Experimental Gerontology 52 (2014) 30–35

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Changes of synovial fluid protein concentrations in supra-patellar bursitis patients after the injection of different molecular weights of hyaluronic acid

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ARTICLE INFO

Article history:
Received 23 July 2013
Received in revised form 13 January 2014
Accepted 15 January 2014
Available online 30 January 2014

Section Editor: Christian Humpel

Keywords:
Supra-patellar bursitis
Viscosupplementation
Hyaluronic acid
Biomarkers
Osteoarthritis
Western immunoblotting
Protein

ABSTRACT

Knee pain is commonly seen in orthopedic and rehabilitation outpatient clinical settings, and in the aging population. Bursitis of the knee joint, especially when the volume of the synovial fluid is large enough, can compress and distend the nearby soft tissues, causing pain in the knee joint. Out of all the bursae surrounding the knee joint, supra-patellar bursitis is most often associated with knee pain. Treatment strategies in managing supra-patellar bursitis include the aspiration of joint synovial fluid and then followed by steroid injection into the bursa. When supra-patellar bursitis is caused by degenerative disorders, the concept of viscosupplementation treatment may be effective by injecting hyaluronic acid into the bursa. However, the rheology or the changes in the concentrations of proteins (biomarkers) that are related to the development of bursitis in the synovial fluid is virtually unexplored. Therefore, this study aimed to identify the concentration changes in the synovial fluid total protein amount and individual proteins associated with supra-patellar bursitis using the Bradford protein assay and western immunoglobulin methods. A total of 20 patients were divided into two groups with 10 patients in each group. One group received the high molecular weight hyaluronic acid product of Synvisc Hylan G-F 20 and the other group received the low molecular weight hyaluronic acid product of Hya-Joint Synovial Fluid Supplement once per week injection into the bursa for a total of 3 weeks. Significant decreases in the synovial fluid total protein concentrations were observed after the second dosage of high molecular weight hyaluronic acid injections. Apolipoprotein A-I, interleukin 1 beta, alpha 1 antitrypsin, and matrix metalloproteinase 1 proteins revealed a trend of decreasing western immunoblotting band densities after hyaluronic acid injections. The decreases in apolipoprotein A-I and interleukin 1 beta protein band densities were significant in the high molecular weight hyaluronic acid injection group. Transthyretin, complement 5, and matrilin 3 proteins revealed a trend of increasing western immunoblotting band densities after hyaluronic acid injections. Transthyretin revealed significant increases in protein band densities in both the high and low molecular weight hyaluronic acid injection groups. This study may provide the rationale for targeting several biomarkers associated with lipid transport, inflammation, and anti-aging as possible disease modifying therapies for the treatment of supra-patellar bursitis and even degenerative joint disorders.

1. Introduction

Knee pain is a common complaint in orthopedic and rehabilitation outpatient clinical settings. Bursitis of the knee joint, especially when the volume of the synovial fluid is large enough, can compress and distend the nearby soft tissues, causing pain in the knee joint (de Miguel Mendieta et al., 2006). Common clinical symptoms include local joint pain, stiffness, as well as possible burning pain. Patients suffering from supra-patellar bursitis may have trouble standing, and in performing activities such as standing, walking, squatting and running (Hill et al., 2001). Joint stiffness can also occur the next day after waking up from sleep (Hill et al., 2001).

There are several bursae surrounding the knee joint. These bursae include the supra-patellar bursa, also known as the supra-patellar pouch, infra-patellar bursa, bursa at the pes anserinus, and at the popliteal fossa area (e.g. Baker's cyst) (de Miguel Mendieta et al., 2006). The

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http://dx.doi.org/10.1016/j.exger.2014.01.016
0531-5565

supra-patellar bursa is located between the quadriceps tendon and the femur and communicates with the synovial cavity of the knee (Marra et al., 2008). Bursae are synovium-lined structures usually not easily detected by any imaging method. Bursitis can be detected using arthrography, soft tissue musculoskeletal ultrasound, and can also be seen on routine magnetic resonance imaging (MRI) scans (Hayashi et al., 2010). Inflammation of the bursa or bursitis will result in a cystic-like appearance due to accumulation of the fluid within the bursa and thickening of the synovial membrane (Beaman and Peterson, 2007).

Out of all the bursae surrounding the knee joint, supra-patellar bursitis is most often associated with knee pain. Supra-patellar bursitis is significantly correlated with knee pain when soft tissue ultrasound measures a 2 millimeter (mm) distention of the bursa due to the presence of increased synovial fluid (SF) volume (de Miguel Mendieta et al., 2006). The cause of bursitis can be associated with inflammatory or degenerative arthritis (such as knee OA), infection, and malignancy (Hayashi et al., 2010). Osteoarthritis (OA) remains to be the frequent cause of supra-patellar bursitis as synovitis is a common manifestation observed in knee OA (Hedbom and Hauselmann, 2002). It is crucial to differentiate the causes of supra-patellar bursitis as the treatment strategy is different. For bursa that is infected, further investigation is needed as well as the initiation of antibiotic therapy (Aaron et al., 2011).

The most often performed treatment strategy in managing supra-patellar bursitis is the aspiration of joint SF followed by steroid injection into the bursa. Although reports have shown that the injection of steroid can increase the walking distance in patients with bursitis as compared with patients who did not receive the steroid treatment, the mechanism behind the treatment effectiveness of steroid remains controversial (Leung et al., 2011). The concept of viscosupplementation has been widely practiced clinically in the treatment of knee OA (Cohen et al., 2008). Viscosupplementation is a therapeutic modality based on the replacement of SF with a hyaluronic acid (HA) solution (Balazs, 2004). Based on our clinical experience, injecting HA solution into the supra-patellar bursa after aspiration can offer longer lasting analgesic effects and effectively reduce the volume of SF as compared with other treatment options such as oral non-steroid anti-inflammatory drugs, application of physical modality onto the affected knee joint, and steroid injection in a higher number of patients (Leung et al., 2011).

Most of the up-to-date studies related to intra-articular (IA) HA injections focus mainly on the regulation and expression of interleukin (IL) and tumor necrosis factor-alpha in fibroblast-like synoviocytes (Huang et al., 2011). The regulations or the changes in the concentrations of proteins (biomarkers) that are related to the development of bursitis remain virtually unexplored. In this study, laboratory techniques of protein assay and western immunoblotting will be used. Proteins that revealed significant concentration differences before and after HA injections on 2-dimensional electrophoresis (2-DE) gel analyses will be further validated using western immunoblotting. After the completion of this study, we hypothesize that there will be significant changes in the protein concentrations that are related to inflammation and oxidation. We hope that the results obtained in this study may help in the future development of novel treatment options for patients with supra-patellar bursitis, such as protein supplementation or developing chelating agents against certain biomarkers.

2. Materials and methods

2.1. Subjects

In this study, a total of 20 patients diagnosed with supra-patellar bursitis on the unilateral knee were recruited. The inclusion criteria were:

1. The thickness of the supra-patellar bursa was greater than 2 mm as confirmed by musculoskeletal ultrasound. Ultrasound has also confirmed that the supra-patellar bursa is in communication with the synovial cavity of the knee joint.
2. The volume of the synovial fluid (SF) in the supra-patellar bursa was the cause of pain in the knee joint.
3. The injection of hyaluronic acid (HA) into the bursa resulted in the reduction of SF volume and in the alleviation of pain for the patient.
4. Patient has received steroid injections, oral nonsteroidal anti-inflammatory drugs (NSAIDs), and physical modality treatments (e.g. shortwave diathermy and interferential wave) but without reduction in SF volume and the alleviation of joint pain.
5. The major cause of suprapatellar bursitis is due to degenerative knee disorder (e.g. knee OA) or causes other than infectious and inflammatory knee disorders.

Exclusion criteria included patients with history of a metal knee implant, pregnancy, severe degeneration of knee joints with total obliteration of joint space, joint and chicken or egg allergy (Tang et al., 2005). Patients with isolated supra-patellar bursa mass mistakenly diagnosed as bursitis was not enrolled in this study. The aspirated SF prior to HA injections was sent for SF analysis. SF showing evidences of crystals suggesting possible gouty arthritis and infection was excluded from the study. All patients signed the informed consent before participating in this study. The institutional ethics committee approved all the protocols involved in this study.

2.2. Sample collection

The aspiration technique followed the standard lateral approach with the knees extended. A strict sterilized procedure was applied to prevent septic infection. The musculoskeletal ultrasound was used to accurately guide the needle into the bursa for the aspiration of SF. Accurate placement of the needle in the bursa avoided poking the needle into the muscle or other soft tissues which may cause SF to be contaminated with blood (Chen et al., 2011a).

Each patient has different volumes of bursa fluid. As much SF as possible was aspirated from the bursa. Approximately 5 to 10 mL of the aspirated SF was sent for biochemical analysis first. Samples showing evidence of active infection or inflammation were not included in this study. SF samples were centrifuged at 2500 rpm for 20 min at 4 °C. The supernatants were stored in 90 μl aliquots with 10 μl of a proteinase inhibitor solution containing 100 mM EDTA (Sigma, St. Louis, MO, USA), 20 mM *N*-ethylmaleimide (Sigma), and 20 mM aminoethylbenzenesulfonyl fluoride (Sigma) added. SF samples free of infection, inflammation, and red blood cells were further aliquoted and stored at −20 °C if they were to be used for experimental analysis within one week. Otherwise the samples were stored at −80 °C. Total protein concentration was calculated for every SF sample.

2.3. Treatment protocols

The recruited 20 patients were divided into two groups with 10 patients in each group. One group received the high molecular weight hyaluronic acid (HMW HA) product of 2 mL Synvisc Hylan G-F 20 (Genzyme Biosurgery, USA) with 6000 kDa). The other group received the low molecular weight hyaluronic acid (LMW HA) product of 2 mL Hya-Joint Synovial Fluid Supplement (SciVision Biotech Inc., Taiwan) with 500–730 kDa. Patients received once per week injection treatment into the bursa for a total of 3 weeks. Ultrasound-guided approach was applied for accurate aspiration of bursa SF samples and the injection of HA into the supra-patellar bursa.

2.4. The calculation of SF total protein concentrations

The standard curve of known bovine serum albumin (BSA) (Sigma, >96% purity) concentrations (1 μg, 0.5 μg, 0.25 μg, and 0.125 μg) was constructed to calculate the total protein concentrations of the SF. The

Bradford method was applied for the calculation of protein concentrations (da Silva and Arruda, 2006). The Bio-Rad Protein Assay dye reagent was used. The dye-proteins were measured by Unicam UV1 spectrophotometer at an absorbance of 595 nm. The SF samples were diluted 30 times first by ddH_2O in order to fit into the linear BSA standard curve for protein concentration calculation.

2.5. Western immunoblotting

In this study, the western immunoblotting method was used to detect the percentage of protein concentration differences before and after the completion of HA injections. Antibodies against the SF proteins that may be associated with degenerative joint diseases such as OA (both polyclonal and monoclonal) were purchased from the ABCAM company (ABCAM, Cambridge, MA, USA). Primary Antibodies to detect the following proteins were purchased: apolipoprotein A-I, transthyretin (TTR), interleukin 1 beta (IL-1 Beta), alpha 1 antitrypsin, matrilin 3, complement 5 (C5), matrix metalloproteinase 1 (MMP1), and prostaglandin D_2 synthase (PGDS). Triplicate western immunoblotting was done for every protein.

SF samples were loaded onto the pre-casted 10% SDS-polyacrylamide gel and were electrophoresed at 60 mV constant voltage until the dye front was near the end. The electrophoresed protein was transferred onto a 0.45 μm thick nitrocellulose membrane, which was saturated for 1 h with 5% (w/v) milk powder in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T). Membranes were incubated with primary antibodies overnight at room temperature. After washing with TBS-T, membranes were incubated for 2 h with horseradish peroxidase-conjugated secondary IgG antibodies. After the final wash, the immune-stained proteins were developed using the ECL developing kit (GE Healthcare BioScience, UK), and detected by the Fluorchem M image system. The primary mouse monoclonal anti-GAPDH antibody (Cell Signaling Technology Inc., Boston, MA, USA) was used as the internal control reference protein bands.

In terms of image analysis, the BIORAD Quantity One 1D analytical software was used to calculate the protein band densities. The band volume and density are in positive correlation with the protein concentration. The higher the detected protein density, the greater the protein concentration. Percentage increase or decrease of protein concentrations was calculated from the band densities.

2.6. Data analysis

The Wilcoxon signed-rank test was used to compare the mean age between patient groups, and the percentage changes in protein concentrations. The SF total protein concentrations were expressed as mean ± standard error of means (SEM). One-way ANOVA with Tukey's post hoc test was used to compare the means of SF total protein concentrations at different time periods before and after HA injections. The Statistical Program for Social Sciences (SPSS) version 13 (SPSS Inc., Chicago) was used for data calculations. Values of $p < 0.05$ were considered statistically significant.

3. Results

The average age of the patients who received LMW HA injection was 63 ± 8 years. The average age of the patients who received HMW HA injection was 64 ± 8 years. There was no significant statistical difference in between the average ages. Tables 1 and 2 describe the gender, the side of knee in which supra-patellar was diagnosed, the duration of the disease, and the previous treatment strategies of the recruited patients.

Protein assay was performed to measure the total protein concentrations in the SF samples. SF samples were diluted by 30 times in order for the measured optical density (OD) values to fit into the linear standard curve of OD value versus known BSA concentrations for total protein concentration calculations. In the HMW HA group, total protein concentrations in the SF samples of "1 week after 2nd HA injection" (22.59 ± 2.16 mg/mL) and "1 week after 3rd HA injection" (22.59 ± 2.16 mg/mL) were significantly lower as compared with the SF samples of "before HA injection" (26.08 ± 2.47 mg/mL) and "1 week after the 1st HA injection" (27.33 ± 3.62 mg/mL) ($p < 0.05$, Table 3).

Triplicate western immunoblotting was performed to test the antibody–antigen reactions to the purchased proteins that may be related to the disease process of arthritis and degenerative joint disorders. Four proteins revealed decreases in concentrations after the completion of HA injections. Apolipoprotein A-I, interleukin 1 beta, and alpha 1 antitrypsin proteins in the HMW HA group revealed the most obvious percentage concentration decreases ($p < 0.05$, Table 4). Apolipoprotein A-I decreased by 57% (Fig. 1A) and interleukin 1 beta by 51% after the completion of HMW HA injections.

Three proteins showed evidence of increased concentration in the SF after HA injections. TTR revealed significant increases in concentrations after the completion of both LMW and HMW HA injections ($p < 0.05$, Table 4). Concentrations of complement 5 and matrilin increased by about 19% and 16% respectively after the completion of HMW HA injections but did not reach statistical significance.

Table 1
Demographic data of supra-patellar bursitis patients who received low molecular weight hyaluronic acid injections.

Age	Sex	Right/left knee	Disease duration	Previous treatment strategy
72	F	Left	3 years	SWD + glucosamine + NSAID
58	M	Right	6 months	None
61	F	Right	6 months	None
59	F	Right	4 months	None
70	F	Left	3 years	IFC + glucosamine + NSAID
72	F	Right	4 years	SWD + glucosamine + NSAID
76	M	Left	4 years	SWD + glucosamine + NSAID
60	F	Left	5 months	Glucosamine
57	M	Right	10 months	None
53	F	Left	10 months	None

M: Male.
F: Female.
SWD: Shortwave diathermy.
IFC: Interferential wave.
NSAID: Nonsteroidal anti-inflammatory drugs.

4. Discussion

Supra-patellar bursitis is a common cause of knee swelling and pain frequently seen in the rehabilitation and orthopedic outpatient clinical setting (Aaron et al., 2011). If the cause of supra-patellar bursitis is due to degenerative disorders such as knee OA, subsequent injection

Table 2
Demographic data of supra-patellar bursitis patients who received high molecular weight hyaluronic acid injections.

Age	Sex	Right/left knee	Disease duration	Previous Treatment Strategy
60	M	Right	1 year	None
70	M	Right	4 years	SWD + glucosamine + NSAID
58	M	Right	6 months	None
57	F	Right	6 months	Glucosamine
73	F	Left	5 years	Glucosamine + NSAID
70	M	Left	10 months	SWD + IFC + glucosamine
66	F	Right	6 months	None
60	F	Right	6 months	None
59	F	Left	1 year	None
75	F	Left	3 years	SWD + glucosamine + NSAID

M: Male.
F: Female.
SWD: Shortwave diathermy.
IFC: Interferential wave.
NSAID: Nonsteroidal anti-inflammatory drugs.

Table 3
Synovial fluid total protein concentrations before and after HA injections.

	Before HA injection A	1 week after 1st HA injection B	1 week after 2nd HA injection C	1 week after 3rd HA injection D	Statistical analyses
Total protein concentration of LMW HA group in mg/mL (n = 10)	27.02 ± 3.11	25.78 ± 2.06	25.48 ± 3.09	23.88 ± 4.51	No statistical differences between all concentrations
Total protein concentration of HMW HA group in mg/mL (n = 10)	26.08 ± 2.47	27.33 ± 3.62	22.59 ± 2.16	20.68 ± 2.71	A VS C = $p < 0.05$ A VS D = $p < 0.05$ B VS C = $p < 0.05$ B VS D = $p < 0.05$

Values expressed as mean ± standard deviation (SD).

of HA after aspiration may be beneficial in alleviating knee pain and preventing future development of bursa SF. The changes in the rheology of SF after HA injection into the bursa are seldom explored.

There were no control or sham treatment groups in this study. This was due to the fact that the proteomic analyses of SF in non-degenerative knee joints were thoroughly documented in our previous study (Chen et al., 2011a). As a result, the differences in the 2-DE SF proteome maps of non-degenerative knee joints and in patients with supra-patellar bursitis are known. Proteins associated with acute inflammatory response and complement activation are shown to be increased in the SF of supra-patellar bursitis patients (data not presented). Therefore, the changes in the protein concentrations observed in this study represent the rheological changes of SF in supra-patellar bursitis patients after hyaluronic acid injections.

Western immunoblotting was the experimental procedure chosen to detect the protein bands and its concentration differences. It is a sensitive visual assay tool to detect antibody-antigen reactions, and is the preferred method for the validation of protein concentrations after proteomic analysis of 2-dimensional electrophoresis (2-DE) (Burnette, 2009; Chen et al., 2011b). Both LMW HA and HMW HA revealed similar trends in the decrease and increase of the examined SF proteins after the completion of injection treatment. It has been documented that higher molecular weight HA may have a better anti-inflammatory effect, whereas low molecular weight HA has superior efficacy for chondroprotection (Huang et al., 2010). Other studies have also shown that hyaluronic acid is capable of slowing aging through its anti-oxidant effect (Zhao et al., 2008). Apolipoprotein A-I revealed the most drop (a decrease of 57%) in protein concentration after HMW HA injections. Other proteins that revealed decreases in concentration percentages included interleukin 1 beta, alpha 1 antitrypsin, and matrix metalloproteinases 1. TTR revealed significant percentage increases in protein concentrations after both LMW and HMW HA injections. Other proteins that revealed increases in concentration percentages included matrilin 3, and complement 5.

Higher apolipoprotein A-I concentrations in the SF indicates that higher lipid levels will enter the knee joint cavity (Oliviero et al., 2012). This will contribute to local joint inflammatory processes and subsequent pain. Interleukin 1 beta protein functions as a catabolic factor, and matrix metalloproteinases are extracellular matrix proteins associated with tissue remodeling and osteochondral changes in degenerative joint disorders (Huang et al., 2011; Kaspiris et al., 2013). The matrix metalloproteinase (MMP) gene family that is believed to be closely associated with degenerative joint disorder is MMP1 (Kaspiris et al., 2013). Both interleukin 1 beta and MMP1 proteins are proven to be up-regulated during the progression of degenerative joint disorders (Huang et al., 2011; Kaspiris et al., 2013). A high concentration of alpha 1 antitrypsin protein in the SF confirms the active progression of cartilage decay and inflammatory process in OA (Olszewska-Slonina et al., 2013). As a result, decreases in the concentrations of these proteins highly indicate that the inflammatory and degenerative processes are reduced after the completion of HA injections, with HMW HA showing the most obvious results.

The association between TTR and degenerative joint disorders is seldom discussed. One study that was done about 3 decades ago mentioned that TTR level is decreased in the serum of patients with active rheumatoid arthritis (Surrall et al., 1987). TTR is present in the serum, and cerebrospinal fluid (CSF) (Chen et al., 2011b). It is a carrier of the thyroid hormone thyroxine (T4) and retinol-binding protein (Chen et al., 2011b). TTR is associated with aging, and its ability to bind to beta-amyloid protein is believed to be able to prevent Alzheimer's disease (Li and Buxbaum, 2011). Interestingly, we have discovered that the TTR concentration in the SF is increased after HA injections. This may suggest that increased TTR level in the SF may have a close association with the anti-aging effect or alleviation of the degenerative process in degenerative joint diseases.

The complement system complements the ability of antibodies and phagocytic cells to clear pathogens from an organism. Studies have suggested that low-grade complement activation may contribute to the development of degenerative diseases, such as macular degeneration, Alzheimer's diseases, and even OA. Complement 5 (C5) is the complement system believed to be associated with degenerative joint disorders (Wang et al., 2011). Therefore, it is logical to think that the SF concentration of C5 should decrease after HA injections. However, results in this study indicated that the SF C5 concentrations are slightly increased after both LMW and HMW HA injections.

Matrilin-3 (MATN3) is a skeletal-specific, tetrameric pericellular protein, localizing to the peri-cellular matrix of chondrocytes, and has both anti-anabolic and pro-catabolic functions. It was believed that MATN3 may be a useful marker to diagnose OA or to evaluate the progression of OA in the SF (Vincourt et al., 2012). Similar to C5, one would think that the concentration of MATN3 protein should decrease after HA injections. In fact, increases in the SF MATN3 concentrations of approximately 10% and 16% were observed after the completion of LMW, and HMW HA injections respectively. As a result, these findings may indicate that SF proteins of C5 and MATN3 may not play crucial roles or not be significantly associated with the development of supra-patellar bursitis.

It was evident that after the second injection dosage of HMW HA, SF total protein concentrations significantly decreased as compared to the SF total protein concentration prior to HMW HA injection. In SF, about 60% to 80% of proteins are high abundant proteins such as albumin and immunoglobulin (Chen et al., 2011a). Results in this study have shown that the concentrations of inflammatory proteins such as apolipoprotein A-I and interleukin 1 beta are greatly reduced after HMW HA injections. This may be the likely cause of the significant decreases in SF total protein concentrations after the second HMW HA injections.

Blood contamination in SF is a serious problem in SF peptide profiling due to similar peptide profile between SF and blood, but with much higher total protein concentrations in the serum (Chen et al., 2011a). Therefore, SF samples were retrieved under ultrasound guidance to avoid blood contamination. One protein that is believed to be associated with inflammation and aging is prostaglandin D_2 synthase (PGDS) (Chen et al., 2009). Monoclonal antibody against human PGDS protein was purchased to explore whether PGDS is associated with the disease entity of supra-patellar bursitis in SF samples. However, triplicate western immunoblotting analysis was not able to detect any PGDS protein bands in SF samples.

Table 4
Percentage changes in the synovial fluid protein concentrations before and after LMW and HMW HA injections.

	1 week after 3rd HA injection LMW HA Group	1 week after 3rd HA injection HMW HA Group
Apolipoprotein A-I	↓ by 33%*	↓ by 57%*
Interleukin 1 beta	↓ by 35%*	↓ by 51%*
Alpha 1 antitrypsin	↓ by 16%	↓ by 37%*
Matrix metalloproteinase 1 (MMP1)	↓ by 13%	↓ by 21%
Transthyretin (TTR)	↑ by 20%*	↑ by 44%*
Complement 5 (C5)	↑ by 10%	↑ by 19%
Matrilin 3	↑ by 10%	↑ by 16%

↑: Increase in SF protein concentration.
↓: Decrease in SF protein concentration.
* $p < 0.05$, as compared with before the hyaluronic acid injections.

In conclusion, this study aimed to identify the concentrations changes in the SF total protein and individual proteins using the Bradford protein assay and western immunoglobulin methods. After the completion of the HA injections to the bursa, proteins associated with lipid transport and inflammation (e.g. apolipoprotein A-I and interleukin 1 beta) are decreased. This may be the likely cause of the observed decrease in SF total protein concentration after the second dosage of HMW HA injection. On the other hand, concentration of the protein with anti-aging effect (e.g. TTR) is significantly increased after both LMW and HMW HA injections. As a result, the rheology of SF in supra-patellar bursitis patients is changed after HA injections, with obvious concentration decreases in inflammatory and lipid transport proteins, and with concentration increases in anti-aging protein. Results obtained in this study may provide the rationale for targeting several biomarkers as a disease modifying therapy for the treatment of supra-patellar bursitis and even degenerative joint disorders.

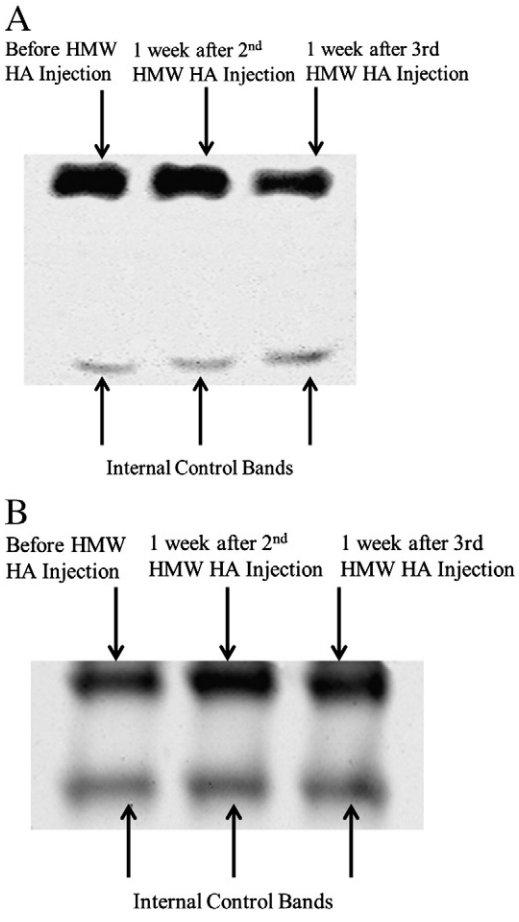

Fig. 1. Representative western immunoblotting protein bands. A. Western immunoblotting protein bands of apolipoprotein A-I, showing a decrease in band density after HMW HA injections. The molecular weight of apolipoprotein A-I was measured to be approximately 28 kDA. B. Western immunoblotting protein bands of TTR, showing an increase in band density after HMW HA injections. The molecular weight of TTR was measured to be approximately 60 kDA.

Conflict of interest

The authors have no conflicts of interests.

Acknowledgments

This study was supported by the grants from the National Science Council, Taiwan (NMRPG3B6031-2, 101-2314-B-182A-060-MY2) and the Chang Gung Memorial Hospital at Linkou Research Project Grant (CMRPG3B0631-2) to Dr. Carl P.C. Chen. The National Science Council grant supported the expense of the consumable products used in this study. The Chang Gung Memorial Hospital Research Project Grant supported the cost of western immunoblotting analyses and the purchase of antibodies. The protein assay and western immunoblotting procedures were done at the Shared Laboratory, Chang Gung Memorial Hospital, Taoyuan Branch, Taiwan.

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