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Original article

Study for investigation of symptomatic improvement and safety of the ingestion of rooster comb degradation product containing low-molecular hyaluronic acid (INJUV) in individuals with knee and lower back pain; open-label trial with no control group

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Abstract

***Objectives*:** The objectives of this study were to verify the efficacy in subjective and objective symptoms and safety of the ingestion of food sample, rooster comb degradation product containing low-molecular hyaluronic acid (INJUV) under an open-label trial with no control group.

***Methods*:** The subjects were 12 healthy adults (6 males and 6 females, age: 57.5 ± 3.5 years old) selected from 30 individuals who suffered with gonalgia and lumbago. INJUV was administered to the subjects by oral ingestion for 8 weeks.

***Results*:** Improvements of gonalgia and lumbago were observed as subjective symptoms. The range of motion in the knee joint was extended as physical findings (left and right average; before 137.4 ± 1.8, 2.9%, $p < 0.05$). Furthermore, diastolic blood pressure was lowered (before 75.7 ± 3.2 mmHg, –8.2%, $p < 0.05$) and HbA1c was decreased (before 5.47 ± 0.08%, –3.3%, $p < 0.01$). No adverse effect was observed during the trial.

***Conclusion*:** The test results showed the improvement in gonalgia and lumbago, and the extension of the range of motion in the knee joint, as well as the safety of INJUV. It was assumed that this low-molecular samples, characterized by a high rate of absorption, exerted beneficial effects on articular cartilage tissues, synovial cells and immunocompetent cells. Further examinations are needed to clarify the efficacy and the mechanism of action.

***KEY WORDS*:** low-molicular hyaluronic acid, arthralgia, lumbago, osteoarthritis, range of motion

Introduction

Locomotive syndrome means the state of lowered mobile or locomotive function due to disorders of locomotoriums such as muscle, bones and joint. In progression of locomotive syndrome (herein after referred as "locomo"), people are forced to walk with a cane and move using a wheelchair, which lowers quality of life (QOL). This could lead to a person being bedridden and requiring long-term care level 5. Japan has been facing a situation where the population is rapidly aging. Countermeasures for the prevention and treatment for locomo could not be achieved without the extension of health expectancy in Japanese society.

This time we focus on osteoarthritis (OA), which is one of the factors inducing locomo. We examined whether or not symptoms of gonalgia and lumbago were mitigated by the ingestion of functional food. It has been reported that glycative stress involves pathogenic mechanisms of rheumatoid arthritis (RA), and OA [1-5]. INJUV, which is a rooster comb degradation product, whose main component is hyaluronic acid was chosen as a test sample in this study [6]. This product is popular among users in post marketing surveillance and is known to have repeat customers.The subjects were healthy males and females who suffered from pain in the knee and lower backin their daily lives. INJUV efficacies on OA and lumbago were examined for 8 weeks; this study was an open trial with no control group.

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Methods

Subjects

With a recruitment notification, males and females aged 55-69 years, with knee and/or low back pain in daily life were asked to participate. The selected subjects were as follows; among the 32 potential individuals who met the eligibility requirements, the inclusion criteria, and did not meet the exclusion criteria, the 12 subjects with the greatest representation in the questionnaire regarding the following index 1) and 2) were selected (male; 6, female; 6, age; 57.5 ± 3.5 years).

1) Japanese Knee Osteoarthritis Measure 7-11): JKOMI. Score in "Degree of keen pain"
2) Japan Lower back pain Evaluation. Questionnaire 12-15): JLEQ I. Score in "Degree of lower back pain"

Inclusion criteria and exclusion criteria were as follows;

Inclusion criteriag

1) Males and females aged 55-69 years.
2) Healthy individuals not receiving any medical disease treatment
3) Individuals with knee and/or lower back pain in daily life (in walking, in sitting with one's legs bent beneath oneself and in ascending and descending stairs)
4) Individuals who are able to sign and provide written consent for participation, after receiving enough explanation of the purpose and details of the study, understanding the study well, and deciding to participate in the study with their own will
5) Individuals who are willing and able to comply with scheduled visits and examinations
6) Individuals judged appropriate for the study by the principal investigator

Exclusion Criteria

1) Individuals recieving medical products for treatment of chronic diseases
2) Individuals receiving outpatient care (except for bonesetters, chiropractors, massagers) for knee and/or lower back pain
3) Individuals with a history of palliative therapy (hyaluronic acid, steroid injection, *etc*.) or surgical therapy (arthroscopic osteotomy, artificial joint, bolt fixation surgery, *etc*.) on knees and/or waist
4) Individuals who have a present or past medical history of psychiatric disease, sleep disorder, high blood pressure, diabetes, dyslipidemia and or other serious diseases
5) Individuals who have or had administration of a drug to treat a disease in the past month (except temporary usage for headache, menstrual pain, common cold and pollenosis)
6) Individuals who have a present or past medical history of serious hepatopathy, renal dysfunction, heart disease, respilatory disease or hyper blood disease
7) Individuals who have a comorbidity and a medical history with gastrointestinal disease (except a medical history of caecum)
8) Individuals with inflammatory disorders such as chronic sinusitis, periodontal disease, bronchitis and othere
9) Individuals with body mass index: BMI ≧ 30.0 kg/m^2
10) Individuals who donated blood over 200 mL in the past month or over 400 mL in the past three months
11) Individuals with serious anemia
12) Individuals who could develop an allergy to the food components contained in the test substance or have a serious allergic reaction to other foods or medical products
13) Individuals with continuous ingestion of food that could affect study results such as health food, food professing improvement of knee and/or low back pain and component expecting effects that may interfere with the study results (especially glucosamine, chondroitin and others) in the past three months, at present or during the examination period, except for general health food for maintaining health
14) Individuals with intake of alcohol 40 mg/day or more, or weekly 200 mg/week or more in average
15) Individuals who are smokers or ex-smokers within one year after quitting
16) Individuals with possible changes of exercise habits or life style during the examination period
17) Individuals who are currently participating at or have participated in other clinical studies in the past 3 months
18) Individuals who are pregnant, are possibly or hope to become pregnant, or are lactating
19) Individuals who are or whose family are engaged in health and functional foods, and/or cosmetic companies for development, manufacture and marketing
20) Individuals judged inappropriate for the study by the principal investigator

Test sample

The test sample was "INJUVcapsule". Useful component injuv (herein after referred as "INJUV") was a powdery material, which was obtained through a freeze-drying process of rooster comb, and is manufactured and sold by Laimu Co., Ltd. (Kohoku-ku, Yokohama, Kanagawa, Japan). INJUV was developed through a unique enzyme process at normal temperature, using rooster comb as a raw material. Products have been sold not only in Japan but also in 11 countries all over the world since 2001, and especially in the United States sales performance is over forty-two million capsules a year. The test samples for this study were sponsored by this company.

This sample is a powder material produced from rooster comb. The raw material, rooster comb was degraded and processed by the food-derived enzyme and this rooster comb enzyme decomposition product was freeze-dried and processed into powder with three times the amount of dextrin (binding food additive) as rooster comb powder [6]. This sample contained 150 mg of INJUV per one capsule, the same as the commercial product does. INJUV is a naturally-derived degradation product with components of a whole rooster comb, which contains HA, collagen peptide, proteoglycan (aggrecan), these precursors, amino acid and vitamins. Generally, oral administration of HA with a high molecular weight of more than a million or a low molecular wight of between one hundred thousand and two hundred thousand has a limited effectiveness. However, this product has been developed by enzyme degradation to be a molecular weight of between 380 and 5,000, which is regarded as an ultra-low molecular weight.

Table 1 shows "Nutrition component of the test product." ***Table 2*** shows "Free amino acid component of the test product" [6]. The weight of the smallest unit of HA is a

Component	Content (%)	Method
Water	2.2～2.6	
Nitrogen	3.84	Semimicro-Kjeldahl method
Protein	3.04	Lowry protein assay
Free amino acid	4.08	Ninhydrin assay
N-Acetylglucosamine	0	Elson-Morgan method
Dextrin (additives)	75.0	

Table 2. Free amino acid component of the test product.

Amino acid	Content (%)	Amino acid	Content (%)
ρ-Serine	1.71	Cystine	2.78
Taurine	3.30	Leucin	2.26
Asparatic acid	2.94	Isoleucine	6.27
Threonine	1.30	Tyrosine	2.65
Serine	2.20	Phenylalanine	3.30
Glutamic acid	2.18	β-amino isobutyric acid	5.45
Glutamine	0.48	Ornithine	1.05
Sarcosine	1.81	Lysine	1.17
Glycine	2.26	1-Methylhistidine	0.78
Alanine	3.52	Anserine	1.92
Citrulline	0.92	Arginine	1.93
α-Amino butyric acid	2.18	Total amino acid identified	57.36
Cysteine	1.03	Unknown amino acid	42.64
Methionine	1.97		

molecular weight of 411, which is a molecular bond of one molecule of glucuronic acid and one molecule of N-acetylglucosamine. Listing the assumed molecular weight of HA contained in the sample, in order of component weight ratio, showed 1,520 (47%), 5,000 (33%), 1,140 (10%), 760 (6%) and 380 (4%), and revealed that the main components are low molecular HA, which have high absorption efficiency. The N-acetylglucosamine content is 0%, as HA degradation product is not contained.

Macromolecule HA has a strong water retention capacity and is utilized for instillation and as a medical agent for intra-articular injection and skin moisturizing lotion. However, there has been only one report about the effects of oral administration of low molecular HA, which was a randomized double-blind clinical trial conducted at Beijing University of Chinese Medicine Dongzhimen Hospital (the People's Republic of China). It was shown that this sample showed an enhanced water retention property in keratin [6].

The safety of the sample, INJUV, was confirmed ($LD_{50} \geqq 5{,}050$ mg/kg body weight) by a 14-day INJUV administration test consigned to Still Meadow Inc. in U.S.A., which corresponded to GLP test.

Trial design

The trial design was as follows; type of control: none, trial blinding schema: open, intervention: oral ingestion of the test product, 8 capsules a day (as INJUV, 1,200 mg/day), which were ingested as 4 capsules twice a day with water or tepid water.

Blood examinations were performed before and 8 weeks after the administration. Medical questionnaire sheet survey, physical examination, specialized blood examination (hs-CRP) and specialized urine examination (pentosidine and creatinine) were performed before, 2 weeks after, 4 weeks after, 6 week after and 8 weeks after the administration. The test period was from March of 2017 to August of 2017.

Assessment items

• Subjective symptoms

Japanese Knee Osteoarthritis Measure (JKOM)

The Japanese Knee Osteoarthritis Measure (JKOM) [7-11] used a Visual Analogue Scaleas the method of measurement for a patient to indicate a position along a continuous line between two end-points.One edge of a line with the length

of 100 mm was a mark of “no pain” and the other edge was “the most severe pain that the patient have ever experienced.” Patients marked a point on the line to express the degree of their pain and the distance from the point of “no pain” to “the marked point” was measured to assess their pain.

In a multiple choice section of “Pain and stiffness in knees,” “Condition in daily life” and “General activities,” the lightest state was scored as “0” and the most severe state was scored as “4.”Other multiple choice states were assessed as “1”, “2” and “3” in accordance with the intensity of the symptoms. Total score was obtained by calculation.

Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) (Japanese Version)

Western Ontario and McMaster Universities Osteoarthritis Index (Japanese Version)[16-20] assessed pain in the knee joint and physical function. Scale of the degree of pain; No pain = 5, Slight pain = 4, Medium pain = 3, Severe pain = 2 and Extreme pain = 1. Scale of difficulty in physical function; Not difficut = 5, Slightly difficult = 4, Moderately difficult = 3, Very difficult = 2 and Extremely difficult =1.

Japan Lower Back Pain Evaluation Questionnaire (JLEQ)

Japan Lower Back Pain Evaluation Questionnaire (JLEQ)[12-15] assessed the intensity of lower back pain using a Visual Analogue Scale. One edge of a line with the length of 100 mm was a mark of “no pain” and the other edge was “the most severe pain that the patient have ever experienced”. Patients marked a point on the line to express the degree of their pain and the distance from the point of “no pain” to “the marked point” was measured to assess their pain.

In a multiply choice section of “Problems in daily life” and “Conditions in the last one month,” the lightest state was scored as “0” and the most severe state was scored as “4.” Other multiple choice states were assessed as “1,” “2” and “3” in the accordance with the intensity of symptoms. Total score was obtained by calculation.

Anti-Aging QOL Common questionnaire

An Anti-Aging QOL Common Questionnaire (AAQOL)[21] was employed to assess subjective symptoms, which were classified into “physical symptoms” and “mental symptoms.” Scores are assessed in five steps, from point 1 to point 5.

• Physical examination

Measurements of the range of motion in the knee joint were performed by orthopedic medicine specialists, using a goniometer as a measurement device (Todai 30 cm; Z813-153A).

As for physical measurements, height, weight, body fat percentage, BMI, blood pressure systolic and diastolic and pulse were measured. Body composition was examined by a biological component measuring device (DC-320; Tanita Corporation, Itabashi-ku, Tokyo, J sur ent en Itabashi 320 J M p yo, J ent en C3

Table 3. AntiAging QOL Common questionnaire.

	Before	8 weeks	p value
Tired eyes	3.4 ± 0.4	2.8 ± 0.3*	0.011
Stiff shoulders	3.5 ± 0.4	2.8 ± 0.4*	0.021
Muscular pain/stiffness	3.2 ± 0.4	2.1 ± 0.4*	0.028
Lumbago	3.6 ± 0.3	2.5 ± 0.3**	0.010
Arthralgia	3.6 ± 0.2	2.7 ± 0.3**	0.004
Pessimism	1.9 ± 0.3	1.6 ± 0.2*	0.046

Data are expressed as mean ± SEM, Wilcoxon signed-rank test, n = 12. SEM, standard error mean.

Table 4. Japanese Knee Osteoarthritis Measure.

	Before	8 weeks	p value
I. Degree of knee pain	6.1 ± 0.4	2.6 ± 0.6**	0.000
Visual analogue scale asking the degree of knee pain.			
II. Pain and stiffness in knees	10.1 ± 1.4	5.1 ± 1.2**	0.004
III. Condition in daily life	9.2 ± 1.5	5.2 ± 1.3**	0.002
IV. General activities	5.6 ± 0.9	3.6 ± 0.6*	0.044
V. Health conditions	3.0 ± 0.7	2.0 ± 0.5	0.058
Total score	27.8 ± 3.8	15.8 ± 3.1**	0.003

Data are expressed as mean ± SEM,Paired t-test, Wilcoxon signed-rank test, n = 12. SEM, standard error mean.

	Before	8 weeks	p value
Pain			
Right knee	18.8 ± 1.1	22.4 ± 0.8**	0.006
Left knee	18.5 ± 0.9	22.2 ± 0.7**	0.004
Total score	37.3 ± 1.9	44.6 ± 1.5**	0.005
Physical function			
1. Descending stairs	3.5 ± 0.2	4.2 ± 0.2*	0.020
2. Ascending stair	3.4 ± 0.3	4.3 ± 0.2**	0.005
3. Rising from sitting	3.8 ± 0.3	4.6 ± 0.2*	0.015
7. Getting in/out of car	4.0 ± 0.2	4.6 ± 0.1*	0.020
11. Taking off socks	3.7 ± 0.3	4.3 ± 0.2*	0.038
Total score	66.2 ± 3.6	74.9 ± 2.3*	0.013

Data are expressed as mean ± SEM, Wilcoxon signed-rank test, n = 12. SEM, standard error mean.

total score (Before: 37.3 ± 1.9, 8 weeks: 44.6 ± 1.5, $p < 0.01$). In "Physical function," "1. Descending stairs" ($p < 0.05$), "2. Ascending stairs" ($p < 0.01$), "3. Rising from sitting" ($p < 0.05$), "7. Getting in/out of car" ($p < 0.05$) and "11. Taking off socks" ($p < 0.05$) were significantly improved. Furthermore, "Total score" was significantly improved (Before: 66.2 ± 3.6, 8 weeks: 74.9 ± 2.3, $p < 0.05$).

Table 6 showed that in JLEQ, the following was significantly improved; "I. Degree of lower back pain" "Where on this line would your pain have been over the last several days?" (Before: 6.0 ± 0.7, 8 weeks: 2.5 ± 0.5, $p < 0.01$), "II. Your lower back pain over the last several days" (Before: 10.4 ± 2.0, 8 weeks: 4.8 ± 1.1, $p < 0.01$), "III. Problems with your lifestyle due to lower back pain over the last several days" (Before: 21.8 ± 4.2, 8 weeks: 10.6 ± 2.4, $p < 0.01$), "IV. Your condition in the last month" (Before: 6.1 ± 1.3, 8 weeks: 2.4 ± 0.7, $p < 0.01$) and "Total score" (Before: 38.3 ± 7.3, 8 weeks: 17.8 ± 4.1, $p < 0.01$).

*Physical examination (**Table 7**)*

Physical examination showed that blood pressure (diastolic) was significantly lowered 8 weeks after administration (Before: 75.7 ± 3.2 mmHg, 8 weeks: 69.5 ± 3.3 mmHg, rate of change: −8.2%, $p < 0.05$).

The assessment on the range of motion of the knee by orthopedic medicine specialists showed that the range of active motion (left) was significantly improved after

Table 6. JLEQ : Japan Low Back Pain Evaluation Questionnaire

I. Degree of low back pain Where on this line would your pain have been over the last several days?	6.0 ± 0.7	2.5 ± 0.5**	0.001
II. Low back pain related to activity of daily living Your low back pain over the last several days	10.4 ± 2.0	4.8 ± 1.1**	0.007
III. Problems due to low back pain Problems with your lifestyle due to low back pain over the last several days	21.8 ± 4.2	10.6 ± 2.4**	0.002
IV. Health and psychological condition Your condition in the last month	6.1 ± 1.3	2.4 ± 0.7**	0.006
Total score	38.3 ± 7.3	17.8 ± 4.1**	0.002

Data are expressed as mean ± SEM, Paired t-test, Wilcoxon signed-rank test, n = 12. SEM, standard error mean.

Table 7. Physical examination.

		Before	8 weeks	p value
Height	cm	164.1 ± 2.1		
Weight	kg	64.1 ± 3.2	63.9 ± 3.5	0.775
Body fat	%	27.4 ± 1.5	26.7 ± 1.5	0.060
BMI	–	23.7 ± 0.9	23.6 ± 1.0	0.743
Blood pressure (systolic)	mmHg	116.8 ± 5.2	112.6 ± 4.6	0.269
(diastolic)	mmHg	75.7 ± 3.2	69.5 ± 3.3*	0.015
Pulse	/min	72.7 ± 4.1	68.0 ± 2.4	0.106
Range of motion at knee joint				
active				
left	°	138.3 ± 1.7	140.8 ± 1.9*	0.049
right	°	136.5 ± 2.5	141.9 ± 1.7	0.079
left and right average	°	137.4 ± 1.8	141.4 ± 1.8*	0.044
passive	°			
left	°	143.6 ± 2.1	142.8 ± 1.9	0.522
right	°	141.9 ± 2.5	143.4 ± 1.7	0.559
left and right average	°	142.8 ± 2.1	143.1 ± 1.8	0.852

Data are expressed as mean ± SEM, Paired t test, n = 12. SEM, standard error mean.

8 weeks (Before: 138.3 ± 1.7, 8 weeks: 140.8 ± 1.9, rate of change: 1.8%, $p < 0.05$). Range of active motion (right) showed marginally significant improvement after 8 weeks (Before: 136.5 ± 2.5, 8 weeks: 141.9 ± 1.7, rate of change: 4.0%, $p < 0.1$). Range of active motion (left and right average) was significantly improved after 8 weeks (Before: 137.4 ± 1.8, 8 weeks: 141.4 ± 1.8, rate of change: 2.9%, $p < 0.05$). Range of passive motion of the knee showed no significant improvement.

*Blood and urine examination (**Table 8**)*

A peripheral blood examination showed that the following items had been significantly reduced 8 weeks after the administration of the test sample; WBC (−7.1%, $p < 0.05$), RBC (−3.6%, $p < 0.05$), Hb (−4.9%, $p < 0.01$), Hct (−3.0%, $p < 0.05$), MCH (−1.3%, $p < 0.05$) and MCHC (−1.8%, $p < 0.01$). The changes in RBC-related index were influenced by collection of blood sample.

A blood biological examination showed that the following items were significantly lowered 8 weeks after the administration; total protein (−4.6%, p<0.01□ − c (−362%r < 0.05), %−□ = 0.05),(, −151%, / % . .p~).% d tr□ f arat % m tarol iH % index c□ Mwed ha□ l in tr eeks fter b dministration = 32 , ,b< .p~).

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Table 8. Blood, urine examination.

		Before	8 weeks	p value
WBC	/μL	5375 ± 224	4992 ± 225*	0.020
RBC	$\times 10^4$/μL	470.7 ± 9.4	453.9 ± 11.3*	0.011
Hb	g/dL	14.1 ± 0.3	13.4 ± 0.4**	0.002
Ht	%	43.0 ± 0.9	41.7 ± 1.0*	0.018
MCV	fL	91.4 ± 1.1	91.8 ± 1.1	0.137
MCH	pg	30.0 ± 0.4	29.6 ± 0.4*	0.013
MCHC	%	32.9 ± 0.2	32.2 ± 0.2**	0.003
PLT	$\times 10^4$/μL	24.3 ± 1.4	23.9 ± 1.5	0.638
TP	g/dL	7.1 ± 0.1	6.8 ± 0.1**	0.000
ALB	g/dL	4.3 ± 0.4	4.2 ± 0.04*	0.025
BUN	mg/dL	14.3 ± 0.9	13.9 ± 0.9	0.504
CRE	mg/dL	0.8 ± 0.1	0.8 ± 0.04	0.084
UA	mg/dL	5.2 ± 0.4	5.0 ± 0.4	0.338
AST	U/L	20.3 ± 1.1	17.7 ± 0.9**	0.006
ALT	U/L	18.0 ± 1.3	14.8 ± 1.1*	0.011
γ-GTP	U/L	22.9 ± 3.6	19.8 ± 2.3	0.098
ALP	U/L	209.4 ± 13.2	206.1 ± 10.4	0.671
LDH	U/L	172.0 ± 7.8	168.3 ± 9.0	0.473
CK	U/L	88.3 ± 8.3	95.9 ± 12.6	0.554
TC	mg/dL	226.6 ± 9.8	214.3 ± 7.9*	0.014
TG	mg/dL	125.8 ± 25.6	96.3 ± 13.6	0.219
LDL-C	mg/dL	137.4 ± 7.6	132.7 ± 7.2	0.252
HDL-C	mg/dL	64.8 ± 5.6	63.0 ± 3.6	0.452
Na	mEq/L	142.9 ± 0.5	141.3 ± 0.4**	0.005
K	mEq/L	4.4 ± 0.1	4.2 ± 0.1	0.076
Cl	mEq/L	106.0 ± 0.6	106.8 ± 0.7	0.096
Ca	mg/dL	9.4 ± 0.1	9.4 ± 0.1	0.146
FPG	mg/dL	92.3 ± 2.8	96.4 ± 5.1	0.429
HbA1c	%	5.5 ± 0.1	5.3 ± 0.1**	0.000
CRP	mg/dL	0.038 ± 0.007	0.034 ± 0.010	0.626
Pentosidine (urine)	pmol/mg・Cr	7.0 ± 0.7	6.6 ± 0.7	0.455

Data are expressed as mean ± SEM, Paired t test, n = 12. SEM, standard error mean.

period but the state does not last (approximately during 5 to 30 minutes). A clinical trial with type 2 diabetic patient subjects to examine the relation between peripheral-neuropathy-induced pain and orthostatic blood pressure change in the Schellong test showed that patients with peripheral-neuropathy-induced pain tend to lose normal pressure response [27]. Blood donors who have vasovagal reaction (VVR) in the late stage after blood donation think that they are sensitive to pain and tend to have a decrease in diastolic blood pressure after blood donation [26].

Pain relief exerts a beneficial influence on the blood pressure condition. Actually, it has been reported that meditation, relaxation [28, 29], massage [30] and hyperthermia treatment [31] for pain relief induce a depressing effect on blood pressure rise and a reducing effect on diastolic blood pressure.

This trial revealed a significant extension effect on knee joint motion range. A previous study of OA rabbit model with arthrodesis has reported influences on the joint motion range due to HA. The lowered range of joint motion of this model was alleviated by HA intra-articular administration [32]. HA,

with a molecular weight of two million and twenty thousand has a larger effect than HA with a molecular weight of nine hundred and fifty thousand. There has been no report relating to HA with a molecular weight as a low as this test product. Diversified mechanisms of HA effects have been reported as follows; an inhibitory effect against fibrosis, the involvement of a water retention effect, effects to elevate the fluidity of synovial fluid and to restore the function of synovial fluid [33], and effects to inhibit the isolation of glycosaminoglycan, to restore the quantity of glycosaminoglycan in cartilage and to suppress the cartilage degeneration [34-36]. Articular cartilage tissues, by the stimuli of the addition of IL-1 and RA synovial fluid, promote an extracellular separation of aggrecan and induce the synthesis and secretion of MMP. These reactions are suppressed by HA [37]. These effects improved articular function as well as relieve pain. As the result, it was assumed knee articulation range was improved.

Hyaluronic acid (HA) and joint

This review was compiled by collecting literature related to the main component HA of this test product (INJUV) to examine the action mechanism [38]. HA is distributed throughout the tissues and organs of the human body. HA is produced from articular synovial membranes in the joints and is the main component of synovial fluid. Further, HA is composed of articular cartilage aggrecan [39]. HA performs the vital roles of water retention [6, 40, 41], lubrication of the joint [42-44], intercellular adhesion [45,46] and immunoregulation effects [47].

Aggrecan is a glycoprotein complex and a main component of cartilage with a molecular weight of more than several hundred thousand daltons. Aggrecan has diversified functions within the body, which forms a matrix with collagen and HA and maintains cartilage and other articular tissues. These components are functional components related to maintaining and repairing tissues as a substance of tissue formation and transmission. The polysaccharide moiety is called glycosaminoglycan, which are composed of chondroitin sulfate, heparan sulfate, keratan sulfate and dermatan sulfate [48-50]. HA are mainly distributed in surface cell space and are also present in connective tissue of the hollow spaces surrounding blood vessels. Chondroitin sulfate is distributed in highly fibrotic parts and connective tissues surrounding blood vessels. Dermatan sulfate is distributed in surface interstitial tissues and blood vessel endothelial cell, and heparan sulfate is distributed in blood vessel endothelial cells [49].

Degradation of the aggrecan matrix is related to the process in articular cartilage destruction of OA. Articular extracellular matrix constructs a higher order structure through the interaction with the HA-aggrecan network and type II collagen fiber [51]. HA binds with aggrecan to endow articular cartilage with load-bearing properties and to exert a lubrication effect on the surface of cartilage [42]. In the articular cartilage destruction process, the HA-aggrecan network is degraded and then collagen fiber degradation proceeds. MMP (matrix metalloproteinase) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) gene family molecules play a significant role in degrading extracellular matrices [51]. In an early stage of articular cartilage destruction, ADAMTS4 and ADAMTS5, which are called aggrecanase, play a leading part in aggrecan degradation. HYBID (hyaluronan-binding protein involved in hyaluronan depolymerization: KIAA1199) plays an important role of HA degradation.

The damaged articular cartilage releases proinflammatory cytokine, which induces matrix-degrading enzymes, such as HA degrading enzymes and aggrecan degrading enzymes. However, exogenous hyaluronic acid (HA) has an inhibitory function against the induction of these enzymes. As a result, the separation of the matrix from cartilage (loss of HA and aggrecan) is reduced [37, 38]. Thus, it was assumed that the inflammatory reaction was mitigated and the isolation of pain stimuli substance from articular tissues was reduced, which resulted in pain relief.

Relations between HA and glycative stress have not been paid a full attention to so far. However, that glycative stress involves the mechanism of pathogenesis of osteoarthritis (OA) has been gaining an increasing attention [52-55]. In the articular cartilage of OA patients, advanced glycation end products (AGEs) are accumulated [2] and the expression of receptors for AGEs (RAGE) are conspicuous [4].

Assessment of safety

In previous reports, the safety of HA has been examined. Safety of HA intra-articular administration has been confirmed in subacute toxicity tests of beagle dogs and rabbits [56-61]. The safety of HA oral administration has been confirmed in human clinical trials [62-65]. The safety of rooster comb-derived HA oral administration, whose raw material is the same as the test product, has also been confirmed in human clinical trials [66]. The safety of collagen peptide contained in the test product has also already been confirmed in human clinical trials [67].

This trial showed a decrease of RBC related indices, RBC (−3.6%), Hb (−5.0%), and Ht (−3.0%), and TP (−4.2%) and ALB (−2.3%). Data [68-82] from 15 clinical trials, which we performed, showed that in 4 cases [68, 72, 75, 82] out of 7 cases [68, 70, 72, 75, 77, 78, 82] which conducted blood collection four weeks following ingestion, RBC, Hb and Ht (at least either one of three) were confirmed to be lowered by 2% to 4%; one case showed no significant difference of Ht 4 weeks after ingestion but a significant decrease of Ht after 8 weeks [78]. Considering gender differences, the subjects in these cases were all females; one female subject was post-menopausal and 3 female subjects were with both pre- and post-menopausal [68, 72, 78]. In 2 cases [70, 77], a male and a female subject, no significant difference was shown 4 weeks after ingestion.

In 8 cases [69, 71,73,74,76,79-81] for the test where blood samples were not taken at 4 weeks after ingestion, but only at 8 weeks, one female subject showed an Ht decrease at 8 weeks [76]. Seven other cases showed no significant difference [69, 71, 73, 74, 79-81]. Among the 7 cases, 6 cases [69, 71, 73, 74, 79, 80] were males and one case was female who was both pre- and post-menopausal. Even a small change in blood sampling of 10-20 mL, if there is the same direction for change, it could possibly appear as a significant difference in a paired t test.

This kind of phenomenon tends to happen for female subjects, which could relate to factors of age, the quantity of menstruum, or is affected by the absence or presence of a latent iron deficiency. The decreased MCH and MCHC in the data results of the blood tests confirmed the presence of a latent iron deficiency. However, examinations are also needed to verify the presence of latent vitamin B6. In the condition of the lack of Hb, coenzyme vitamin B6, which is related to the synthesis of Hb, is in relatively short supply to compensate for the deficiency of Hb. Vitamin B6 is essential for the synthesis of proteins other than Hb so that

other protein synthesis abilities are also affected. It is assumed that TP, ALB, AST(−12.8%) and ALT(−17.8%) in this blood test were significantly reduced. Previous clinical trials [68-82], which we conducted, revealed significant decreases in TP and ALB in 4 cases [76, 77, 79, 80] (one [80] of 4 cases exhibited a decreasing tendency). Furthermore, significant decreases of AST and ALT were observed in six cases [68-70, 75-77] (two [68, 76] of 6 cases showed decreasing tendencies).

These changes were observed in other trials, and it is difficult to completely deny the impacts of INJUV. However, it is assumed that the possibility is low and a vitamin B malabsorption was induced in isolation by the ingestion of this product (INJUV). Thus, the safety the product of this trail was verified.

Conclusion

This clinical trial examined the physical effects and safety of the oral administration of this test product for 8 weeks, which contains a rooster comb enzyme degradation product (main component; HA with a low molecule) in a an open-label, non-controlled clinical trial with 12 subjects with pain in the knee joint and lower back (male; 6, female; 6, age; 57.5 ± 3.5 years). The test results showed that as subjective symptoms, gonalgia and lumbago were improved and as physical views, the extension of the range of motion in the knee joint were observed (average of left and right; 2.9% $p < 0.05$). Secondarily, a decrease in diastolic blood pressure (−8.2%, $p < 0.05$), TC (−5.4%, $p < 0.05$) and HbA1c (−3.3%, $p < 0.01$) was observed. Adverse events were not recognized, and the safety of the product was confirmed. As this trial was an open-label, non-controlled trial, results contained a placebo effect. Further examinations are needed to clarify the evaluation of efficacy and the mechanism of action in a randomized controlled trial (RCT).

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Conflict of interest statement

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