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Evaluation of the effect of N-acetyl-glucosamine administration on biomarkers for cartilage metabolism in healthy individuals: a randomized double-blind placebo-controlled clinical study

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ABSTRACT

Background: A randomized double-blind placebo-controlled clinical study was conducted to evaluate the chondroprotective action of N-acetyl-glucosamine (GlcNAc) supplement on healthy individuals without arthritis.

Methods: Healthy subjects (n=120, 54.3 ± 8.1 years (mean ± SD)) without arthritis were randomly assigned to receive 500 mg GlcNAc (n=60, GlcNAc group) or a placebo (n=60, placebo group) once a day for 16 weeks, and cartilage metabolism was evaluated by analyzing

type II collagen degradation (C2C) and type II collagen synthesis (PIICP) markers, and the ratio of type II collagen degradation to type II collagen synthesis (C2C/PIICP).

Results: Among 120 eligible subjects, 114 subjects completed the study. First, all the subjects with a body mass index of ≥25 kg/m^2 and Kellgren and Lawrence grade of ≥1 were analyzed. However, the changes in the C2C and PIICP levels and the C2C/PIICP ratios from the baseline were not significantly different between the placebo and GlcNAc groups during 16 weeks and 8 weeks after the intervention. Next, to make the effect of GlcNAc even clearer, the subjects with body mass index of <25 kg/m^2 and Kellgren and Lawrence grade of 0 were analyzed. The changes in the C2C levels from the baseline were significantly decreased in the GlcNAc group compared with the placebo group at 8 and 12 weeks during the intervention. In contrast, the changes in the PIICP levels from the baseline levels were almost constant during and after the intervention in both groups. Based on these findings, the changes in the C2C/CIICP ratios from the baseline slightly decreased in the GlcNAc group compared to the placebo group at 8 and 12 weeks during the intervention. Furthermore, no test supplement-related adverse events were observed during and after the intervention.

Conclusion: These observations suggest that oral administration of GlcNAc at a dose of 500 mg/day exerts a chondroprotective effect in subjects without arthritis. This effect was achieved by improving cartilage metabolism (reducing type II collagen degradation), without causing apparent adverse effects.

Keywords: N-acetyl-glucosamine, biomarker, cartilage metabolism, joint health, randomized clinical study

BACKGROUND

Osteoarthritis develops due to the progressive destruction of articular cartilage, being the most common joint disease, which often results in physical dysfunction and pain in elderly people [1-3]. In osteoarthritis, knee joints are particularly impaired, because they are weight-bearing

joints. Previous studies in experimental osteoarthritis models have indicated that changes in the chemical and metabolic properties of cartilage matrix are detectable at early stages of arthritis [4]. Thus, various molecular markers have been developed to measure cartilage metabolism in joint disorders [5-9]. Such biomarkers are used to evaluate the actions of disease-modifying drugs and chondroprotective supplements on the cartilage as they specifically reflect alterations in cartilage metabolism [10].

Type II collagen is a major constituent of articular cartilage and represents 90-95% of the cartilage collagens [7]. Thus, the components of type II collagen have been used as biomarkers for cartilage metabolism. A C-terminal crosslinking peptide (CTX-II) is cleaved during degradation of type II collagen [11], while a neoepitope (C2C) is generated by intrahelical cleavage at the C terminus of the 3/4 piece of degraded type II collagen [12,13]. Thus, CTX-II and C2C are both used as markers for type II collagen degradation. In contrast, a C-terminal type II procollagen propeptide (PIICP or CPII), which is cleaved during the processing of newly synthesized type II procollagen, can be used as a marker for type II collagen synthesis [14].

Various nutritional supplements, including glucosamine, chondroitin, and collagen, are used to promote joint health. They are also specifically used to treat or prevent cartilage disorders such as osteoarthritis [15,16]. Glucosamine suppresses the degradation and stimulates the synthesis of glycosaminoglycans [17, 18]. Glucosamine also suppresses the expression of collagen-degrading enzymes, such as matrix metalloproteinases (MMPs) and stimulates type II collagen synthesis in chondrocytes [19, 20]. Thus, these studies suggested that glucosamine may exert chondroprotective action in cartilage disorders by maintaining glycosaminoglycans and type II collagen levels in the articular cartilage [21-23]. More importantly, we evaluated the effect of glucosamine on the cartilage metabolism in healthy individuals using soccer players by analyzing the levels of CTX-II and CPII [24]. The results indicated that oral administration of glucosamine exhibits a chondroprotective action on soccer players by preventing type II collagen degradation (CTX-II) and maintaining type II collagen synthesis (CPII).

Furthermore, N-acetylglucosamine (GlcNAc), a derivative of glucosamine, is expected to exert chondroprotective action in cartilage disorders because GlcNAc stimulates the hyaluronan

synthesis via the upregulation of hyaluronan synthase-2 in chondrocytes [25] and the intra-articular injection of GlcNAc protects animals from experimental osteoarthritis [26, 27]. In fact, 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 either 500 mg or 1000 mg/day similarly exhibits a chondroprotective action by lowering the C2C/PIICP ratio, which indicates the relative reduction of type II collagen degradation by GlcNAc [28]. However, the effect of GlcNAc was not significantly different from that of a placebo diet, because the number of subjects analyzed in each group was small (n=7~10) [28]. Thus, in the present study, in order to make the effect of a GlcNAc clearer we increased the sample size in each group (n≥50). The effect of oral administration of GlcNAc (a dose of 500 mg/day) on the cartilage metabolism was evaluated by analyzing type II collagen degradation (C2C) and synthesis (PIICP) markers, and C2C/PIICP ratio.

METHODS

Study design

A prospective randomized double-blind placebo-controlled, parallel-group comparative study was designed to evaluate the effects of GlcNAc and a placebo diet on the cartilage metabolism (type II collagen synthesis and degradation) in healthy individuals without findings of joint disorders. Additionally, the safety of the test supplement was evaluated throughout the study. The study was registered at the UMIN Clinical Trials Registry (Trial No. UMIN000020589), performed by TTC Co., Ltd., Tokyo, Japan from January 2016 to October 2016 and involved three clinical service organization centers in Japan (Tana Orthopedic Surgery, Kanagawa, Kitashinyokohama Orthopedic Surgery, Kanagawa and Shirayuri Dermatology and Orthopedic Surgery). The study protocol with the title of “Evaluation of the effect of N-acetly-glucosamine-containing diet on cartilage markers” (protocol number: 27048) 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 8-week, run-in (screening) period, a 16-week intervention period, and an 8-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 8 weeks after the intervention for the enrolled subjects.

Subjects

The primary endpoint was the difference in the C2C/PIICP ratio between the groups. Considering data from the literature [28], it was assumed that the mean difference (MD) would be 1.64, with a SD of 3.0. To detect this difference, with a power of 80% and a significance level of 5% and taking into account that 20% of the subjects will be lost to follow-up, it was calculated that 54 subjects would be needed to be studied in each group. Thus, finally a total of 120 subjects were recruited as eligible subjects (60 subjects in each group; GlcNAc and placebo groups). Inclusion criteria were as follows: i) Japanese males or females aged 40-74 years; ii) without joint pain; iii) radiographic severity of knee joints grade 0 or 1, based on Kellgren and Lawrence grades [29] (grade 0, no radiographic features of osteoarthritis; grade 1, doubtful joint space narrowing and possible osteophytic lipping) without diagnosis of osteoarthritis by orthopedist.

Exclusion criteria were the folloeing: i) a diagnosis of gout/hyperuricemia or rheumatoid arthritis; ii) surgical treatment of joint(s) performed or required; iii) clinical history of bone or cartilage disorders including fracture and distortion within one year prior to enrollment; iv) routine use of dietary supplements containing hyaluronic acid, N-acetyl glucosamine, glucosamine, chondroitin sulfate, collagen peptides or any other constituents of the test supplement within three months prior to enrollment; v) hypersensitivity or allergy to the test component; vi) diagnosis or current medication of disorders including malignancies, hypertension (atherosclerosis), cardiac, renal, thyroid, lung and hepatic disorders, or cerebral infarction with medication of warfarin; vii) routine use of external medicine including poultices and taking prescribed medicine (>3 days/week); viii) intra-articular injections of either

corticosteroids or hyaluronic acid within one year prior to enrollment; ix) subjects, who are regularly performing excessive exercise, which places loads on the joints; x) daily drinking of >60 g alcohol/day; xi) pregnant women, nursing mothers or women intending to have children in the future; xii) participation in any other clinical studies within one month prior to enrollment; and xiii) the presence of any clinical conditions judged by the medical investigators to preclude the participation of subjects in the study.

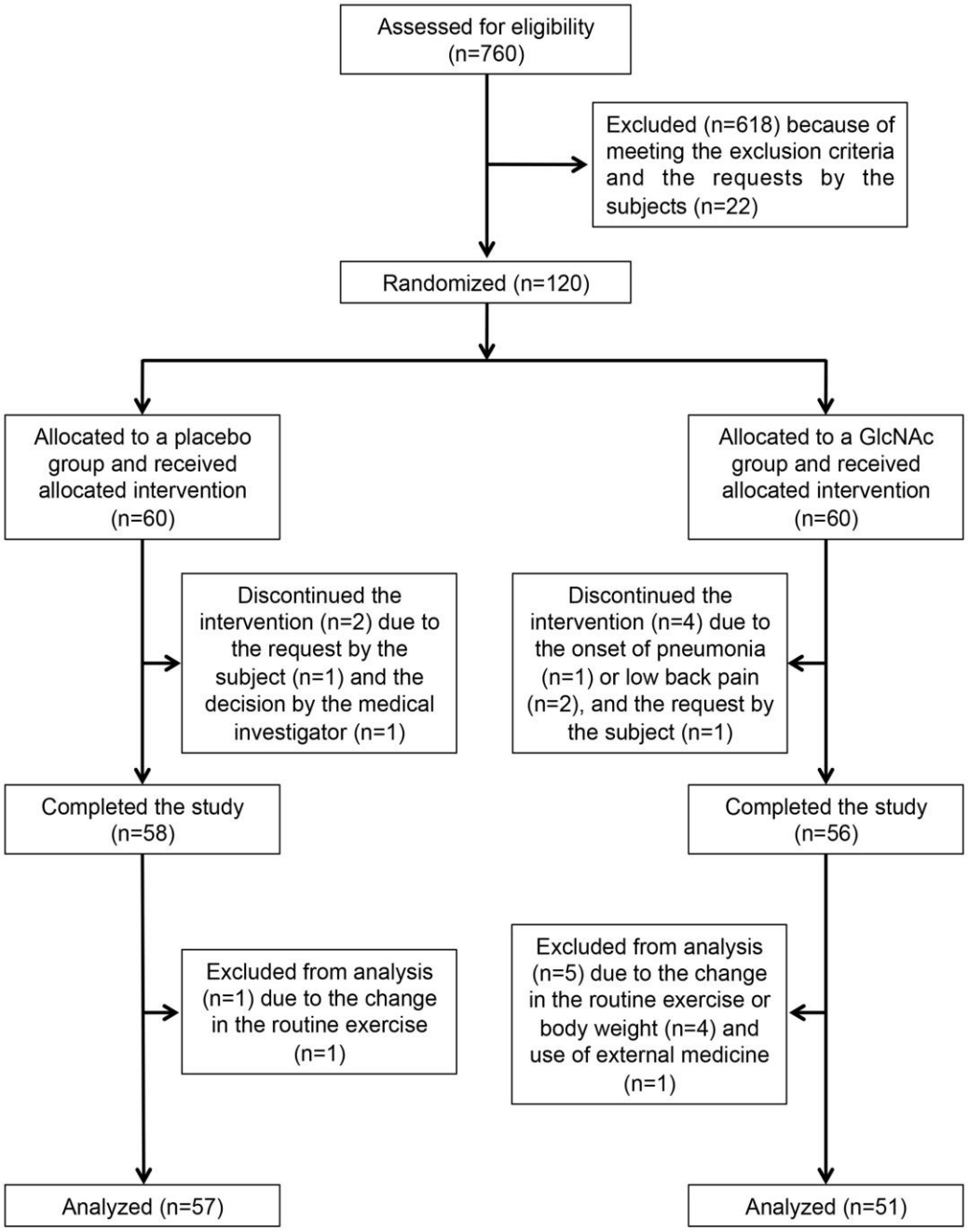

Figure 1. Flow diagram of the subjects who participated in the study.

Following the assessment of 760 subjects for eligibility, 618 subjects were excluded based on the exclusion criteria and 22 subjects declined to participate of their own volition. Finally, 120 Japanese adults (48 males and 72 females; aged 40-74 years; mean age, 54.3 ± 8.1 years (mean ± SD)) without knee joint pain and radiographic evidence of knee osteoarthritis (Kellgren and Lawrence grades 0-1, mainly 0) [29] were enrolled as eligible subjects. The research co-coordinators randomly assigned the eligible subjects to receive a placebo (60 subjects in a placebo group) and GlcNAc (60 subjects in a GlcNAc group; Figure 1) using a table of random numbers. The allocation table was sealed, and all research staffs (which included medical investigators, clinical service staffs, analyzers of urine and blood samples, and data, and staffs of TTC and other research services) and participants were blinded to the allocation during the test period. Following completion of the study, the allocation table was made available for analysis of the data. During the intervention, 6 subjects discontinued the study of their own volition due to the onset of pneumonia or low back pain and the decision of the medical investigator (2 subjects in a placebo group and 4 subjects in a GlcNAc group). Thus, 114 subjects completed the study. Furthermore, 6 subjects were excluded by the medical investigator, because of the change in the routine exercise or body weight and the use of external medicine (poultice) for knee joint pain due to varices in the lower during the intervention, which may affect the efficacy of test supplement (1 subject in a placebo group and 5 subjects in a GlcNAc group). As a result, 108 subjects (mean age 54.0 ± 7.9 years; 57 subjects in a placebo group and 51 subjects in a GlcNAc group) were finally judged to be eligible for assessment of the efficacy of test supplement (Figure 1 and Table 1). Moreover, to improve clarity regarding the effect of the test supplement, further assessments focused on healthier subjects, as determined by their body mass indices in addition to Kellgren and Lawrence grades [29], since being overweight (joint loading) is an important determinant for the developing of joint destruction [4]. Thus, the subjects with body mass index of ≥25 kg/m^2 and Kellgren and Lawrence grade of ≥1 (17.6 % of the 108 subjects) were excluded,and 89 subjects (mean age, 53.4 ± 7.9 years; 48 subjects in placebo group, 41 subjects in GlcNAc group) with body mass index of <25 kg/m^2 and Kellgren and Lawrence grade of 0 were evaluated (Table 2).

Intervention and subject assignment

The participants were instructed not to change their lifestyles, including eating habits, sleep habits, drinking of alcohol, and especially exercise habits. They were also instructed to not start taking any dietary supplements during the test period or to stop taking dietary supplements if they already started taking dietary supplements. Furthermore, the participants were instructed to visit clinical service centers for medical examinations and laboratory tests in the morning on the appointed time with fasting state (from 22 pm of the preceding day of the visit) and abstinence from alcohol (from the preceding day of the visit). The test supplement was manufactured in a powder-form preparation containing 500 mg GlcNAc or 500 mg maltodextrin (as a placebo) by Q'sai Co., Ltd. (Fukuoka, Japan).

Subjects were randomly assigned to receive 500 mg GlcNAc (GlcNAc group) or 500 mg maltodextrin (placebo group) per day. 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 (500 mg/day) was determined based on the results of a previous study [28]. Adherence to the intervention was evaluated on the basis of consumption record in the study diary recorded by the enrolled subjects, and <80% of adherence was considered a protocol violation.

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

Evaluation of cartilage metabolism

To evaluate the effect of the test supplement on cartilage metabolism, the levels of type II collagen degradation (C2C) and synthesis (PIICP) markers were evaluated in serum samples collected at baseline, weeks 8, 12, and 16 during the intervention and 8 weeks after the intervention, using Collagen Type II Cleavege ELISA (IBEX Pharmaceuticals Inc., Montreal, Canada) and ELISA Kit for Procollagen II C-Terminal Propeptide (Cloud-Clone Corp., USA), respectively. Additionally, the C2C/PIICP ratios were calculated and compared between the GlcNAc and placebo groups.

Safety evaluation

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

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Regarding the baseline characteristics of subjects, the distributions of males and females and Kellgren and Lawrence grades were analyzed using Pearson's χ^2 test with other parameters being analyzed using the Student's *t*-test between the GlcNAc and placebo groups. Safety data, the changes of C2C and PIICP levels (secondary endpoints), and the C2C/PIICP ratios (the primary endpoint) from the baseline during and after the intervention were compared between the GlcNAc and placebo groups using the Student's *t*-test. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Characterization of study groups

Table 1 presents the baseline characteristics of the 108 subjects (57 subjects in a placebo group; 51 subjects in a 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, and levels of biomarkers for type II collagen metabolism (C2C, PIICP, and C2C/PIICP ratio). There were no significant differences in these parameters between the placebo and GlcNAc groups at the baseline. Adherence to the allotted supplement was ≥93% among the 114 subjects who completed the study.

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

Variables	**Placebo (n=57)**	**GlcNAc (n=51)**	**P value**
Ages (years)	53.7 ± 7.7	54.5 ± 8.2	0.5
Male/female (number of subjects)	26/31	16/35	0.2
Height (cm)	163.2 ± 7.7	161.6 ± 8.0	0.3
Weight (kg)	56.5 ± 9.0	56.8 ± 9.9	0.9
Body mass index (kg/m^2)	21.1 ± 2.0	21.6 ± 2.4	0.2
Systolic blood pressure (mmHg)	121.6 ± 12.2	121.8 ± 13.5	0.9
Diastolic blood pressure (mmHg)	75.3 ± 8.9	74.9 ± 8.9	0.8
Pulse rate (beats/min)	70.8 ± 8.9	70.4 ± 8.6	0.8
Kellgren and Lawrence grade, 0:1			
Right knee (number of knees)	53:4	48:3	>0.9
Left knee (number of knees)	51:6	47:4	0.7
C2C (ng/ml)	275.8 ± 27.7	277.6 ± 28.4	0.7
PIICP (ng/ml)	33.7 ± 7.1	33.3 ± 6.6	0.8
C2C/PIICP ratio	8.6 ± 2.2	8.6 ± 1.8	0.8

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

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

In addition to the markers for type II collagen degradation (C2C) and synthesis (PIICP), the ratio of type II collagen degradation to synthesis can be used to predict the progression of joint damage in patients with knee osteoarthritis [30, 31]. Thus, the effect of GlcNAc on cartilage metabolism was evaluated by measuring the C2C/PIICP ratio in addition to levels of C2C and PIICP using sera collected at baseline, weeks 8, 12, and 16 during the intervention and 8 weeks after the intervention.

C2C levels (ng/ml) significantly decreased from the baseline levels of 275.8 ± 27.7 and 277.6 ± 28.4 to 219.6 ± 43.6 and 223.2 ± 43.8 at 16 weeks during the intervention ($P<0.01$), and slightly increased to 251.7 ± 47.7 and 246.9 ± 46.8 at 8 weeks after the intervention in the placebo and GlcNAc groups respectively, although the C2C levels were still decreased compared with the baseline levels at 8 weeks after the intervention ($P<0.01$). Thus, the changes in the C2C levels from the baseline were decreased both in the placebo (-56.7 ± 48.7) and GlcNAc (-54.8 ± 44.3) groups at 16 weeks during the intervention, and slightly returned to the baseline levels in the placebo (-24.6 ± 47.2) and GlcNAc (-31.3 ± 41.4) groups at 8 weeks after the intervention, although there was no significant difference between the two groups (Fig. 2A).

By contrast, PIICP levels did not essentially change, both in the placebo and GlcNAc groups during 16 weeks and 8 weeks after the intervention, although PIICP level (ng/ml) significantly increased in the placebo group from the baseline level of 33.7 ± 7.1 to 37.3 ± 11.6 at 16 weeks during the intervention ($P<0.05$). Thus, the changes in the PIICP levels from the baseline PIICP levels were almost constant during and after the intervention both in the placebo and GlcNAc groups, except that the change slightly increased in the placebo group (3.5 ± 10.0) compared with the GlcNAc group (1.1 ± 8.9) at 16 weeks during the intervention (Fig. 2B). However, there was no significant difference between the two groups.

Based on these findings, C2C/PIICP ratios significantly decreased from the baseline levels of 8.6 ± 2.2 and 8.6 ± 1.8 to 6.6 ± 2.6 and 6.9 ± 2.3 at 16 weeks during the intervention ($P<0.01$), and increased to the baseline levels (8.7 ± 3.2 and 8.0 ± 2.9) at 8 weeks after the intervention in the placebo and GlcNAc groups respectively. Thus, the changes in the C2C/PIICP ratios from the baseline were slightly decreased in the placebo (-2.0 ± 2.1) and GlcNAc (-1.6 ± 2.4) groups at 16 weeks during the intervention and returned to the baseline levels at 8 weeks after the intervention, although there was no significant difference between the two groups (Fig. 2C).

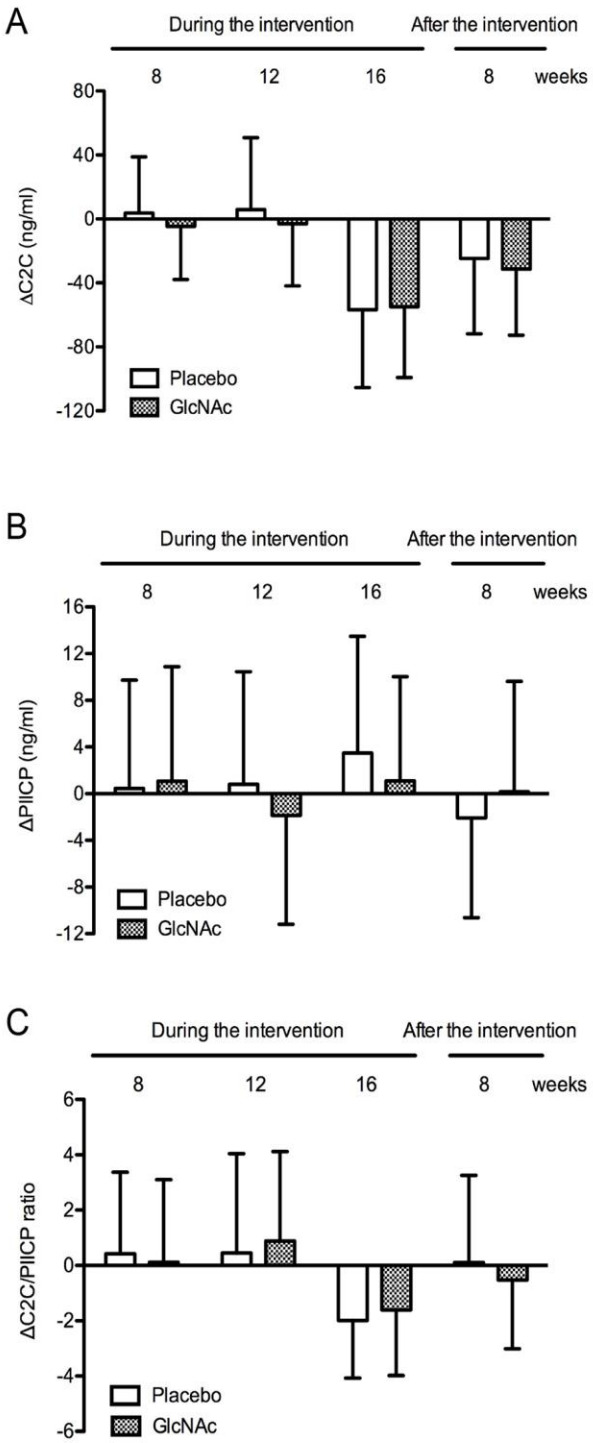

Figure 2. Cartilage metabolism in the placebo and GlcNAc groups during and after the intervention. C2C and PIICP levels were measured, and the C2C/PIICP ratios were calculated, using sera collected from the subjects in the placebo groups of placebo (n=57) and GlcNAc (n=51) at baseline, weeks 8, 12, and 16 during the intervention and 8 weeks after the intervention. The data of C2C (A), PIICP (B), and C2C/PIICP ratio (C) are expressed as the changes from the baseline at weeks 8, 12, and 16 during the intervention and 8 weeks after the intervention. Values are expressed as the mean ± SD.

Assessment of cartilage metabolism in subjects without overweight and radiographic feature of osteoarthritis

In order to improve clarity regarding the effect of the test supplement, further assessments focused on healthier subjects, as determined by their body mass indices in addition to Kellgren and Lawrence grades [29], since overweight (joint loading) is an important determinant for the developing of joint destruction [4]. Thus, the subjects with body mass index of ≥25 kg/m^2 and Kellgren and Lawrence grade of ≥1 (17.6 % of the 108 subjects) were excluded, and 89 subjects (mean age, 53.4 ± 7.9 years; 48 subjects in placebo group, 41 subjects in GlcNAc group) with body mass index of <25 kg/m^2 and Kellgren and Lawrence grade of 0 were evaluated. Table 2 presents the baseline characteristics of these subjects, including demographic characteristics, physiological characteristics, and levels of biomarkers. There were no significant differences in these parameters between the placebo and GlcNAc groups at the baseline. Thus, the effect of GlcNAc on cartilage metabolism was evaluated using these subjects at baseline, weeks 8, 12, and 16 during the intervention and 8 weeks after the intervention.

Table 2. Baseline characteristics of the subjects with body mass index of <25 kg/m^2 and Kellgren and Lawrence grade of 0 in the placebo and GlcNAc groups.

Variables	Placebo (n=48)	GlcNAc (n=41)	P value
Ages (years)	53.0 ± 7.6	53.8 ± 8.2	0.7
Male/female (number of subjects)	21/27	13/28	0.3
Height (cm)	162.7 ± 7.7	161.5 ± 7.9	0.5
Weight (kg)	55.6 ± 8.4	55.1 ± 8.1	0.8
Body mass index (kg/m^2)	20.9 ± 1.8	21.0 ± 1.9	0.7
Systolic blood pressure (mmHg)	121.3 ± 12.5	120.3 ± 13.9	0.7
Diastolic blood pressure (mmHg)	74.8 ± 8.8	74.3 ± 9.1	0.8
Pulse rate (beats/min)	70.3 ± 9.0	70.8 ± 9.2	0.8
C2C (ng/ml)	276.1 ± 26.2	276.7 ± 30.4	0.9
PIICP (ng/ml)	34.2 ± 6.7	33.2 ± 6.4	0.5
C2C/PIICP ratio	8.4 ± 2.1	8.6 ± 1.9	0.6

Values are expressed as the mean ± SD, except the distributions of males and females.

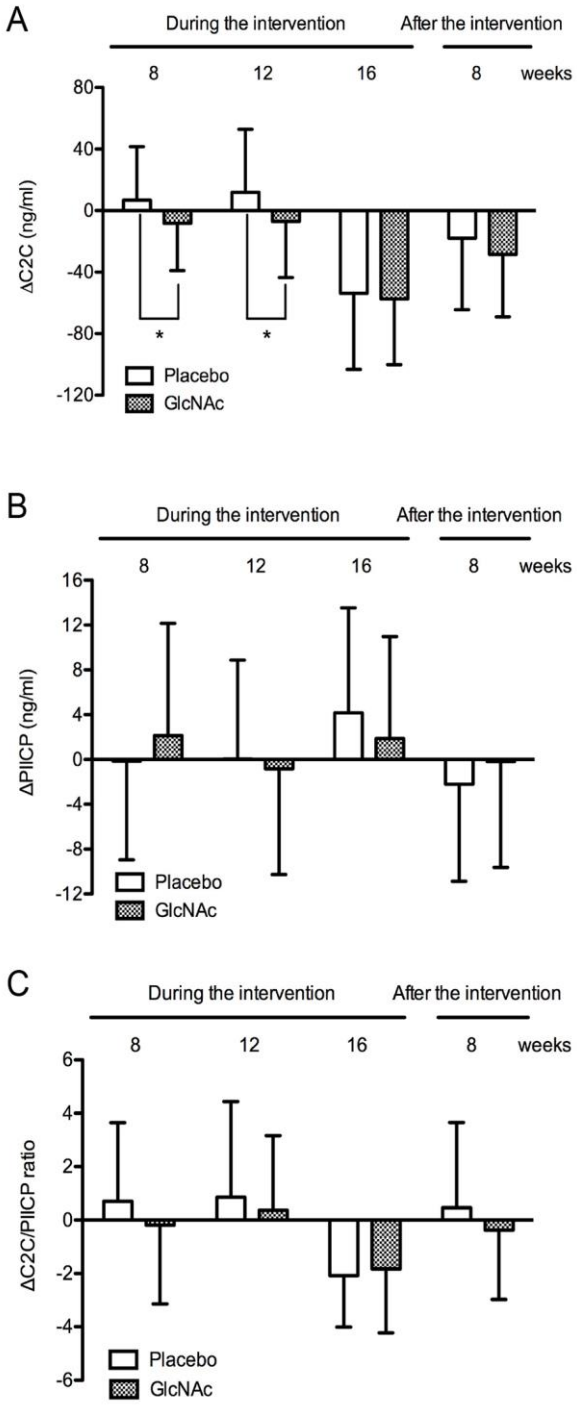

Figure 3. Cartilage metabolism in subjects with body mass index of <25 kg/m^2 and Kellgren and Lawrence grade of 0. C2C and PIICP levels were measured, and the C2C/PIICP ratios were calculated, using sera collected from the subjects in the placebo groups of placebo (n=48) and GlcNAc (n=41) at baseline, weeks 8, 12, and 16 during the intervention and 8 weeks after the intervention. The data of C2C (A), PIICP (B), and C2C/PIICP ratio (C) are expressed as the changes from the baseline at weeks 8, 12, and 16 during the intervention and 8 weeks after the intervention. Values are expressed as the mean ± SD, and compared between the placebo and GlcNAc groups. *P<0.05.

Thus, the effect of GlcNAc on cartilage metabolism was evaluated using these subjects at baseline, weeks 8, 12, and 16 during the intervention and 8 weeks after the intervention. C2C levels (ng/ml) significantly decreased from the baseline levels of 276.1 ± 26.2 and 276.7 ± 30.4 to 223.1 ± 43.5 and 219.3 ± 39.5 at 16 weeks during the intervention ($P<0.01$) and slightly increased to 258.9 ± 46.2 and 248.3 ± 46.3 at 8 weeks after the intervention in the placebo and GlcNAc groups respectively, although the C2C levels were still decreased compared with the baseline levels at 8 weeks after the intervention ($P<0.05$). Thus, the changes in the C2C levels from the baseline were decreased both in the placebo (-53.7 ± 49.6) and GlcNAc (-57.4 ± 42.7) groups at 16 weeks during the intervention and slightly increased to the baseline levels in the placebo (-17.9 ± 46.5) and GlcNAc (-28.4 ± 40.7) groups at 8 weeks after the intervention, although there was no significant difference between the two groups (Fig. 3A). However, C2C levels slightly increased from the baseline levels to 282.9 ± 32.2 and 288.7 ± 44.0 in the placebo group, but decreased to 268.4 ± 32.7 and 269.7 ± 37.2 in the GlcNAc group at 8 and 12 weeks during the intervention. Thus, the changes in the C2C levels from the baseline were significantly decreased in the GlcNAc group (-8.3 ± 30.7 and -7.1 ± 36.5) compared with the placebo group (6.8 ± 34.8 and 11.9 ± 40.9) at 8 and 12 weeks during the intervention ($P<0.05$) (Fig. 3A).

In contrast, PIICP levels did not essentially change in the placebo and GlcNAc groups during 16 weeks and after 8 weeks of the intervention, although PIICP level (ng/ml) significantly increased in the placebo group from the baseline level of 34.2 ± 6.7 to 38.5 ± 10.7 at 16 weeks during the intervention ($P<0.01$). Thus, the changes in the PIICP levels from the baseline PIICP levels were almost constant during and after the intervention, both in the placebo and GlcNAc groups, except that the change slightly increased in the placebo group (4.2 ± 9.4) compared with the GlcNAc group (1.9 ± 9.1) (Fig. 3B). However, there was no significant difference between the two groups.

Based on these findings, C2C/PIICP ratios significantly decreased from the baseline levels of 8.4 ± 2.1 and 8.6 ± 1.9 to 6.3 ± 2.3 and 6.8 ± 2.3 at 16 weeks during the intervention ($P<0.01$), and increased to the baseline levels (8.9 ± 3.4 and 8.2 ± 3.0) at 8 weeks after the intervention in the placebo and GlcNAc groups respectively. Thus, the changes in the C2C/PIICP ratios from the baseline were slightly decreased both in the placebo (-2.1 ± 1.9) and GlcNAc (-1.8 ± 2.4) groups

at 16 weeks during the intervention and returned to the baseline levels at 8 weeks after the intervention (Fig. 3C). However, at 8 and 12 weeks during the intervention, the changes in the C2C/CIICP ratios from the baseline were slightly decreased in the GlcNAc group (-0.2 ± 3.0 and 0.4 ± 2.8) compared with the placebo group (0.7 ± 3.0 and 0.9 ± 3.6), although there was no significant difference between the two groups.

Assessment of safety and tolerability

Out of all 120 enrolled subjects, 15 subjects (25.0%) in the placebo group and 32 subjects (53.3%) in the GlcNAc group experienced one or more adverse events during the intervention period. Major adverse events reported from the subjects belonging to the placebo and GlcNAc groups were symptoms of a common cold (sore throat, cough, and/or bronchitis), diarrhea, knee pain, back pain, neck/shoulder pain, and headache. The total number of adverse events reported was 21 and 58 in the placebo and GlcNAc groups respectively, and the frequency of adverse events occurring was significantly higher in the GlcNAc group than the placebo group ($P<0.01$). However, all adverse events were of mild or moderate intensity and judged by the medical investigator to be unrelated to the intervention.

Furthermore, changes in 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) were minimal and within the reference values during the intervention in both groups, except for one subject in the GlcNAc group, in whom the levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and γ-glutamyl transpeptidase were increased over the reference values. However, the levels were still increased after the completion of intervention. Thus, the event was judged by the medical investigator to be unrelated to the intervention.

DISCUSSION

Accumulating evidence indicates that biomarkers for cartilage metabolism, particularly type II collagen metabolism, can be used to screen for individuals at risk of progressive joint destruction, and for monitoring the effects of structure-modifying agents or dietary supplements on joint diseases like osteoarthritis [8, 9]. For example, previous studies have demonstrated the use of

type II collagen degradation biomarkers, including CTX-II and C2C, to evaluate the effects of chondroprotective agents such as glucosamine [32, 33] and chondroitin sulfate [34]. Furthermore, type II collagen synthesis biomarkers, such as CPII (PIICP), have been used alone or in combination with type II collagen degradation biomarkers (CTX-II and C2C) to monitor the disease state and progression of osteoarthritis, since the ratio of type II collagen degradation to synthesis has been demonstrated to be useful for monitoring the effect of chondroprotective agents [30,31]. Based on these findings, in this study a randomized double-blind placebo-controlled clinical trial was performed in order to evaluate the effect of oral Gl cNAc administration (500 mg/day) on joint health of healthy individuals without arthritis. The effect of GlcNAc on cartilage metabolism in healthy middle-aged adults (mean age, 54.3 ± 8.1 years) was investigated by analyzing type II collagen degradation (C2C) and synthesis (PIICP) markers, and C2C/PIICP ratio.

The results indicated that the changes in the C2C levels from the baseline were decreased both in the placebo and GlcNAc groups at 16 weeks during the intervention, and slightly increased to the baseline levels in both groups at 8 weeks after the intervention (Fig. 2A). In contrast, the changes in the PIICP levels from the baseline levels were almost constant during and after the intervention both in the placebo and GlcNAc groups (Fig. 2B). Based on these findings, the changes in the C2C/PIICP ratios from the baseline were slightly decreased both in the placebo and GlcNAc groups at 16 weeks during the intervention and returned to the baseline levels at 8 weeks after the intervention (Fig. 2C). However, there were no significant differences in the changes of C2C, PIICP levels, and C2C/PIICP ratios from the baseline between the placebo and GlcNAc groups during and after the intervention. To further elucidate the effect of the GlcNAc supplement, the subjects with body mass index of $\geq$25 kg/m^2 and Kellgren and Lawrence grade of $\geq$1 were excluded, and the subjects with body mass index of <25 kg/m^2 and Kellgren and Lawrence grade of 0 were evaluated. Notably, the changes in the C2C levels from the baseline were significantly decreased in the GlcNAc group compared with the placebo group at 8 and 12 weeks during the intervention ($P<0.05$) (Fig. 3A). In contrast, the changes in the PIICP levels from the baseline levels were almost constant during and after the intervention both in the

placebo and GlcNAc groups (Fig. 3B). Based on these findings, the changes in the C2C/CIICP ratios from the baseline were slightly decreased in the GlcNAc group compared with the placebo group at 8 and 12 weeks during the intervention, although there was no significant difference between the two groups (Fig. 3C). Lastly, no test supplement-related adverse events were observed during and after the intervention. Together, these observations suggest that oral administration of GlcNAc at doses of 500 mg/day exhibits a chondroprotective effect on the healthy individuals without any apparent adverse effect, possibly by improving cartilage metabolism via the reduction of type II collagen degradation.

The mechanism by which the GlcNAc exerts a protective effect on the cartilage metabolism still needs to be clarified. In this context, it is interesting to note that GlcNAc stimulates hyaluronan synthesis via the upregulation of hyaluronan synthase-2 in chondrocytes [25]. Hyaluronan is reported to inhibit IL-1β-induced MMP-13 expression via its principal receptor, CD44, and subsequent signaling of p38 mitogen-activated protein kinase (MAPK) in arthritic chondrocytes [35]. Additionally, hyaluronan suppresses aggrecan degradation by downregulating IL-1α-induced expression a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4, an aggrecanase, through the CD44 signaling in osteoarthritic chondrocytes [36]. Hyaluronan also suppresses the IL-1β-induced expression of MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 in osteoblasts [37]. Notably, GlcNAc inhibits the IL-1β-mediated expression of inducible NO synthase, cyclooxygenase-2 and IL-6 via the inhibition of MAPKs including c-jun N-terminal kinase, extracellular signal-related kinase and p38MAPK activation in chondrocytes [38]. Therefore, GlcNAc may improve cartilage metabolism by reducing type II collagen degradation due to its chondroprotective and anti-inflammatory effects based on the suppression of cartilage degrading enzymes such as MMPs and ADAMTSs, potentially via the production of hyaluronan. However, a detailed mechanism outlining how GlcNAc acts on cartilage metabolism via type II collagen degradation still needs to be clarified.

The present study had a limitation. In the initial analysis (Fig. 2), all the subjects containing those with body mass index of ≥25 kg/m^2 and Kellgren and Lawrence grade of ≥1 were analyzed, 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 body mass index of <25 kg/m^2 and Kellgren and Lawrence grade of 0 were analyzed, and consequently the test supplement (GlcNAc) was revealed to be significantly effective for improving cartilage metabolism (suppressing type II collagen degradation) compared with the placebo. These observations indicate that some adequate criteria (such as body mass index and radiographic feature of osteoarthritis) should be introduced for strictly screening the subjects enrolled in the randomized double-blind placebo-controlled clinical trials to demonstrate the chondroprotective effect of dietary supplements on the cartilage metabolism in healthy individuals without arthritis.

To the best of our knowledge, this study was the first to demonstrate the potential effect of oral GlcNAc administration on cartilage metabolism in healthy individuals. However, it has previously been demonstrated that the intra-articular injection of GlcNAc exhibits chondroprotective effects on experimental osteoarthritis models [26,27], and the administration of a GlcNAc-containing beverage improved the symptoms of patients with knee osteoarthritis, possibly by relatively increasing type II collagen synthesis and reducing the ratio of CTX-II/CPII [39]. Moreover, the oral administration of GlcNAc-containing green tea supplement exerts a chondroprotective action on the healthy individuals by lowering the C2C/PIICP ratio, which demonstrates the relative reduction of type II collagen degradation and increase of type II collagen synthesis [40]. The efficacy and safety of GlcNAc demonstrated in the present study indicates that GlcNAc supplement can be safely administered, as it exerts a potent chondroprotective effect on healthy individuals by improving the type II collagen metabolism (suppressing type II collagen degradation) in the cartilage without any major adverse effects. Therefore, GlcNAc supplement as a functional food [41] can be a potential candidate for maintaining or improving joint health of healthy individuals without arthritis.

CONCLUSION

The present study has demonstrated that the oral administration of GlcNAc at a dose of 500 mg/day exerts a chondroprotective action on the healthy individuals by suppressing type II collagen degradation without apparent adverse effects.

List of Abbreviations: GlcNAc, N-acetyl-glucosamine; C2C, type II collagen degradation; PIICP, type II collagen synthesis; CTX-II, C-terminal crosslinking peptide; MMPs, matrix metalloproteinases; MD, mean difference; SD, standard deviation.

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