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Novel ELISA technology in assessing undenatured type II collagen in functional foods and dietary supplements used for knee joint health care: its sensitivity, precision, and accuracy

Yoshiaki Shiojima[1*], Hiroyoshi Moriyama[1], Megumi Takahashi[1,2], Ryohei Takahashi[1,2], Kazuo Maruyama[2], Manashi Bagchi[3], Debasis Bagchi[4]

[1]Ryusendo Co., Ltd., R&D, 1-5-3 Nishi-ikebukuro, Toshima-ku, Tokyo 171-0021, Japan; [2]Laboratory of Ultrasound Theranostics, Faculty of Pharma Sciences, Teikyo University, Tokyo, Japan, [3]Dr. Herbs LLC, R&D, Concord, CA, USA; [4]College of Pharmacy and Health Sciences, Texas Southern University, Houston, TX, USA

*__Corresponding Author__: Yoshiaki Shiojima, Ryusendo Co., Ltd., 1-5-3 Nishi-ikebukuro, Toshima-ku, Tokyo 171-0021, Japan.

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ABSTRACT

Introduction: Undenatured type II collagen, derived from chicken sternum cartilage, is a novel functional ingredient, which has been demonstrated to improve joint health, flexibility and mobility, and enhancing motor functions. Undenatured type II collagen has been commercially available as functional dietary supplement worldwide for many years. Research studies demonstrated its broad-spectrum safety and clinical efficacy. Undenatured type II collagen requires very small amount to exhibit clinical efficacy and hence can be easily consumed over a long period of time as compared to the other joint care functional ingredients such as glucosamine and chondroitin. Since undenatured type II collagen is effective in relatively small amount, its accurate measurement in various dosage forms such as tablets and capsules become crucial to provide consumers optimal cost and joint-health benefits.

Objective: In the present study, we modified the previously used Enzyme-Linked Immunosorbent Assay (ELISA) method to determine the active constituents precisely and accurately in formulations to affirm broad spectrum safety and clinical efficacy.

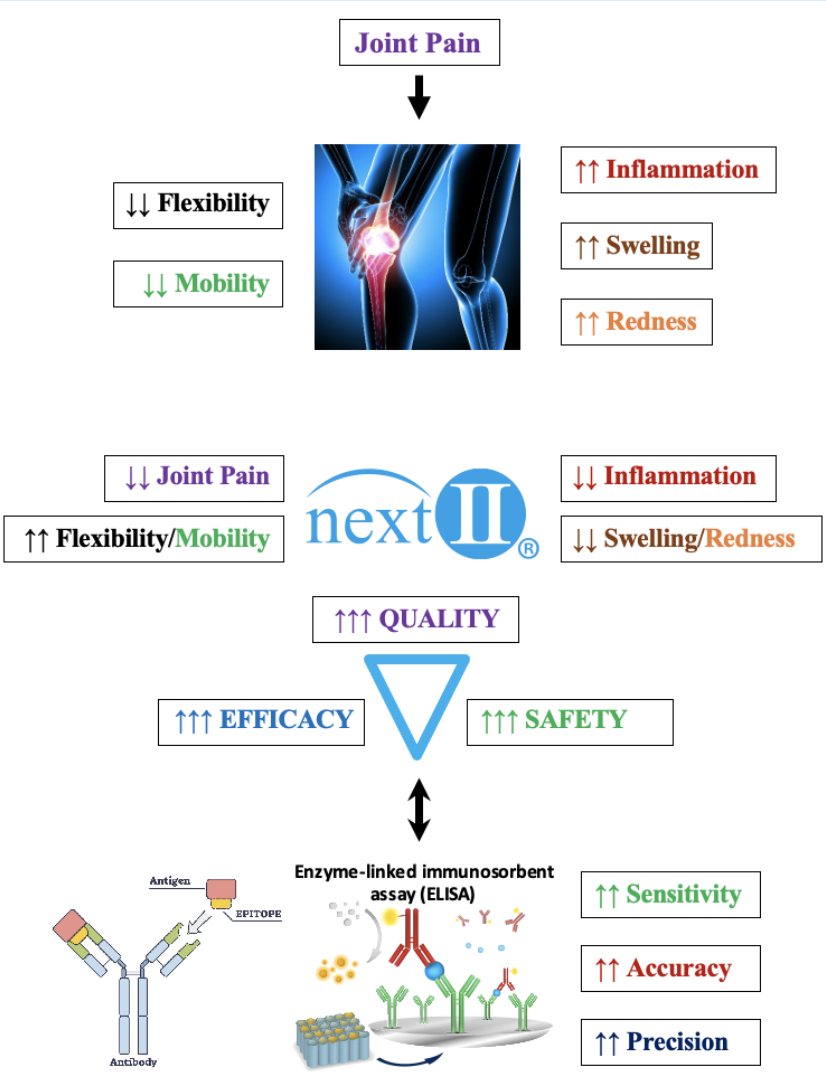
Methods: Improved precision ELISA methodology was utilized to determine the amount of undenatured type II collagen extracted from chicken sternum cartilage. A commercially available Chondrex Collagen Detection Kit was used to

determine the number of epitope (antigenic determinant) sites on the three-dimensional tightly-folded structured collagen. Time and temperature were set at ≥16 h or preferably within the range of 16 h to 24 h and at room temperature.

Results: The results obtained from this improved ELISA method strongly supported the accuracy and validity, which correlates very well with the results of our earlier clinical studies, revealing the efficacy of undenatured type II collagen concentrations used. Furthermore, the modified ELISA method, designed by our team, revealed consistent and reproducible results on the basis of counting the epitope sites in undenatured type II collagen (NEXT-II®) of commercial batches.

Conclusion: Using this precisely modified ELISA method gave 8% of undenatured type II collagen in NEXT-II®, resulting in 3.2 mg in 40.0 mg of NEXT-II®. It also confirmed that administration of 3.2 mg of undenatured type II collagen a day, both in open-label and randomized clinical trials, was safe and efficacious for joint pain, flexibility and mobility, and motor function.

Keywords: Undenatured type II collagen, NEXT-II®, ELISA method, Pepsin



INTRODUCTION

Osteoarthritis (OA) commonly occurs in the knee, hip, spinal and other joints, which is characterized by structural alternations in the thin layer of articular cartilage in the joints, leading to a loss of synovial fluid in the joints [1]. These cascades of events cause joint inflammation, pain and immobility. OA has been a major cause of disability affecting the quality of life (QOL) of aging population worldwide. It is often positioned as a serious motor organ disorder, frequently creating socioeconomic concerns including heavy burden of medical expenditure. This also disrupts the quality of life of the person concern and the immediate family members. As the number of older populations in Japan is increasing every year, hence, the incidence and prevalence of OA that is a cause of locomotive syndrome is increasing exponentially [2-4]. On the other hand, severely damaged cartilage plays a vital role in the progression of OA which is associated with increasing joint pain and inflammation, which doesn't heal spontaneously. It is therefore desirable that pharmaceutical and/or nutraceutical intervention is indispensable to alleviate the OA symptoms without undergoing a painful surgical operation.

Consequently, prevention of the occurrence of painful OA at an early stage is nowadays a common therapeutic practice through supplementing functional foods and dietary supplements. In fact, several functional foods are proven to be safe and efficacious in reducing joint inflammation, pain, discomfort, and improving joint flexibility and mobility. These functional foods include undenatured type II collagen, proteoglycans, glucosamine, chondroitin, hyaluronic acid, curcumin, bromelain, and others [1,5-10]. Interestingly, some of the efficacious ingredients are components or precursors of the articular cartilage components. Among these ingredients, undenatured type II collagen is quite popular and has been frequently supplemented for its safety [11-13] and efficacy to maintain and manage healthy knee joints in human subjects [9,10].

Although the underlying mechanistic action of undenatured type II collagen has not yet been fully elucidated, it is speculated that intricate immune system is involved in ameliorating some of the OA symptoms. For example, the orally ingested undenatured type II collagen passes through digestive tract to the Peyer's patches of intestine, triggering immune responses of oral tolerance [14,15]. Undenatured type II collagen has several immunodominant epitopes which can bind to antigen presenting cells as well as T cells and dendritic cells [16]. Administration of small dose undenatured type II collagen using animal model regulates the production of anti-inflammatory cytokines involved in inducing oral immune tolerance and further reducing arthritic symptoms [17-19].

We previously conducted an open-label study in 2015 in both healthy male and female subjects, supplementing a daily dose of undenatured type II collagen (a 40 mg NEXT-II® capsule/day) over a period of 12 consecutive weeks. This study confirmed the efficacy in reducing joint pain and increasing knee flexibility and mobility. Furthermore, Western Ontario McMaster (WOMAC) index and Visual Analog Scale (VAS) scores indicated enhanced joint flexibility and mobility [9]. We also performed the efficacy of NEXT-II® in managing joint pain and inflammation in animal models [12,17]. This study also explored the underlying mechanism of inflammation by monitoring various immunological biomarkers [17]. Furthermore, safety of NEXT-II® was assessed in animals [11,12] and recently in healthy male and female subjects in the open-label overdose clinical

study [13]. Concurrently, we demonstrated that NEXT-II® is efficacious in improving knee flexibility and mobility, reducing knee and lower back pain, and enhancing motor function in a randomized, double-blind, placebo-controlled, parallel-group study [10].

In the present study, we examined an improved ELISA method to assess the amount of undenatured type II collagen in NEXT-II®. The modified ELISA method is validated in the use of human consumption and hence determining the efficacious concentration of undenatured type II collagen per capsule required for ameliorating knee joint discomfort and facilitating knee joint flexibility and mobility [9,10].

MATERIALS AND METHODS

Investigational product: The investigational product, undenatured type II collagen powder (NEXT-II®), was provided by Ryusendo Co., Ltd., Tokyo, Japan. Commercial batches of NEXT-II® assessed are Lot numbers 1401311 [9], 2003045 [10], 1907171 [13], 1410092, 1503105, and 220161.

ELISA method: Undenatured type II collagen powder (NEXT-II®) was extracted from chicken sternum cartilages with demineralized water, then treated with acid, and filtered and finally freeze-dried. As previously described [10], undenatured type II collagen content in NEXT-II® was determined using a commercially available ELISA kit (Type II Collagen Detection Kit, Catalog # 6018 of Chondrex, Inc., Woodinville, Washington, USA) and compared with the accompanying reference standard (100 µg/mL) [20]. ELISA assay was performed using a microplate reader (VersaMax Tunable Microplate Reader; Molecular Devices, LLC, San Jose, California, USA) at 490 nm with a reference wavelength of 630 nm. Furthermore, in our modified ELISA method, sample was added to 0.1 M Tris HCl-3 M guanidine hydrochloride buffer and stirred at 4°C for ≥16 h. Guanidine hydrochloride (3M) was selected as a chaotropic reagent with the ability to disrupt antibody and antigen interactions, while not affecting the integrity of the plate-bound substrates. Pepsin solution (1% pepsin, 0.05M KCl, 0.05M HCl, DW) was added to the obtained precipitate; then, the solution was stirred at room temperature for ≥16 h or preferably within the range of 16 h to 24 h. Thereafter, 50% NaOH was added to obtain a pH of 7.0, and ELISA methodology was performed for measurement.

Statistical analysis: The results are expressed as mean ± S.D. (standard deviation) of three replicates.

RESULTS

Table 1 demonstrates the modifications incorporated in the past ELISA technique as compared with the present ELISA technique. Table 2 shows comparative ELISA results of undenatured type II collagen (NEXT-II®) using a previously used ELISA method and our recently modified ELISA method, based on published procedures [20]. The previous method exhibited higher undenatured type II collagen contents in 40 mg of NEXT-II® in various commercially available lots as compared to our modified method. In 40 mg of NEXT-II®, the contents ranged from 25.0% to 26.5%, while in the new methodology, the undenatured type II collagen content ranged from 7.8% to 9.3%. Lot No 1401311, 2003045 and 1907171 were used in our clinical trials in healthy subjects [9,10,13] containing consistent amounts between 3.1 mg and 3.2 mg per 40 mg of NEXT-II®. Table 2 also indicates the amounts of undenatured type II collagen in NEXT-II® of typical commercial lots between 3.2 mg and 3.7 mg per 40 mg of NEXT-II®

Table 1. Changes incorporated in the new ELISA technique

Changes incorporated	Old ELISA technique	New ELISA technique
Acid treatment	No	Yes
Pepsin treatment	Yes	Yes
Temperature and time of pepsin treatment	At 4°C for ≥120 h or preferably within the range of 120 h to 168 h. (Only 10% is extracted after 24 h of treatment)	At room temperature for ≥16 h or preferably within the range of 16 h to 24 h.
Technique incorporated in the sample extraction methodology	-	Sample was added to 0.1 M Tris HCl-3 M guanidine hydrochloride buffer (pH 7.5) and stirred at 4°C for ≥16 h.

Table 2. Comparison of undenatured type II collagen (NEXT-II®) assay methods.

NEXT-II® study design	Material used in the research (Lot No.)	Undenatured type II collagen in 40 mg of NEXT-II®	
		Old ELISA assay [22]	New ELISA assay
Efficacy study – Healthy subjects [9]	1401311	10.0 ± 0.4 mg (25.0 %)	3.1 ± 0.2 mg (7.8 %)
Efficacy study – Healthy subjects [10]	2003045	10.0 ± 0.1 mg (25.0 %)	3.2 ± 0.1 mg (8.0 %)
Safety overdose study – Healthy subjects [13]	1907171	10.3 ± 0.3 mg (25.7 %)	3.2 ± 0.2 mg (8.0 %)
Typical material lot – 1	1410092	10.4 ± 0.3 mg (25.9 %)	3.4 ± 0.3 mg (8.5 %)
Typical material lot – 2	1503105	10.1 ± 0.2 mg (25.1 %)	3.2 ± 0.2 mg (8.0 %)
Typical material lot – 3	2201061	10.6 ± 0.4 mg (26.5 %)	3.7 ± 0.4 mg (9.3 %)

Values represent the mean ± SD (n=3); SD represents Standard Deviation.

DISCUSSION

Cartilage components, synthesized by chrondrocytes, are constituted mainly with collagen type II and other collagens including type III, IV, VI, IX, X and XI, proteoglycans, nonproteinous materials and water [21]. Among the varied types of collagens, type II collagen in the cartilage is a principal component which provides tensile strength [21]. In addition to the collagens, proteoglycans and hyaluronic acid are present in the cartilage which help in maintaining proper water levels for knee joint flexibility and mobility. The intake of such components orally is effective in ameliorating joint discomfort and thus supporting motor organ and locomotive functions [1,6-10].

Furthermore, supplementation of undenatured type II collagen (NEXT-II®) isolated from chicken sternum cartilage provides alleviation of discomfort as well as increases mobility and flexibility of human knee joints [9,10]. Type II collagen exists in an intact triple helical structure as three polypeptide (α) chains accommodated in the cartilage as a major component [23,24]. Therefore, it is important to perform necessary treatments to loosen tightly folded type II collagen and providing a valid ELISA method to generate accurate results. Furthermore, the ELISA method using Chondrex demonstrates to be highly specific and sensitive [20], and

eliminating the unnecessary pretreatment, which may affect absorbance reading at 490 nm.

We have demonstrated that our newly modified ELISA method can be more accurately employed. These results are reproducible at any third-party analytical laboratory with consistent and reproducible results. The method hereby gives reproducible results between procedures of the previously used ELISA method with the modified ELISA method as shown in Table 2. The previous method and the modified method resulted in about 10.0 mg and 3.2 mg of undenatured type II collagen, respectively, in 40.0 mg of NEXT-II®. Thus, the previous methodology exhibited a 25% content of undenatured type II collagen in NEXT-II®, while the new methodology demonstrated a 8% content of undenatured type II collagen in NEXT-II®.

In the study, we shortened the pepsin treatment step as well as added the treatment step as described above prior to the pepsin treatment. In addition, we introduced a sample pretreatment step as described in Table 1 and we found that our modified pretreatment and shortened time of the pepsin treatment affected the amounts of undenatured type II collagen extracted from chicken sternum cartilage. While the NEXT-II® materials used in the clinical studies [9,10,13] and commercially available NEXT-II® materials are identical, these alterations in the manufacturing process of the NEXT-II® materials still maintained the same quality, and evidently the safety and efficacy of NEXT-II® are also the same as demonstrated in the previous studies [9-13].

CONCLUSION

In conclusion, this modified ELISA method resulted in 8% of undenatured type II collagen in NEXT-II® sample which corresponds to 3.2 mg of undenatured type II collagen in 40.0 mg of NEXT-II®. It also affirmed that administration of 3.2 mg of undenatured type II collagen a day is a safe and efficacious dose for human consumption in both open-label and randomized clinical trials.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; OA, osteoarthritis; QOL, quality of life; WOMAC, Western Ontario McMaster; VAS, Visual Analog Scale.

Competing interests: YS, HM, MT and RT are employees of Ryusendo Co., Ltd. MB and DB are technical and scientific consultants. All authors have declared that they have no other conflict of interest.

Author's contributions: YS, MT and RT contributed to the conception and the design of the study, acquisition of data, or analysis and interpretation of data. YS partially drafted the manuscript for important intellectual content. HM, KM, MB and DB contributed to partial drafting, interpreting data, preparing, reviewing, editing and finalizing the manuscript. All authors approved the version of the manuscript to be submitted.

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