthermore, to evaluate walking habit of the participants, step counts were recorded by using a pedometer (OMRON HJ-105, OMRON Corporation, Kyoto, Japan) for 3 days before the visit at baseline, weeks 4, 8, 12, and 16 during the intervention and 4 weeks after the intervention; an average step count (steps/day) was also calculated.

Subjects

Major exclusion criteria contained the following: gout/hyperuricemia or rheumatoid arthritis; surgical treatment of joint(s) performed or its necessity; clinical history of bone or cartilage disorders including fracture and distortion within one year before the enrollment; routine use of dietary supplements containing hyaluronic acid, GlcNAc, glucosamine, chondroitin sulfate, collagen peptides or any other constituents of the test supplement within 3 months before the enrollment; hypersensitivity or allergy to constituents of the test supplement; diagnosis or current medication of disorders such as malignancies, hypertension, cardiac diseases, renal diseases, thyroid diseases, hepatic disorders and cerebral infarction; routine use of external medicine including poultices, and taking prescribed medicine (>3 days/week); intra-articular injections of either corticosteroids or hyaluronic acid within one year before the enrollment; severe exercise, which loads on the joints; daily drinking of >60 g alcohol/day; pregnant women, nursing mothers or women of child-bearing potential during the study period; participation in other clinical studies within 1 month before the enrollment; and the presence of any clinical conditions judged

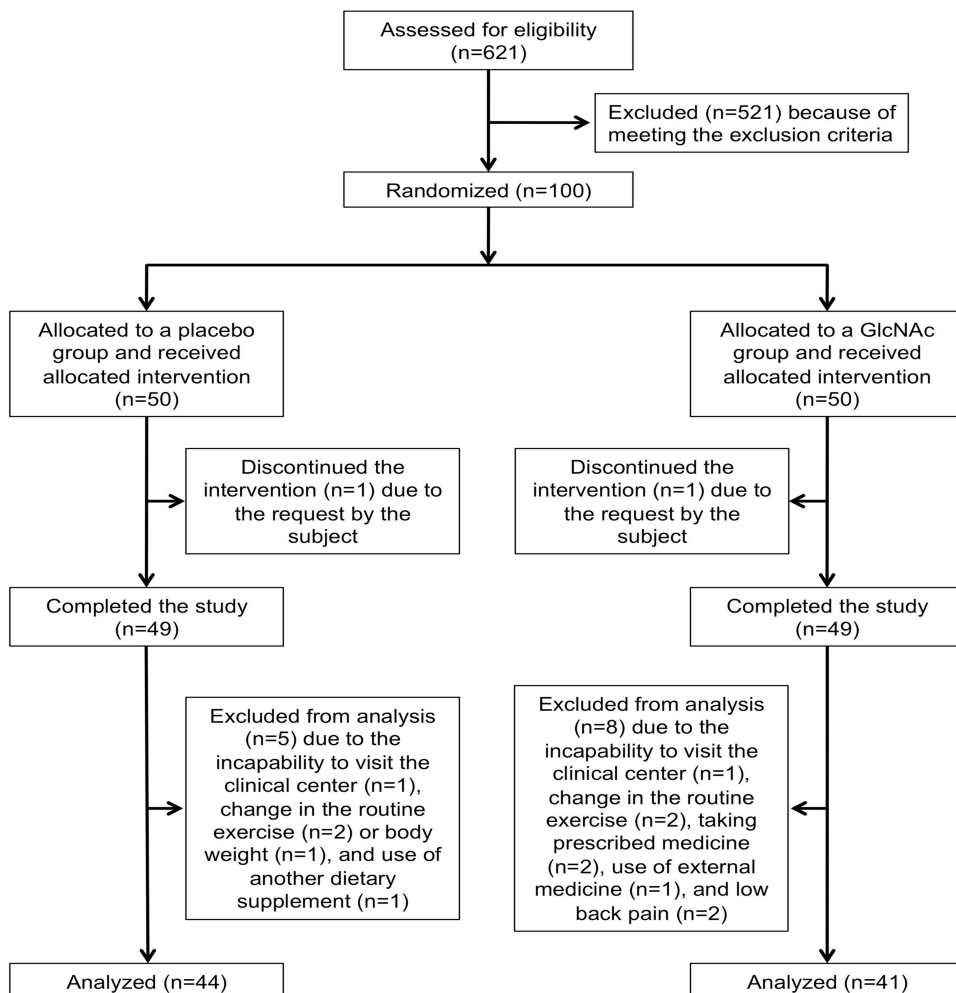


Figure 1. Flow diagram of the subjects who participated in the

by the medical investigator to preclude the participation of subjects in the study.

After the assessment of 621 subjects for eligibility, 521 subjects were excluded based on the exclusion criteria, and 100 Japanese adults (44 males and 56 females, aged 20-64 years, mean age 51.3 ± 1.0 years (mean \pm SE)) without clinical and radiographic evidence of knee osteoarthritis (Kellgren and Lawrence grades 0-1, mainly 0) [29] were enrolled as eligible subjects. The research coordinators created an allocation table and randomly assigned the eligible subjects to receive a GlcNAc-containing diet (n=50, GlcNAc group) or a placebo diet (n=50, placebo group) (Figure 1).

The allocation table was sealed, and all of the research staff and participants were blinded to the allocation during the test period. After completion of the study, the allocation table was made available for analysis of the data. During the intervention, 2 subjects discontinued the study due to their own requests (one subject in a placebo group and one subject in a GlcNAc group). In total, 98 subjects completed the study. Furthermore, 13 subjects were excluded by the medical investigator, because of an inability to visit the clinical centers, change in the routine exercise or body weight, use of another dietary supplement, use of prescribed medicine (antifungal or anti-allergic agent), use of external medicine (poultice), and low back pain (lumbago) during the intervention, which may affect the efficacy of test supplement (5 subjects in a placebo group and 8 subjects in a GlcNAc group). Thus, 85 subjects (mean age 51.6 ± 1.1 years (mean \pm SE); 44 subjects in a placebo group and 41 subjects in a GlcNAc group) were finally judged to be eligible for assessment of the efficacy of test supplement (Table 1). Moreover, to make the effect of test supplement even more clear, we focused on the subjects with joint loading and impaired cartilage metabolism, based on the step counts and the type II collagen degradation and synthesis markers respectively. Thus, 39 subjects (19 subjects in the placebo group and 20 subjects in the GlcNAc group) with decreased step counts (<6700 steps/day), lowered type II collagen degradation (<260 ng/ml C2C), and enhanced type II collagen synthesis (≥ 50 ng/ml PIICP) were excluded, while 46 subjects (25 subjects in the placebo group and 21 subjects in the GlcNAc group) with increased step counts (≥ 6700 steps/day), enhanced type II collagen degradation (≥ 260 ng/ml C2C), and lowered type II collagen synthesis (<50 ng/ml PIICP) were evaluated (Table 2).

Intervention and subject assignment

The test supplement was manufactured in a powder form (3.5 g in an aluminum pack) by Satoen Co., Ltd. (Shizuoka, Japan) and contained 1200 mg chitin degradation products (corresponding to 1000 mg GlcNAc) and a vehicle (2.3 g) consisting of 1800 mg dextrin, 400 mg green tea/green

tea extract and 100 mg rice, whereas the placebo diet (3.5 g) contained only a vehicle (3.5 g) consisting of 3000 mg dextrin, 400 mg green tea/green tea extract and 100 mg rice.

Subjects were randomly assigned to receive a 1000 mg GlcNAc-containing diet (GlcNAc group) or a placebo diet containing only vehicles (placebo group). All subjects were instructed to take the test supplement or placebo (dissolved in 100 ml water) once a day for 16 weeks. The daily dose of GlcNAc (1000 mg/day) was determined based on the results of the previous study [28]. Adherence to the intervention was evaluated on the basis of consumption record in the study diary, and <80% of adherence was considered a protocol violation.

Serum and second void of morning urine were collected from the subjects in a fasting state at baseline, weeks 4, 8, 12, and 16 during the intervention, and 4 weeks after the intervention. Serum and urine samples were immediately used for routine laboratory tests; alternatively, sera were aliquoted and stored at -80°C until the assays of C2C and PIICP.

Evaluation of efficacy and safety

To evaluate the effect of test supplement on the cartilage metabolism, serum samples collected at baseline, weeks 8, 12, and 16 during the intervention and 4 weeks after the intervention, were used for the assays for type II collagen degradation (C2C) and synthesis (PIICP) markers. Serum C2C and PIICP were measured using Collagen Type II Cleavage ELISA (IBEX Pharmaceuticals Inc., Montreal, Canada) and ELISA Kit for Procollagen II C-Terminal Propeptide (USCN Life Science Inc., Wuhan, China), respectively. Additionally, the C2C/PIICP ratios were calculated and compared between the GlcNAc and placebo groups.

Safety and tolerability were assessed throughout the study on the basis of the incidence and severity of intervention-related adverse events (side effects), as well as abnormal changes in physical parameters (such as blood pressure and pulse rate) and laboratory tests, including hematology, biochemical profile and urinalysis. Change in physical conditions and use of pharmaceutical products were also recorded in a diary.

Statistical analysis

Values are expressed as the means \pm standard errors (SE), unless otherwise noted. In the baseline characteristics of subjects, the distributions of males and females, and Kellgren and Lawrence grades were analyzed by the Pearson's chi-square test, and other parameters were analyzed by Student's *t*-test between the GlcNAc and placebo groups. Additionally, safety data were compared between the placebo and GlcNAc groups by Student's *t*-test; the changes of C2C and PIICP levels, and the C2C/PIICP ratios from the baseline during and after the intervention were

compared in the GlcNAc and placebo groups, and between the GlcNAc and placebo groups by Student's *t*-test. P values <0.05 were considered significant.

RESULTS

Characterization of study groups

Table 1 shows the baseline characteristics of 85 subjects (44 subjects in the placebo group; 41 subjects in the GlcNAc group), who completed the study and fulfilled the eligibility criteria. The baseline characteristics included demographic characteristics (age, and distribution of male and female subjects), physiological characteristics (body height, body weight, body mass index, systolic blood pressure, diastolic blood pressure and pulse rate), distribution of Kellgren and Lawrence grades, step counts per day, and levels of biomarkers for type II collagen metabolism (C2C, PIICP and C2C/PIICP ratio). Between the placebo and GlcNAc groups, these parameters were not essentially different. Adherence to the allotted dietary supplement (a placebo diet or a GlcNAc-containing diet) was 92-100% among the 98 subjects who completed the study.

Table 1. Baseline characteristics of the subjects in the placebo and GlcNAc groups

Variables	Placebo (n=44)	GlcNAc (n=41)	P value
Ages (years)	52.0 ± 1.4	51.1 ± 1.6	0.683
Male/female (number of subjects)	19/25	16/25	0.826
Height (cm)	164.00 ± 1.28	162.39 ± 1.22	0.369
Weight (kg)	58.30 ± 1.37	54.92 ± 1.40	0.089
Body mass index (kg/m ²)	21.62 ± 0.37	20.72 ± 0.35	0.084
Systolic blood pressure (mmHg)	118.8 ± 2.2	117.2 ± 1.9	0.582
Diastolic blood pressure (mmHg)	75.0 ± 1.3	72.1 ± 1.4	0.146
Pulse rate (beats/min)	69.0 ± 1.2	69.3 ± 1.1	0.830
Kellgren and Lawrence grade, 0:1			
Right knee (number of knees)	39:5	41:0	0.056
Left knee (number of knees)	39:5	40:1	0.204
Step counts (steps/day)	8876.6 ± 579.5	9351.8 ± 750.0	0.615
C2C (ng/ml)	301.01 ± 5.25	307.48 ± 6.48	0.437
PIICP (ng/ml)	40.64 ± 1.16	40.57 ± 1.17	0.967
C2C/PIICP ratio	7.75 ± 0.32	7.91 ± 0.34	0.737

Values are expressed as the mean ± SE, except the distributions of males and females, and Kellgren and Lawrence grades.

Assessment of cartilage metabolism using type II collagen degradation and synthesis markers

It has been demonstrated that the ratio of type II collagen degradation to synthesis can be used for the prediction of the progression of joint damage in patients with knee osteoarthritis [30, 31]. Based on this concept, to evaluate the effect of GlcNAc-containing supplement on cartilage metabolism, the C2C/PIICP ratios as well as C2C and PIICP were measured using sera collected at baseline, weeks 8, 12, and 16 during the intervention and 4 weeks after the intervention. C2C levels were gradually decreased from the baseline levels both in the placebo and GlcNAc groups during the intervention; C2C levels (ng/ml) were decreased from the baseline levels of 301.01 ± 5.25 and 307.48 ± 6.48 to 284.26 ± 5.83 ($P < 0.01$ compared with the baseline) and 292.82 ± 7.16 ($P < 0.05$) at 16 weeks during the intervention, and maintained almost the same levels of 286.27 ± 6.31 ($P < 0.01$) and 295.64 ± 6.26 ($P < 0.01$) at 4 weeks after the intervention in the placebo (n=44) and GlcNAc (n=41) groups respectively. However, there was no significant difference between the two groups in the changes of C2C levels. In contrast, PIICP levels were almost constant during and after the intervention both in the placebo and GlcNAc groups, and there was no significant difference between the two groups (data not shown). Consequently, as shown in Fig. 2A, the C2C/PIICP ratios were gradually decreased from the baseline both in the placebo and GlcNAc groups. The C2C/PIICP ratios were decreased from the baseline levels of 7.75 ± 0.32 and 7.91 ± 0.34 to 7.26 ± 0.38 and 7.21 ± 0.34 at 16 weeks during the intervention, and maintained almost the same ratios of 7.36 ± 0.35 and 7.29 ± 0.28 ($P < 0.01$ compared with the baseline) at 4 weeks after the intervention in the placebo (n=44) and GlcNAc (n=41) groups respectively. Consistent with these findings, the changes in the C2C/PIICP ratios from the baseline were decreased both in the placebo and GlcNAc groups. However, the ratios were slightly suppressed in the GlcNAc group (-0.70 ± 0.38 and -0.62 ± 0.22) compared with those in the placebo group (-0.50 ± 0.28 and -0.39 ± 0.28) at weeks 16 during the intervention and 4 weeks after the intervention respectively, although there was no significant difference between the 2 groups (Fig. 2B).

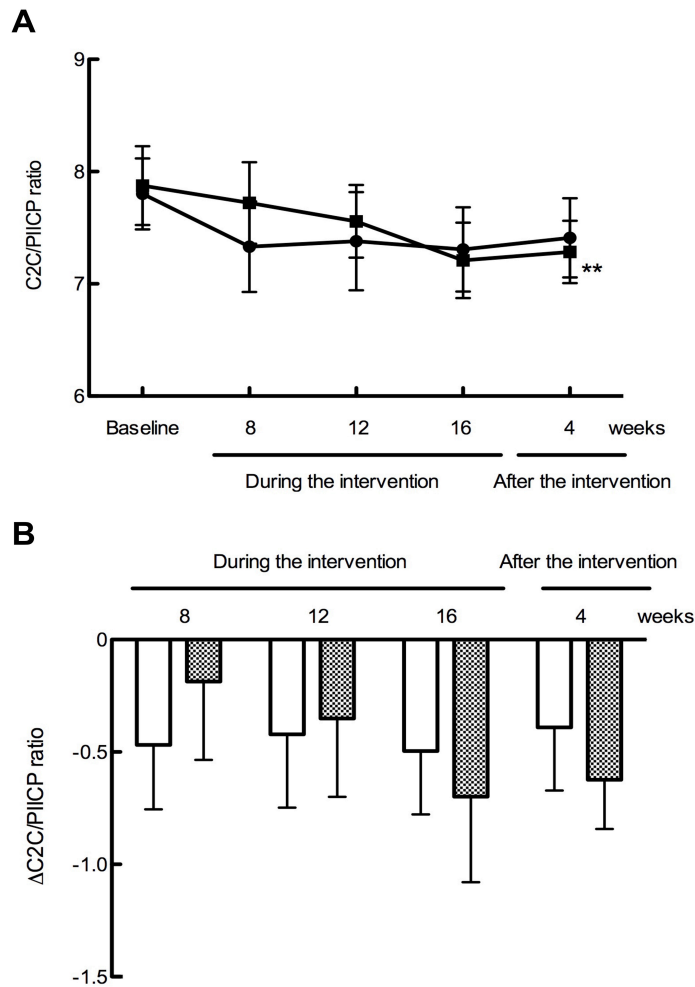


Figure 2. Changes of the C2C/PIICP ratios in the subjects of the placebo and GlcNAc groups during and after the intervention. C2C and PIICP levels were measured, and the C2C/PIICP ratios were calculated, using sera collected from the subjects in the groups of placebo (n=44; closed circles) and GlcNAc (n=41; closed squares) at baseline, weeks 8, 12, and 16 during the intervention and 4 weeks after the intervention (A). Moreover, the changes of the C2C/PIICP ratios from the baseline were calculated and expressed as Δ C2C/PIICP ratio in the groups of placebo (open column) and GlcNAc (hatched column) at weeks 8, 12, and 16 during the intervention and 4 weeks after the intervention (B). Values are expressed as the mean \pm SE. The C2C/PIICP ratios are compared with the baseline during and after the intervention in the placebo and GlcNAc groups (A). **P<0.01.

Next, to make the effect of the test supplement even more clear, we focused on the subjects with joint loading and impaired cartilage metabolism. Joint loading is an important determinant for the development of joint destruction [4]. Thus, joint loading was evaluated by the step counts of subjects, and the step counts of 6700 steps/day was used as a criterion for the joint loading. Additionally, cartilage metabolism was assessed with the type II collagen degradation and

synthesis markers. Thus, the subjects with decreased step counts (<6700 steps/day, 33% of subjects with the decreased level of walking), lowered type II collagen degradation (<260 ng/ml C2C, 10% of subjects with the decreased level of C2C) and enhanced type II collagen synthesis (≥ 50 ng/ml PIICP, 10% of subjects with the increased level of PIICP) were excluded, while 46 subjects (mean age 52.0 ± 1.3 years (mean \pm SE), 25 subjects in the placebo group and 21 subjects in the GlcNAc group) with increased step counts (≥ 6700 steps/day), enhanced type II collagen degradation (≥ 260 ng/ml C2C) and lowered type II collagen synthesis (<50 ng/ml PIICP) were evaluated. Table 2 shows the baseline characteristics of these subjects, including demographic characteristics, physiological characteristics, distribution of Kellgren and Lawrence grades, step counts per day and levels of biomarkers for type II collagen metabolism. Between the placebo (n=25) and GlcNAc (n=21) groups, these parameters were not significantly different, except for C2C level.

Table 2. Baseline characteristics of the subjects with ≥ 6700 steps/day of step counts, ≥ 260 ng/ml of C2C and <50 ng/ml of PIICP in the placebo and GlcNAc groups

Variables	Placebo (n=25)	GlcNAc (n=21)	P value
Ages (years)	51.2 ± 1.9	53.0 ± 1.7	0.499
Male/female (number of subjects)	11/14	8/13	0.769
Height (cm)	164.08 ± 1.83	162.53 ± 1.62	0.539
Weight (kg)	58.44 ± 1.98	54.58 ± 1.48	0.137
Body mass index (kg/m ²)	21.62 ± 0.51	20.60 ± 0.31	0.111
Systolic blood pressure (mmHg)	120.1 ± 2.6	119.6 ± 2.2	0.884
Diastolic blood pressure (mmHg)	76.2 ± 1.9	74.3 ± 1.8	0.503
Pulse rate (beats/min)	69.4 ± 1.8	69.5 ± 1.6	0.962
Kellgren and Lawrence grade, 0:1			
Right knee (number of knees)	21:4	21:0	0.114
Left knee (number of knees)	21:4	21:0	0.114
Step counts (steps/day)	10723.9 ± 658.7	11541.9 ± 967.6	0.477
C2C (ng/ml)	299.38 ± 4.98	322.42 ± 8.11	0.016
PIICP (ng/ml)	40.12 ± 1.17	38.25 ± 1.64	0.347
C2C/PIICP ratio	7.67 ± 0.32	8.82 ± 0.52	0.057

Values are expressed as the mean \pm SE, except the distributions of males and females, and Kellgren and Lawrence grades.

C2C level was not essentially changed in the placebo group during the intervention (16 weeks) and after the intervention (4 weeks) (data not shown). In contrast, C2C level (ng/ml) was decreased in the GlcNAc group (n=21) from the baseline of 322.42 ± 8.11 to 306.19 ± 10.70 at 16 weeks during the intervention, and maintained almost the same levels of 309.70 ± 8.21 ($P < 0.05$ compared with the baseline) at 4 weeks after the intervention. However, there was no significant difference between the two groups. Interestingly, PIICP level was increased in the GlcNAc group from the baseline of 38.25 ± 1.64 to 42.35 ± 2.22 at 16 weeks during the intervention, and maintained almost the same levels of 42.39 ± 2.22 ($P < 0.05$ compared with the baseline) at 4 weeks after the intervention. In contrast, PIICP level was almost constant in the placebo group during and after the intervention (data not shown). However, there was no significant difference between the two groups in the changes of PIICP levels during and after the intervention.

Based on these findings, the C2C/PIICP ratio was decreased in the GlcNAc group from the baseline of 8.82 ± 0.52 to 7.70 ± 0.51 at 16 weeks during the intervention, and maintained almost the same ratio of 7.71 ± 0.44 ($P < 0.01$ compared with the baseline) at 4 weeks after the intervention, whereas the C2C/PIICP ratio was almost constant in the placebo group during and after the intervention (Fig. 3A). However, there was no significant difference in the changes of C2C/PIICP ratios between the two groups. Interestingly, the changes in the C2C/PIICP ratios from the baseline were decreased in the GlcNAc group by -1.13 ± 0.61 at week 16 during the intervention and maintained almost the same level (-1.12 ± 0.32) at 4 weeks after the intervention, although the changes in the C2C/PIICP ratios from the baseline were not essentially changed in the placebo group during and after the intervention (Fig. 3B). Consequently, the changes in the C2C/PIICP ratios from the baseline were significantly suppressed in the GlcNAc group compared with the placebo group at weeks 16 during the intervention ($P < 0.05$) and 4 weeks after the intervention ($P < 0.05$). Notably, the difference between the placebo and GlcNAc groups was slightly decreased at 4 weeks after the intervention (placebo group, 0.25 ± 0.39 vs. GlcNAc group, -1.12 ± 0.32) compared with that at weeks 16 during the intervention (placebo group, 0.34 ± 0.35 vs. GlcNAc group, -1.13 ± 0.61).

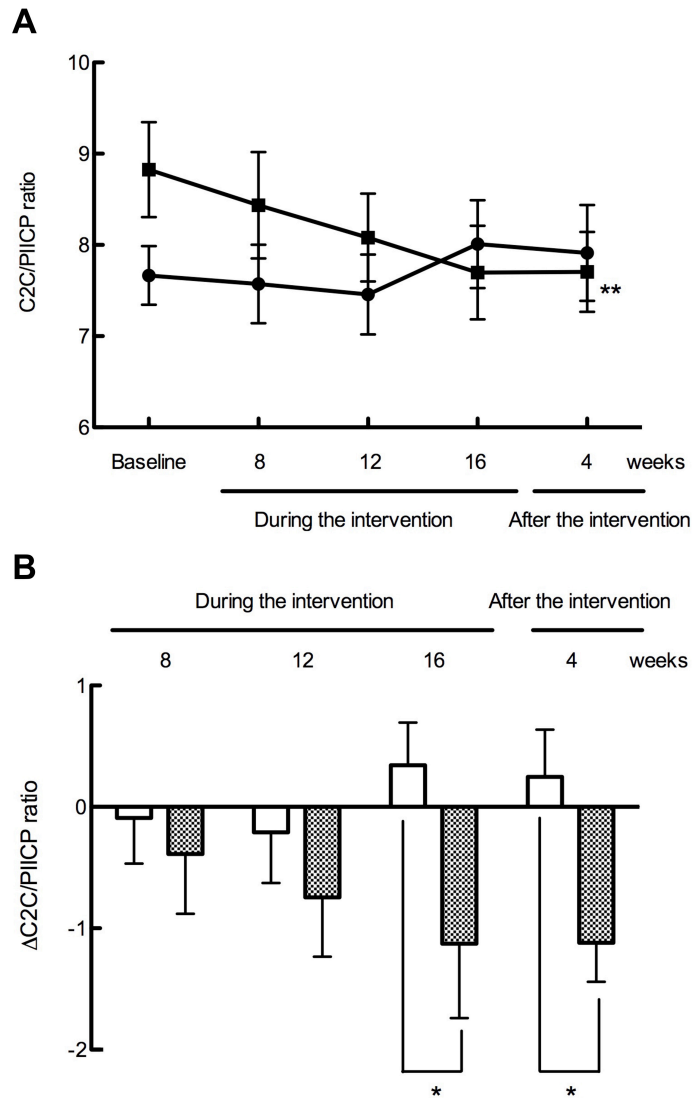


Figure 3. Changes of the C2C/PIICP ratios in the subjects with ≥ 6700 steps/day of step counts, ≥ 260 ng/ml of C2C and < 50 ng/ml of PIICP in the placebo and GlcNAc groups during and after the intervention. C2C and PIICP levels were measured, and the C2C/PIICP ratios were calculated, using sera collected from the subjects in the groups of placebo (n=25; closed circles) and GlcNAc (n=21; closed squares) at baseline, weeks 8, 12, and 16 during the intervention and 4 weeks after the intervention (A). Moreover, the changes of the C2C/PIICP ratios from the baseline were calculated and expressed as Δ C2C/PIICP ratio in the groups of placebo (open column) and GlcNAc (hatched column) at weeks 8, 12, and 16 during the intervention and 4 weeks after the intervention (B). Values are expressed as the mean \pm SE. The C2C/PIICP ratios are compared with the baseline during and after the intervention in the placebo and GlcNAc groups (A), or compared between the placebo and GlcNAc groups (B). *P<0.05, **P<0.01.

Together these observations suggest that the oral administration of test supplement containing GlcNAc exhibit a protective action on the cartilage metabolism against the subjects with joint loading and impaired cartilage metabolism among the healthy individuals without symptoms of joint disorders, by improving the C2C/PIICP ratio, which indicates relative reduction of type II collagen degradation and increase of type II collagen synthesis.

Assessment of safety and tolerability

Fifteen subjects (30%) in the placebo group (n=50) and 24 subjects (48%) in the GlcNAc group (n=50) experienced one or more adverse events during the intervention period among 100 enrolled subjects. The total number of adverse events reported was 23 in the placebo group and 49 in the GlcNAc group, and there was no significant difference in the frequency between the two groups (P=0.10). Major adverse events reported from the subjects of the placebo and GlcNAc groups were symptoms of common cold (sore throat, cough, rhinorrhea and/or fever), back pain, joint pain (knee), lassitude/fatigue, and neck pain. All adverse events were of mild intensity and judged by the medical investigator to be unrelated to the intervention.

Furthermore, the physical measurement parameters (body weight and body mass index), physiological examinations (systolic and diastolic blood pressures, and pulse rate), and laboratory tests (urinalysis, hematology and blood chemistry) did not show any significant changes from the baseline during and after the intervention in the two groups.

DISCUSSION

The biomarkers for cartilage metabolism, especially type II collagen metabolism, can be used for screening a risk of progressive joint destruction and also for monitoring the effects of structure-modifying agents or dietary supplements on osteoarthritis [8]. For example, the actions of chondroprotective agents, i.e., glucosamine and chondroitin sulfate, have been evaluated by using type II collagen degradation biomarkers such as CTX-II and C2C [14, 32-34]. Moreover, type II collagen synthesis biomarkers, such as CPII (PIICP), have been used in combination with type II collagen degradation biomarkers (e.g., CTX-II and C2C) for monitoring the disease state and progression of osteoarthritis, because the combination of type II collagen degradation and synthesis biomarkers (calculating the ratio of type II collagen degradation to synthesis) has been demonstrated to be more effective for predicting the progression of osteoarthritis or monitoring the action of chondroprotective agents on cartilage metabolism in osteoarthritis [30, 31]. Based on these concepts, in order to evaluate the action of GlcNAc on joint health of healthy subjects without symptoms of arthritis in the present study, we have conducted a randomized

double-blind placebo-controlled clinical trial and evaluated the effect of oral administration of GlcNAc (at a dose of 1000 mg/day) on the cartilage metabolism in healthy individuals (mostly middle-aged adults, an average age of 51 years) by investigating the ratio of type II collagen degradation to synthesis using type II collagen degradation (C2C) and synthesis (PIICP) markers.

The results indicated that the changes in the C2C/PIICP ratios from the baseline were slightly suppressed in the GlcNAc group compared with those in the placebo group at weeks 16 during the intervention and 4 weeks after the intervention in the initial analysis. However, there was no significant difference between the two groups (Fig. 2B). To make the effect of GlcNAc even more clear, the subjects with joint loading (≥ 6700 steps/day) and impaired cartilage metabolism (≥ 260 ng/ml C2C and < 50 ng/ml PIICP) were evaluated. Subjects with a walking habit of 6700 steps/day are estimated to walk 4 km every day, on the assumption of a stride being 60 cm. Interestingly, the changes in the C2C/PIICP ratios from the baseline decreased in the GlcNAc group at week 16 during the intervention and 4 weeks after the intervention, whereas the changes in the C2C/PIICP ratios from the baseline were not essentially changed in the placebo group during and after the intervention (Fig. 3B). Thus, the changes in the C2C/PIICP ratios from the baseline were significantly suppressed in the GlcNAc group compared with the placebo group at week 16 during the intervention ($P < 0.05$) and 4 weeks after the intervention ($P < 0.05$). Interestingly, the difference between the placebo and GlcNAc groups was slightly decreased at 4 weeks after the intervention compared with that at week 16 during the intervention, suggesting that the effect of GlcNAc is possibly reversible and can disappear after withdrawal of the administration of GlcNAc. Moreover, test supplement-related adverse events were not essentially observed during and after the intervention. Altogether, these observations and results likely suggest that the oral administration of GlcNAc at a dose of 1000 mg/day exhibits a chondroprotective action on the healthy people without apparent adverse effect, by lowering the C2C/PIICP ratio, which indicates relative reduction of type II collagen degradation and increase of type II collagen synthesis, and improving the cartilage metabolism. In the present study, GlcNAc exhibited a chondroprotective action on individuals with joint loading (walking habit) by improving C2C/PIICP ratio. Since walking habit gives the load on joints in thigh and lower leg (including hip, knee, and ankle joints), GlcNAc is speculated to protectively act on these joints. However, the changes in the serum levels of type II collagen degradation and synthesis markers reflect the cartilage metabolism in joints of the whole body [5]. Thus, GlcNAc is expected to exert a chondroprotective action on other joints (such as shoulder and elbow joints) than the above-mentioned joints in thigh and lower leg.

The mechanism by which the GlcNAc-containing dietary supplement exerts a protective action on the cartilage metabolism remains to be clarified. In this context, it is interesting to note that GlcNAc stimulates hyaluronan synthesis by upregulating hyaluronan synthase-2 in chondrocytes [23]. Importantly, hyaluronan inhibits the IL-1 β -induced MMP-13 expression in arthritic chondrocytes via the action on the principal receptor CD44 [35]. Moreover, hyaluronan suppresses the aggrecan degradation by downregulating IL-1 α -induced expression of ADAMTS (a disintegrin-and metalloproteinase with thrombospondin motifs)-4, an aggrecanase, through the CD44 signaling in osteoarthritic chondrocytes [36], and also suppresses the IL-1 β -induced expression of MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 in osteoblasts [37]. Notably, GlcNAc inhibits the IL-1 β -mediated expression of inducible NO synthase, cyclooxygenase-2 and IL-6 via the inhibition of MAPK (mitogen-activated protein kinase) signaling in chondrocytes [24]. More recently, we have revealed with a DNA microarray analysis that GlcNAc inhibits the degradation of type II collagen possibly by downregulating the expression of periostin and upregulating the expression of lipocalin 2 in a rat osteoarthritis model [38]; periostin is involved in the degradation of cartilage through the expression of inflammatory cytokines and MMPs [39-42], whereas lipocalin 2 regulates the proliferation and differentiation of chondrocytes [43]. Based on these findings, it is interesting to speculate that GlcNAc induces the production of hyaluronan and modulates the expression of various inflammatory and chondroprotective molecules (such as MMPs, ADAMTSs, periostin and lipocalin 2) involved in the degradation and maintenance of cartilage, thereby reducing the C2C/PIICP ratio, which indicates relative decrease of type II collagen degradation and increase of type II collagen synthesis, and improving the cartilage metabolism. However, the detailed mechanism for the chondroprotective action of GlcNAc in humans remains to be elucidated in the future.

Nevertheless, the present study has a limitation. In the initial analysis (Fig. 2), the subjects with various conditions of joint loading and cartilage metabolism were enrolled, and the statistically significant effect of the test supplement (GlcNAc) on the cartilage metabolism could not be detected. Thus, in the second analysis (Fig. 3), the subjects with joint loading and impaired cartilage metabolism were analyzed, and consequently the test supplement (GlcNAc) was revealed to be significantly effective for improving the cartilage metabolism. These observations suggest that some valuable criteria (such as joint loading and the levels of biomarkers) should be introduced for screening the enrolled subjects in the randomized double-blind placebo-controlled clinical trials to demonstrate the potential effect of dietary supplements on the cartilage metabolism in healthy individuals without symptoms of arthritis.

It has already been demonstrated that the intra-articular injection of GlcNAc exhibits the chondroprotective actions on experimental osteoarthritis models [25, 26], and the administration of GlcNAc-containing beverage improves the symptoms of patients with knee osteoarthritis possibly by increasing type II collagen synthesis [27]. However, the convincing effect of GlcNAc on healthy individuals has not been established. Consequently, this is the first study to demonstrate the significant effect of oral administration of GlcNAc on the cartilage metabolism in healthy individuals. The efficacy and safety of GlcNAc observed in the present study suggest that a dietary supplement containing GlcNAc can be safely administered and potently exerts a chondroprotective action on the healthy people without symptoms of arthritis by improving the type II collagen metabolism in the cartilage. Thus, GlcNAc-containing supplement can be considered a potential candidate for maintaining or caring the joint health of healthy people.

CONCLUSIONS

The present study has revealed that the oral administration of GlcNAc at a dose of 1000 mg/day possibly exerts a chondroprotective action on the healthy individuals by lowering the C2C/PIICP ratio, which indicates relative reduction of type II collagen degradation and increase of type II collagen synthesis without apparent adverse effects.

Competing interests: All authors have no financial interests or conflict of interests.

Authors' Contributions: HI, TH and MO designed the research; AT and MF performed the clinical study; AT and IN analyzed the data; IN prepared the manuscript.

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