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The influence of platelet rich plasma on synovial fluid volumes, protein concentrations, and severity of pain in patients with knee osteoarthritis

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ABSTRACT

Knee pain is commonly seen in orthopedic and rehabilitation outpatient clinical settings. Patients with knee osteoarthritis (OA) are often complicated with joint soreness, swelling, weakness, and pain. These complaints are often caused by the excessive amount of synovial fluid (SF) accumulated in the bursae around the knee joint. This study was aimed to evaluate the effectiveness of platelet rich plasma (PRP) in treating patients with minor to moderate knee osteoarthritis (OA) combined with supra-patellar bursitis using a proteomic approach and clinical evaluation tool. In this study, 24 elderly patients with minor to moderate knee OA combined with supra-patellar bursitis were recruited. Musculoskeletal ultrasound was used for accurate needle placement for the aspiration of SF followed by subsequent PRP injections. Three monthly PRP injections were performed to the affected knees for a total of 3 months. Approximately after the 2nd PRP injection, significant decreases in SF total protein concentrations, volumes, and Lequesne index values were observed. SF proteins associated with chelation and anti-aging physiological functions such as matrilin, transthyretin, and complement 5 increased at least 2-fold in concentrations. Proteins associated with inflammation, such as apolipoprotein A-I, haptoglobin, immunoglobulin kappa chain, transferrin, and matrix metalloproteinase decreased at least 2-fold in concentrations. Therefore, at least two monthly PRP injections may be beneficial for treating patients with minor to moderate knee OA combined with supra-patellar bursitis.

1. Introduction

Patients with knee osteoarthritis (OA) are often complicated with joint soreness, swelling, weakness, and pain (Kon et al., 2011). These complaints may be caused by the excessive amount of synovial fluid (SF) accumulated in the bursae around the knee joint. Out of all the bursae surrounding the knee joint, supra-patellar bursitis is most frequently associated with knee pain (de Miguel Mendieta et al., 2006). Bursa is a fluid contained structure that is located between the tendon and bone. Its main function is to reduce friction between the adjacent moving soft tissue structures. Inflammation of this fluid-filled structure is called bursitis (Aaron et al., 2011).

Supra-patellar bursa is located between the quadriceps tendon and the femur. Bursitis can be detected using imaging tools such as magnetic resonance imaging (MRI) or arthrography (Beaman and Peterson, 2007). But with the advantage of radiation free, musculoskeletal ultrasound can be used in an outpatient clinical setting to diagnose bursitis (Chu et al., 2012). Supra-patellar bursitis is correlated with knee pain when ultrasound image measures >2 mm (mm) distention of the bursa due to increased SF volume (de Miguel Mendieta et al., 2006). OA remains to be the most frequent cause of supra-patellar bursitis as synovitis is a common manifestation observed in knee OA (Heidari et al., 2016).

Treatment of knee OA includes taking oral nonsteroidal anti-inflammatory drugs (NSAIDs), physical modalities, strengthening exercise, shoe modification, and oral supplements such as glucosamine (Wang et al., 2016). The concept of viscosupplementation has been widely applied in the treatment of knee OA. It is a therapeutic modality based on the replacement of SF with hyaluronic acid (HA) (Cohen et al., 2008). But in recent years, a more regenerative treatment concept has been used in the treatment of knee OA. The concept uses the application of blood derivatives, especially platelet-rich plasma (PRP), in treating knee OA. Studies have stated that the effect of autologous PRP in treating knee OA is superior to that of HA (Laudy et al., 2015).

The number of platelets in human blood is not concentrated enough and is mixed with red blood cells and leukocytes. Red blood cells and leukocytes in the blood tend to impede the gathering of platelets. Purification procedure is needed to concentrate these platelets. The biological rational behind PRP treatment is that pools of growth factors such as

Abbreviations: PRP, platelet rich plasma; OA, osteoarthritis; SF, synovial fluid.

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platelet-derived growth factor (PDGF) and transforming growth factor (TGF-β) are stored in platelet α-granules and are believed to take part in the repair and regeneration of articular cartilage (Khoshbin et al., 2013). Several studies on pain scales and functional indexes have shown that PRP injections can improve pain and functional scales in patients with early knee OA. The effect may last up to a period of 12 months (Laudy et al., 2015).

However, studies on the changes of SF protein concentrations and supra-patellar bursa SF volumes before and after PRP injections are limited. In this study, the severity of knee pain will be evaluated by Lequesne index before and after PRP injections (da Silva et al., 2015). SF volumes can be measured after ultrasound-guided aspiration procedures. Protein assay using the Bradford method and 2-dimensional electrophoresis (2-DE) proteomic technique will be used to calculate the changes in total and individual SF protein concentrations (Olson, 2016). The 2-DE technique is preferred as hundreds of protein spots can be detected and compared on gel images (Gorg et al., 2004). Proteins showing at least two-fold concentration changes after PRP injections will be further validated by Western immunoblotting. We hypothesize that SF volumes, total protein concentrations, and inflammatory proteins are decreased after PRP injections. The extent of knee pain as measured by Lequesne index can be improved after PRP injections. Results obtained in this study may help us understand the physiological functions of SF proteins related to knee OA and the occurrence of bursitis. These findings may be used to develop protein supplements or drugs that can be used to treat degenerative joint disorders.

2. Materials and methods

2.1. Subjects

In this study, a total of 24 elderly patients (>65 years of age, 14 female and 10 male patients) diagnosed with supra-patellar bursitis were recruited. They signed the informed consent before participating in this study. This study was conducted in a tertiary hospital rehabilitation outpatient clinic, and was approved by the local medical ethics and the human clinical trial committee (Chang Gung Memorial Hospital, Taiwan, IRB: 102-5939A3).

The inclusion criteria were:

1. Thickness of the supra-patellar bursa was measured to be >2 mm (mm) as confirmed by musculoskeletal ultrasound.
2. Patients had history of receiving knee joint steroid injections, physical modality treatments, and taking oral NSAIDs but without any obvious reduction in SF volumes and knee pain.
3. The major cause of supra-patellar bursitis was due to knee OA, not caused by infectious or inflammatory knee disorders.
4. Patients were affected by chronic (for at least 6 months) pain or swelling of the knee and with image findings of grades 1 to 3 on the Ahlbäck scale (Kijowski et al., 2006).
5. Ultrasound confirmed that the supra-patellar bursa was in communication with the synovial cavity of the knee joint. This was to rule out the diagnosis of isolated cystic lesion at the supra-patellar region.

2.2. Treatment protocol

The "REGEN" environmental chamber for storage of platelet concentrate kit (REGENLAB, RegenACR-C Classic, Switzerland) was used for the preparation of autologous PRP. After harvesting about 10 mL of blood from the patient, it was then injected into the PRP test tube. The test tube was then centrifuged for 15 min under the speed of 2900 rounds per minute (rpm). Five mL of supernatant (PRP) was then aspirated from the test tube and was ready to be injected.

Musculoskeletal ultrasound was used to accurately guide the needle into the supra-patellar bursa site for the aspiration of SF first followed by intra-articular (IA) knee joint injection of PRP on a once per month basis for a total of three months.

The Philips iU22 ultrasound system with a bandwidth of 7–12 MHz transducer was used (Philips Healthcare, Andover, MA, USA). Accurate placement of the needle into the bursa can avoid poking of the needle into the muscle or other soft tissues that may cause blood contamination in the SF. The standard lateral approach with the knee extended was applied for ultrasound-guided IA aspiration and injection procedures. The exact volume of the SF was measured from the syringe after aspiration. The interpretation of ultrasound images and the measuring of SF volumes were done by the same physiatrist to avoid experimental bias.

2.3. Calculation of synovial fluid total protein concentrations

The standard curve of known bovine serum albumin (BSA) (Sigma, >96% purity) concentrations (2 μg, 1 μg, 0.5 μg, 0.25 μg, and 0.125 μg) was constructed to calculate the total protein concentration of SF samples. The Bradford method was applied for the calculation of protein concentrations (Olson, 2016).

2.4. 2-dimension electrophoresis (2-DE)

The proteomic technique of 2-DE was used to detect significant increases and decreases in protein spot intensities before and after PRP injections. The exact steps of 2-DE and subsequent protein identification using mass spectrometry (MS) can be read in our previous published article (Chen et al., 2009). In this study, good quality SYPRO Ruby stained SF 2-DE gel images were obtained from each patient. Three (triplicate) gel images were constructed from SF samples gathered before the 1st PRP injection, 3 gel images from after the 2nd PRP injection, 3 gel images from after the 3rd PRP injection, and 6 gel images from the follow up time periods of 3 months and 6 six months after the completion of the 3rd PRP injection). The ProXPRESS™ 2D Proteomic Image System (PerkinElmer Life and Analytical Sciences) was used to scan the SYPRO Ruby stained SF 2-DE gels. The gel images were acquired as digital TIF files and analyzed using the PDQuest Basic 8.0.1 Analytical software (BioRad).

2.5. Western immunoblotting

Western immunoblotting method was used to validate the protein spots that revealed significant increases or decreases in concentrations 2-DE analyses. Polyclonal and monoclonal antibodies were purchased from the ABCAM company (ABCAM, Cambridge, MA, USA). Primary antibodies to detect the proteins of apolipoprotein A-I (APOA1), complement 5 (C5), haptoglobin (HPT), immunoglobulin kappa chain (IGKC), matrilin (MATN), transferrin (TRFE), transthyretin (TTR), and matrix metalloproteinase (MMP) were purchased. The exact experimental steps of Western immunoblotting can be seen in our previous published article (Chen et al., 2014).

2.6. Lequesne index

The Lequesne Functional Index for degenerative knee joint was used to evaluate the extent of knee pain, and the changes of knee functional status before and after PRP injections. The Lequesne Functional Index was evaluated by the same experimenter to prevent inter-tester variability. Index value of >7 is highly indicative of degenerative knee disorder (da Silva et al., 2015).

2.7. Statistical analysis

The Wilcoxon signed-rank test was used to compare the age difference between patients. The statistical tool of repeated measures ANOVA with Bonferroni correction was used to compare the SF total protein and individual protein concentrations, SF volume changes, and changes in Lequesne Functional Index values before and after PRP

injections. The protein concentrations, SF volumes, and functional index values were calculated and measured at the time periods of 1st PRP injection, 2nd PRP injection, 3rd PRP injection, and the follow up time periods of 3 and 6 months. The Statistical Program for Social Sciences (SPSS) version 13 (SPSS Inc., Chicago) was used for data calculations. Values with $p < 0.05$ were considered statistically significant.

3. Results

The mean age of the recruited fourteen female and ten male patients was 70 ± 3.1 years. Wilcoxon signed-rank test revealed no significant statistical differences in their age. The average SF total protein concentration was calculated to be 34.89 ± 3.62 μg/μL before the 1st PRP injection. The average SF total protein concentrations were then calculated to be 31.20 ± 2.14 μg/μL during the time of the 2nd PRP injection, 23.74 ± 2.41 μg/μL during the time of the 3rd PRP injection, 20.07 ± 1.77 μg/μL 3 months and 23.14 ± 2.81 μg/μL 6 months after the 3rd PRP injection. Significant decreases in SF protein concentrations were observed after the 2nd PRP injection ($p < 0.05$) (Table 1).

The average aspirated SF volume was measured to be 18.47 ± 4.67 mL before the 1st PRP injection. The average aspirated SF volumes were then measured to be 17.38 ± 3.48 mL during the time of 2nd PRP injection, 8.28 ± 1.43 mL during the time of 3rd PRP injection, and 5.27 ± 1.37 mL 3 months and 6.81 ± 2.66 mL 6 months after the 3rd PRP injection. Similarly, significant decreases in the aspirated SF started to show after the completion of the 2nd PRP injection ($p < 0.05$) (Table 1).

In the assessment of knee pain severity using Lequesne Functional Index, the average index was recorded to be 12.21 ± 2.44 before the 1st PRP injection. The average index values were then recorded to be 11.15 ± 1.62 during the time of the 2nd PRP injection, 5.52 ± 1.57 during the time of the 3rd PRP injection, 4.50 ± 2.01 at 3 months, and 4.33 ± 0.78 at 6 months after the 3rd PRP injection.

The 2-DE gel images of SF samples revealed at least 2-fold increases in protein intensities of matrilin, transthyretin, and complement 5 (Fig. 1). Proteins with at least 2-fold decreases in protein intensities were apolipoprotein A-I, haptoglobin, immunoglobulin kappa chain, transferrin, and matrix metalloproteinase. These proteins were further validated by Western immunoblotting (Table 2 states the names of SF proteins and Fig. 2 reveals the images of proteins that were identified by 2-DE).

4. Discussion

Young patients were not recruited in this study because the occurrence of knee OA at a young age is most likely due to trauma, overuse or other pathological factors. Hyaluronan plays an essential role in the SF lubrication quality at the cartilage-cartilage interface. Its concentration decreases with age, and hence increases the likelihood of developing knee OA. Changes in the concentrations of SF hyaluronan will affect the SF proteomic findings in different age groups (Temple-Wong et al., 2016). Recruiting older patients with knee joint narrowing and bursitis ensure that their pain was due to degenerative OA. The extent of supra-patellar bursitis has been shown to be associated with cartilage loss. In other words, the higher the supra-patellar bursa SF volume, the more the severity of knee cartilage loss (Heidari et al., 2016). It may be difficult to aspirate adequate knee SF for proteomic study if knee OA patients without bursitis were recruited. As a result, this study recruited patients >65 years of age who were confirmed with the diagnosis of knee OA and supra-patellar bursitis.

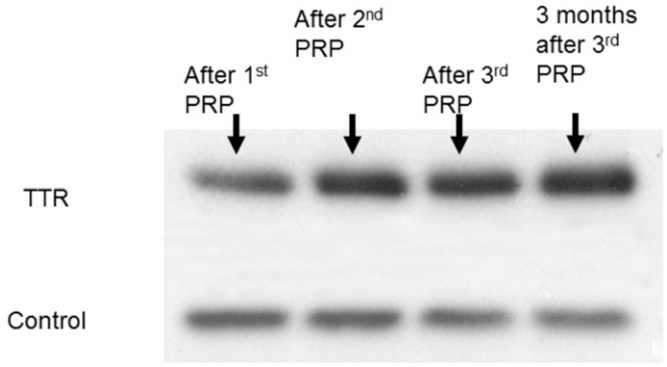

Fig. 1. Western immunoblotting protein bands of transthyretin showing increased intensities after PRP injections.

In this study, a control group involving the injection of normal saline placebo, steroid or HA into the knee joint were not included. The main reason was that we have done SF proteomic analyses for knee OA patients after the injection of these injectants in our previous studies. Our data did not reveal any significant changes in SF protein concentrations before and after normal saline and steroid injections. Proteins associated with inflammation, such as the complement system and immunoglobulin G also did not reveal any significant changes in their concentrations (data not presented). Our previously published paper showed that IA knee joint injection of hyaluronic acid in the treatment of knee OA revealed SF protein concentration changes different from this study (Chen et al., 2014). In this study, significant decrease in the concentration of SF protein haptoglobin was observed. Haptoglobin has been shown to be highly associated with knee degeneration and can serve as a candidate biomarker for knee OA (Liao et al., 2015). Therefore, the SF proteomic results obtained in this study may truly resemble the treatment effectiveness of PRP in knee OA patients.

Studies that look into the changes of SF proteins after PRP injections in elderly knee OA patients are limited. This study looked thoroughly into the SF total and individual protein concentration differences before and after PRP injections. SF total protein concentration decreased significantly after the second monthly PRP injection. This was associated with at least two-fold concentration decreases in the proteins of apolipoprotein A-I, haptoglobin, immunoglobulin kappa chain, transferrin, and matrix metalloproteinase. In the SF, abundant proteins of albumin and immunoglobulin constitute about 60 to 80% of the total protein concentration (Chen et al., 2014). Therefore, the significant decrease in the protein concentration of immunoglobulin may be the main cause of decreased SF total protein concentration after PRP injections.

Literature search was done for proteins showing significant concentration changes after PRP injections. Studies have shown that the extent of inflammation in the synovial membrane of knee OA is not as obvious as in rheumatoid arthritis (RA). However, inflammation is definitely an

Table 1
Differences in synovial fluid protein concentrations, volumes, and LeQuesne index before and after PRP injections.

	Before PRP injections (1st PRP injection)	1 month after 1st PRP injection (2nd PRP injection)	1 month after 2nd PRP injection (3rd PRP injection)	3 months after 3rd PRP injection	6 months after 3rd PRP injection
Average SF total protein concentration in μg/μL	34.89 ± 3.62	31.20 ± 2.14	23.74 ± 2.41*	20.07 ± 1.77*	23.14 ± 2.81*
Average aspirated SF volume in mL	18.47 ± 4.67	17.38 ± 3.48	8.28 ± 1.43*	5.27 ± 1.37*	6.81 ± 2.66*
Lequesne functional index	12.21 ± 2.44	11.15 ± 1.62	5.52 ± 1.57*	4.50 ± 2.01*	4.33 ± 0.78*

*: significant statistical differences at the time of 3rd PRP injection (patients have received 2 PRP injections), 3 months and 6 months after the completion of the 3rd PRP injection as compared with the first 2 PRP injections ($p < 0.05$). Values expressed as mean ± standard error of means (SEM).

Table 2
Eight representative proteins from human SF identified by MS after 2-DE separation.

Spot name[a]	Description	Access no[a]	Mr(kDa)[b]	pI[b]	No. of matched[c]	Seq cov (%)[d]
APOA1	Apolipoprotein A-1	P02647	30.00	5.56	13	46
C5	Complement 5	P06684	112.75	8.90	18	67
HPT	Haptoglobin	P00738	45.80	6.13	20	44
IGKC	Immunoglobulin kappa chain	P01834	11.61	9.10	47	66
MATN	Matrilin	P21941	54.00	8.31	36	51
MMP	Matrix metalloproteinase	P14780	92.00	5.70	17	38
TRFE	Transferrin	P02787	79.29	6.81	24	36
TTR	Transthyretin	P02766	15.87	5.52	10	73

[a] Protein name and accession number according to the SwissProt and TrEMBL databases.
[b] Predicted molecular weights in kilodaltons and pI according to protein sequence and Swiss-2DPAGE databases.
[c] Number of peptide masses matching the top hit from MS-Fit PMF.
[d] Amino acidic sequence coverage for the identified proteins.

ongoing progressive process in knee OA as cartilage destruction can be observed (Chang et al., 2009). APOA1 is a plasma derived high density apolipoprotein that plays a key role in the transport and delivery of lipids. Dyslipidemia or dysregulated lipid profile in the SF is known to be an underlying cause of inflammation in OA joint cavity and the development of OA. The pro-inflammatory properties of APOA1 were confirmed in an in vitro environment. APOA1 has also been proven to have a potential role in inducing strong matrix metalloproteinases (MMPs) expressions (Oliviero et al., 2009). MMPs can aggravate inflammation in OA by degrading extracellular matrix macromolecules and decreasing expression of chondrocyte proteins resulting in severe joint pain, loss of movement, and irreversible dysfunction (Lu et al., 2015). As a result, significant decreases in APOA1 and MMP concentrations after PRP injections may attenuate the joint inflammatory response in knee OA.

The IGKC is one of the two types of light chains that is involved in antibody production as secreted by B lymphocytes. Increased levels of free Ig light chains can be detected in inflammation (Chang et al., 2009). HPT on the other hand, is a major acute phase glycoprotein with a molecular weight of 43 kilodaltons. When its concentration increases by twofold or more in the SF or plasma, it is correlated with conditions such as inflammation, injury or malignancy (Park et al., 2013). TRFE is an iron-binding plasma glycoprotein that is responsible with the control of free iron in biological fluids. A study has shown that transferrin, immunoglobulin, and albumin protein concentrations are increased in OA and RA joints (Izai et al., 1992). Therefore, significant decreases in IGKC, HPT, and TRFE proteins may further indicate that the extent of inflammation is decreased in knee OA joints after PRP injections.

Not only seen in degenerative brain disorders, amyloid deposits are prevalent in knee OA joints, and is a risk factor in the development of OA. TTR protein can chelate amyloid, further preventing the deposition of amyloid. TTR is a homotetrameric protein synthesized mainly in the choroid plexus and liver. In age-related studies, TTR can be detected predominantly at the cartilage surface of aged normal cartilage samples (Akasaki et al., 2015). Therefore, the significant increase in SF TTR concentration after PRP injections may signify a healthier knee joint as more amyloid deposits can be chelated.

The matrilins, especially matrilin 3, are noncollagenous extracellular matrix proteins that are believed to play a crucial regulatory role in maintaining cartilage microenvironment. In matrilin-3 knock-out mice, we can observe increased chondrocyte hypertrophy, which is

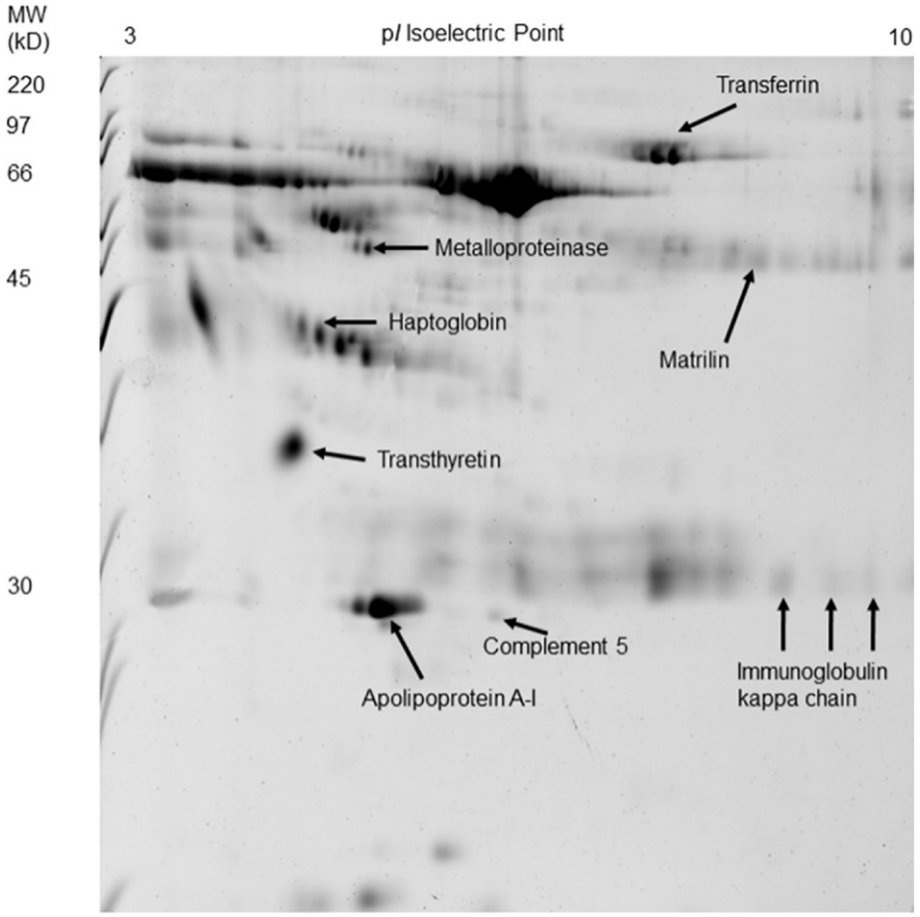

Fig. 2. SYPRO Ruby stained 2-DE gel image of SF sample with MS identified protein spot names and their molecular weights (MW) in kilodaltons (KD).

associated with cartilage degeneration seen in human knee OA. As a result, increased matrilin concentration after PRP injections may inhibit chondrocyte hypertrophy, and hence attenuate cartilage degeneration (Yang et al., 2014). The complement system complements the ability of antibodies and phagocytic cells to clear pathogens from organisms. C5 is believed to be associated with degenerative joint disorders. Some studies on the complement system actually contradict with each other. For example, some studies suggested that low-grade complement activation may contribute to OA. But one study indicated that the activation of complement is abnormally high in human OA joints (Wang et al., 2011). Our results indicated that C5 concentration is significantly increased after PRP injections.

In summary, proteomic findings in this study suggested that for patients with minor to moderate knee OA, the completion of 3 monthly PRP injections results in significant decreases of SF protein concentrations associated with inflammation (eg/APOA1, HPT, IGKC, TRFE, and MMP), and increases in the protein concentrations associated with chelation and inhibition of chondrocyte hypertrophy (eg/TTR and MATN). These proteomic findings are associated with significantly improved index of knee OA severity, and decreased supra-patellar bursa SF volumes. As a result, based on the data obtained in this study, drugs targeted at the inflammatory SF proteins such as haptoglobin and apolipoprotein to decrease their concentrations in the SF may be developed to treatment knee OA. In future related research, our goal will be to conduct studies with larger sample sizes, hoping that more SF proteins or biomarkers related to knee OA can be discovered.

5. Conclusions

This study aimed to identify the changes of SF protein concentrations, supra-patellar bursa SF volumes and the severity of knee pain in patients with knee OA during and after PRP injections. After receiving 2 monthly PRP injections, the rheology of SF started to change with significant drops in total protein concentrations. Proteins associated with inflammation (eg/APOA1, HPT, IGKC, TRFE, and MMP) decreased and proteins associated with chelation and anti-aging physiological functions (eg/TTR and MATN) increased significantly. These changes were combined with clinical improvements of decreased SF volumes and index of OA severity. From proteomic perspective, SF environment may become less susceptible to degeneration after PRP injections. As a result, multiple monthly PRP injections (eg/at least 2 PRP injections) are recommended and may be beneficial for treating minor to moderate knee OA patients with supra-patellar bursitis.

Compliance with ethical standards

Conflict of interest statement

All six authors declared that they have no conflict of interests in this study.

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Ethical approval

This study was approved by the local medical ethics and the human clinical trial committee (Chang Gung Memorial Hospital, IRB: 102-5939A3 & 103-1829A3).

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