



Identification of early response to hypertonic dextrose prolotherapy markers in knee osteoarthritis patients by an inflammation-related cytokine array

Po-Jung Pan^{a,b}, Jia-Chi Wang^{b,c}, Chih-Chun Tsai^d, Hsing-Chun Kuo^{e,f,g,h,*}

^aDepartment of Physical Medicine and Rehabilitation, National Yang Ming Chiao Tung University Hospital, Yilan, Taiwan, ROC;

^bDepartment of Medicine, National Yang Ming Chiao Tung University University, Taipei, Taiwan, ROC; ^cDepartment of Physical

Medicine and Rehabilitation, Taipei Veterans General Hospital, Taipei, Taiwan, ROC; ^dDepartment of Mathematics, Tamkang

University, Taipei, Taiwan, ROC; ^eDepartment of Nursing, Division of Basic Medical Sciences, Chang Gung University of Science and

Technology, Chiayi, Taiwan, ROC; ^fResearch Fellow, Chang Gung Memorial Hospital, Chiayi, Taiwan, ROC; ^gResearch Center for

Food and Cosmetic Safety, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan, Taiwan, ROC;

^hChronic Diseases and Health Promotion Research Center, CGUST, Chiayi, Taiwan, ROC

Abstract

Background: Osteoarthritis (OA) is one of the most common forms of arthritis, and hypertonic dextrose prolotherapy has long been used clinically to treat knee OA. The aim of this study was to investigate the inflammation-related protein-expression profile characterizing the efficacy of the hypertonic dextrose prolotherapy in knee OA as prognostic markers.

Methods: OA patients over the age of 65 were recruited for Western Ontario McMaster University Osteoarthritis (WOMAC) index, knee X-ray evaluation and knee joint synovial fluid analysis before and after hypertonic dextrose prolotherapy. The expressions of inflammation-related factors were measured using a novel cytokine antibody array methodology. The cytokine levels were quantified by quantitative protein expression and analyzed by ELISA using the patients' knee-joint synovial fluid.

Results: The WOMAC Index and minimum joint space width before receiving the intra-articular injection and at 2-week intervals were compared. Twelve patients who received OA intervention were enrolled and finally a clinical evaluation of 12 knee joints and knee synovial fluid samples were analyzed. In this study, after receiving hypertonic dextrose prolotherapy, the OA patients clearly demonstrated a significant improvement in WOMAC index and increasing tendency in the medial minimum joint space width after intervention. Meanwhile, we observed a significantly associated tendency between hypertonic dextrose treatment of knee OA and the upregulation of MMP2, TIMP-1, EGF, CXCL9 and IL-22.

Conclusion: The findings provide knee OA patients receiving hypertonic dextrose prolotherapy, which is accompanied by the improvement of knee symptoms and associated tendency of upregulation of MMP2, EGF, CXCL 9 and IL-22.

Keywords: CXCL9; EGF; Hypertonic dextrose prolotherapy; IL-22; MMP2; Osteoarthritis; TIMP-1; WOMAC Index

1. INTRODUCTION

Knee osteoarthritis (OA) is a common chronic degenerative disease. About 33.6% of elders over 65 years of age will have knee OA, which is manifested with joint pain, stiffness, and limited range of motion and progressively results in functional impairment.^{1,2} OA knee as a disease is characterized by joint synovial reaction with articular cartilage and subchondral bone destruction. The choice of clinical treatment options depends on the severity of the knee OA. Non-pharmacological management

may include weight loss, therapeutic exercise, electric modality physiotherapy, and use of a knee brace and an insole fit.³⁻⁵ Pharmacological management may include oral analgesics, anti-inflammatory drugs, a glucosamine supplement, and joint injections with corticosteroid at the acute joint effusion stage and a viscosupplementation of hyaluronic acid.⁶⁻⁸ Surgical intervention with osteotomy or total knee replacement will be considered if medical treatment fails. Prolotherapy, known as a complementary treatment, involves the injection of an irritant solution and hypothetically will induce a local inflammatory reaction, thus facilitating the regeneration of connective tissue.⁹ Hypertonic dextrose is the most commonly injected solution, and its efficacy in knee OA has been reported in recent years.¹⁰⁻¹² However, the detailed mechanism is not well elucidated, leading to our interest in the knee joint's synovial membrane and cartilage response to hypertonic dextrose prolotherapy.

Previous studies revealed the protein expression in the synovial fluid underlying the pathogenesis of knee OA.^{13,14} The mechanism of prolotherapy for knee OA needs to be elucidated, especially with respect to the inflammation and regeneration of the related pathway. All cell functions, including cell proliferation, cell death and differentiation, as well

*Address correspondence. Dr. Hsing-Chun Kuo, Department of Nursing, Division of Basic Medical Sciences, Chang Gung University of Science and Technology, Chiayi, Taiwan, ROC. E-mail address: guscsci@gmail.com (H.-C. Kuo).

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as maintenance of health status and disease development, are controlled by many genes and signaling pathways. However, almost all cell functions are executed by proteins, which cannot be studied by DNA and RNA alone. The experimental analysis clearly shows a disparity between the relative expression levels of mRNA and their corresponding proteins.¹⁵ Therefore, it is critical to analyze the protein profile by Human Cytokine Antibody Array. Furthermore, cytokines play an important role in innate immunity, apoptosis, angiogenesis, cell growth and differentiation in most disease processes, including cancer and cardiac diseases.¹⁶ The interaction between cytokines and the cellular immune system is a dynamic process. The interactions of positive and negative stimuli and positive as well as negative regulatory loops are complex and often involve multiple cytokines. Combining cytokine analysis with clinical investigation, we conducted a study to provide some molecular evidence involving the hypertonic dextrose prolotherapy of knee OA. The aim of this study was to investigate the early response biomarkers, inflammation-related protein-expression characterizing the efficacy of the hypertonic dextrose prolotherapy in knee OA.

2. METHODS

2.1. Patient recruitment

In this single-arm study, patients who were over 65 years of age and who had been suffering from at least 6 months of symptomatic knee OA met the clinical criteria of the American Rheumatological Association for moderate or moderate-to-severe knee OA (grade II or III according to the radiological classification of knee OA defined by the Kellgren-Lawrence system) and were recruited from the Physical Medicine and Rehabilitation out-patient service at National Yang-Ming University Hospital in January 1, 2016 to June 30, 2017.¹⁷ The exclusion criteria were patients who had severe knee OA (grade IV), a history of rheumatoid or other inflammatory arthritis, received physiotherapy during the previous 1 to 2 weeks before the treatment, received oral corticosteroids or anticoagulant, and any knee intra-articular injections during the previous 1 month before the treatment, had poorly controlled diabetes mellitus with fasting blood sugar greater than 200 mg/dL, or a history of knee surgery, dementia or psychological disease. The aim and whole course of the study were explained orally to all 12 patients. Procedural risks were also explained and patients could quit any time. Written informed consents were obtained from all study patients. The study protocol was sent to the Institution Review Board of the Yang-Ming University Hospital and was approved (IRB No. 2015B004).

2.2. Intervention

Each patient received five intra-articular injections at 2-week intervals in weeks 0, 2, 4, 6, and 8. During the procedure, each

patient sat on a bed with the knee flexed at 90°, and the injection site was marked at the anteromedial or anterolateral part of the knee. After proper sterilization, 25% dextrose 6 mL was injected into the knee joint via the anteromedial or anterolateral approach by an expert physiatrist using a 22-gauge needle.¹⁰ Corticosteroid or NSAID anti-inflammatory drugs were avoided after injections (Fig. 1).

2.3. Outcome measurement

For clinical outcome measurements, we compared the validated Western Ontario McMaster University Osteoarthritis (WOMAC) Index and the standing knee X-ray before the first injection and 2 weeks after the last injection. The WOMAC questionnaire consists of 24-items in three subscales for measuring pain (WOMAC_A 5 items), joint stiffness (WOMAC_B 2 items) and physical function (WOMAC_C 17 items). The answer to each question was scored in 100-mm visual analogue format. Standard standing posterior-anterior view radiographs were taken with the patient's knee joint fully extended. The minimum joint space widths of the respective medial and lateral compartment of the tibia-femoral joint were assessed by the same radiologist using the picture archiving and communication system.¹⁸

2.4. Sample collection and preparation

Each patient was placed in a supine position with the knee flexed at 10 to 15°. Under ultrasound guidance, a sample of about 1 mL of synovial fluid was aspirated from the suprapatella bursa via the superomedial or superolateral approach before the first injection and 2 weeks after the last injection.¹⁹ The synovial fluid samples were stored at -30°C for further analysis.

2.5. Secreted human chemokine by cytokine antibody array

The patients' secretion of inflammatory factors was evaluated in duplicate using a protein array method (RayBio Human Angiogenesis Antibody Array, RayBiotech C Series 1000; RayBiotech, Inc., Norcross, GA). This assay can simultaneously detect 270 different inflammatory factors (spotted in sub-arrays) with high specificity. The sensitivity of the antibodies in the arrays ranged from 1 to 2000 pg/mL. Each array was incubated with 1.0 mL of synovial fluid at 4°C overnight, and the bound antigens were detected according to the manufacturer's instructions. The membranes were then analyzed according to the manufacturer's instructions, followed by incubation with the sample at 4°C overnight and incubation with a biotinylated antibody cocktail with gentle shaking. After another round of washing, membranes were incubated with horseradish peroxidase-streptavidin. Finally, signals on the membranes were detected by a chemiluminescent detection system (Bio-Rad, Hercules, CA, USA). To determine the relative concentrations of the inflammatory factors in the synovial fluid samples, Quantitative analysis of fluorescence assays was conducted using ImageGauge 3.46 software (Fujifilm Co., Tokyo, Japan), as previously described.²⁰

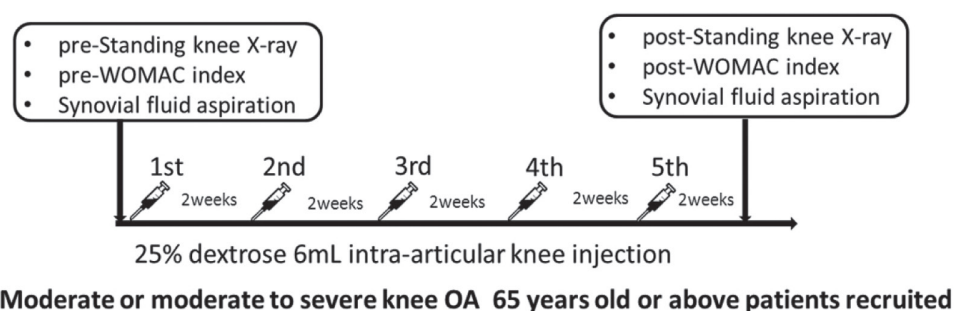


Fig. 1 Evaluation and treatment protocol of hypertonic dextrose prolotherapy in knee osteoarthritis patients.

2.6. Cytokine bead array

The synovial fluid was tested for MMP2, TIMP-1, EGF, IL-10 and IL-22 using a cytokine bead array (CBA) kit from BD Biosciences, as per the manufacturer's protocol. Concentrations of MMP2, TIMP-1, EGF, IL-10 and IL-22 were measured using a LEGENDplex Human Inflammation Panel CBA (Biolegend). The LEGENDplex CBA was optimized so that each test could be performed using half of the manufacturer's recommended volumes for samples and reagents, and all other aspects of the assay were performed following the manufacturer's instructions. Samples were acquired on a FACSCanto II using FACSDiva software and analyzed using FlowJo software or LEGENDplex software (Biolegend).

2.7. Statistical analysis

Statistical analysis was carried out with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The Wilcoxon signed ranks test was applied to examine the clinical outcome between pretreatment and posttreatment. The level of two-tailed statistical significance was 0.05. All data are expressed as mean \pm standard error. Student t-test (unpaired, two-tailed) was used for a comparison of continuous data between the experimental groups. Differences in the distribution of the staining score between the groups were assessed using the Mann-Whitney U test. Probability values (*p*) less than 0.05 were considered as statistically significant.

3. RESULTS

3.1. WOMAC index and measurement in medial minimum joint space width

Twelve participants were initially recruited for this study and two declined during the treatment period due to intolerable knee pain after injection or an unsatisfactory injection effect and two lost follow-ups. Finally, 12 knee joints WOMAC indexes from eight patients and 10 knee joints X-ray medial and lateral minimum joint space widths from seven patients were evaluated (Fig. 1).

In Table 1, significant improvements were noted between the pretreatment and posttreatment groups in the composite and all subscale WOMAC scores (53.2 to 27.0 in WOMAC, 10.3 to 4.8 in WOMAC_A, 4.2 to 2.7 in WOMAC_B, and 38.8 to 19.5 in WOMAC_C, respectively). There was an increasing tendency in the medial minimum joint space width (2.8 to 3.4 mm), but no statistically significant difference in the medial and lateral minimum joint space widths (Table 1).

3.2. Inflammation-related cytokine biomarker screening

At last, eight synovial fluid samples from seven patients were collected completely. This observation suggested the presence of dextrose intervention in the medial minimum joint space width between the pretreatment and posttreatment groups. To explore this mechanism of paracrine regulation, the human chemokine

and cytokine antibody array (Ray Biotech Inc., Norcross, GA, USA), including 274 specific antibodies, was used. Potential biomarkers in the synovial fluid for evaluating cytokines were screened using a cytokine array. Among these candidate cytokines identified, we found that transforming growth factor β (TGF- β 1), soluble M-CSF, MDC, BMP6, CCL23, CNTF and EGF individually showed a 1.5-fold greater inducible expression than the pretreatment groups in the knee-joint samples compared to the human cytokine antibody array C6 (Fig. 2A), with Flt-3 and MIG showing the decreasing levels by 1.5-fold. Interestingly, TIMP-1 and soluble MIP-1a and TRAIL-R3 synergistically increased the effects of the post-treatment groups from array C7. Importantly, we found that Adipsin, Furin, Galectin 7, MICA, MMP-2, LYVE-1, Maraosin, MICB, GH1, IL10, BCAM, IL-22, MMP-10, Siglec 9, Trappin 2, PAI-1, TREM-1, TSH, VCAM-1 and GDF-15 acted as autocrine factors from array C9. Additionally, we showed that recessively expressed the paracrine modulation of Leptin, ALCAM, CD80, CXCL16, IL-2, MIP1F-1 and TIMP-4 from array C8 acting on the posttreatment groups, and other factors, including PSA, CA-XI, AFP and IL-17C, dominantly increased the effects of the posttreatment groups from array C10. As shown in Fig. 2B, which illustrates the results of the 47 differences observed after dextrose intervention, including the upregulation of 37 proteins and the downregulation of 10 proteins, in the posttreatment groups of knee OA patients.

3.3. Biomarker verification

This result indicates that increased concentrations of these cytokines in synovial fluid were associated with the 2-week hypertonic dextrose prolotherapy's being useful as biomarkers. Therefore, these cytokines were further examined by quantitative detection. All samples from synovial fluid from the seven knee OA patients between the pretreatment and posttreatment groups, individually assessed was collected, and a CBA was performed to evaluate the secretion of select cytokines by quantitative analysis. The secretion of pro-inflammatory cytokine matrix metalloproteinase-2 (MMP-2), TIMP metalloproteinase inhibitor 1 (TIMP-1), epidermal growth factor (EGF), interleukin-10 (IL-10), and interleukin-22 (IL-22) were observed to be higher in the post-treatment groups compared to their fresh counterparts (Fig. 3).

4. DISCUSSION

Our study showed similarly improved results of WOMAC scales compared to the previous studies reported by Dumais in 2012, Eslamian in 2015, and Rabago in 2012, 2013 and 2015. For disclosing the cytokine markers of early response to hypertonic dextrose prolotherapy, the follow-up period was 10 weeks post first injection in this study, a shorter period than in previous studies. Nevertheless, the clinical outcomes including pain, joint stiffness and physical function were also improved. This finding indicated that the hypertonic dextrose intra-articular injections for OA knee patients have a clinical effect lasting from weeks to months posttreatment.^{20,21}

Table 1

Clinical evaluation in WOMAC index and X-ray knee joint space width

	WOMAC (score)		WOMAC_A (score)		WOMAC_B (score)		WOMAC_C (score)		Medial MJS (mm)		Lateral MJS (mm)	
	Mean \pm SD	n	Mean \pm SD	n	Mean \pm SD	n	Mean \pm SD	n	Mean \pm SD	n	Mean \pm SD	n
Pretreatment	53.2 \pm 14.7	12	10.3 \pm 3.1	12	4.2 \pm 1.3	12	38.8 \pm 10.9	12	2.8 \pm 1.6	10	6.7 \pm 1.8	10
Posttreatment	27.0 \pm 11.6	12	4.8 \pm 1.9	12	2.7 \pm 1.8	12	19.5 \pm 9.0	12	3.4 \pm 1.3	10	6.7 \pm 1.8	10

WOMAC Western Ontario McMaster University Osteoarthritis Index (WOMAC composite 24-items in three subscales for pain (WOMAC_A 5 items), joint stiffness (WOMAC_B 2 items) and physical function (WOMAC_C 17 items) measurements), M JS minimal joint space, Wilcoxon Signed Ranks Test.

^a*p* < 0.05.

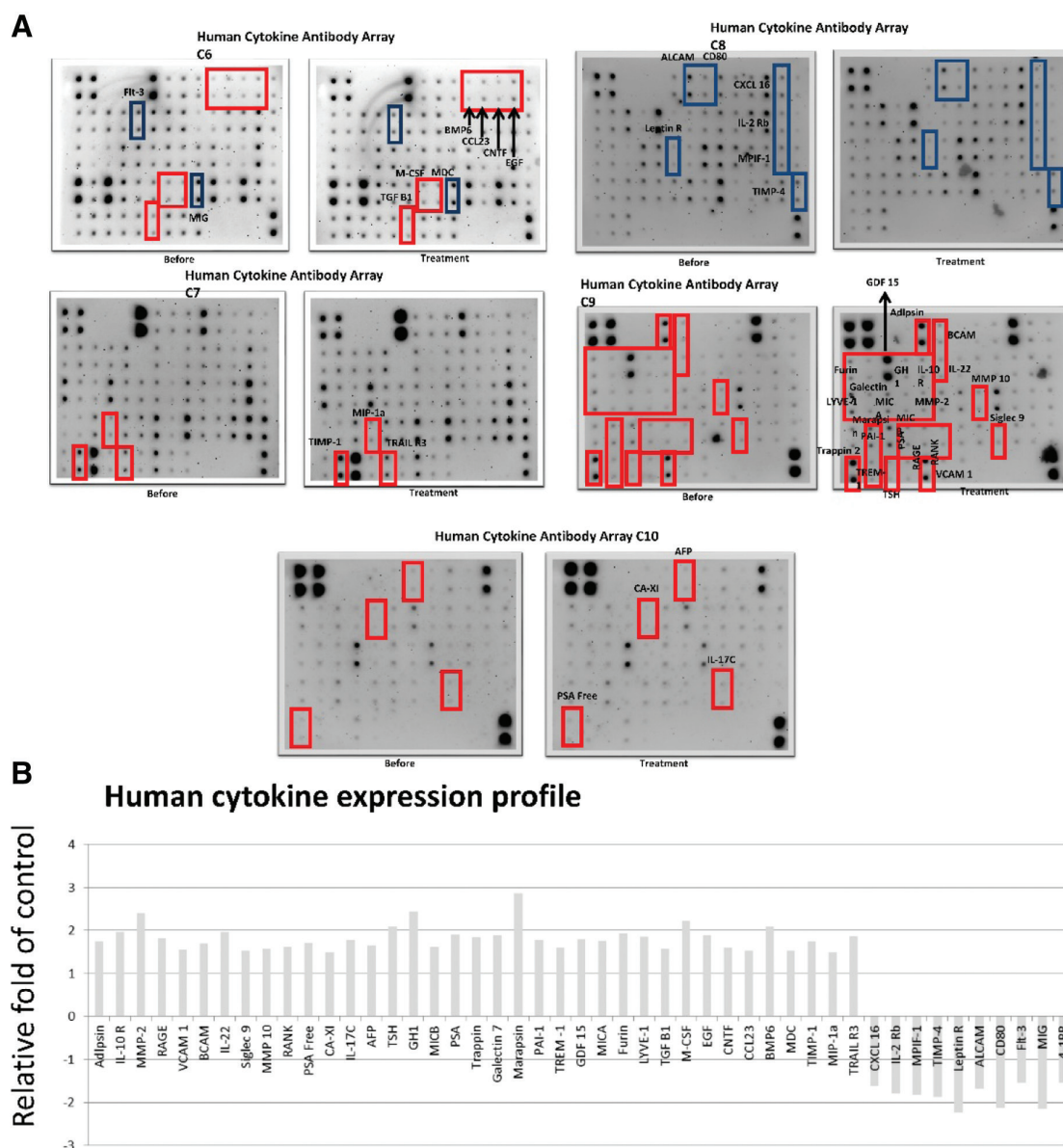


Fig. 2 Cytokine antibody array and hypertonic dextrose prolotherapy treatment of knee osteoarthritis. Human 274 cytokines were blotted onto a membrane and arrayed three times following the manufacturer's protocol. The intensities of the relative expression levels of cytokines were quantified by densitometry (VersaDoc imaging system; Bio-Rad). The density value of each test sample was normalized to as described from Human Cytokine Antibody Array C6, C7, C8, C9, C10 (A) and graphed (B). Antibody arrays were used to examine the growth factors secreted. The arrays were scanned and quantified, and the levels were normalized to those of the positive controls.

Few studies follow the joint space width as an outcome measurement after hypertonic dextrose prolotherapy for knee OA. Our study tried to survey the knee-joint cartilage structural change in addition to functional evaluation. Though there was no statistically significant improvement, the medial minimum joint space width increased in most of the knee joints after five injections. Knee joints X-ray from a 74-year old female in this study was shown in Fig. 4. This result is compatible with the chondrogenic effect of intra-articular hypertonic dextrose in knee OA as published by Topol et al in 2016.²²

Dextrose prolotherapy is an alternative to surgery for knee OA patients. The potential mechanism of dextrose prolotherapy relevant pain-intensity reduction is associated with the hyperpolarization of nociceptive pain fibers by opening the potassium channels.²¹ Prolotherapy simulates the normal tissues' healing

and repair response, which includes the three stages of inflammation, proliferation, and tissue remodeling.²³ Hypertonic dextrose solutions induce inflammation and stimulate local healing in injured articular tissue through attracting immune cells. In addition, some in vitro and in vivo studies indicated that dextrose also augments the growth of ligaments and tendons, fibroblastic proliferation, and the restoration of the extracellular matrix and articular cartilage by triggering the production of growth factors.^{20,24,25}

The biological mechanisms of dextrose have been indicated in cell and animal models. However, the question of which proteins that participate in tissue repair and the healing process will be regulated by hypertonic dextrose in human knee joints remains ambiguous. Therefore, we used a human cytokine antibody array to evaluate the expression of dextrose-induced cytokine in

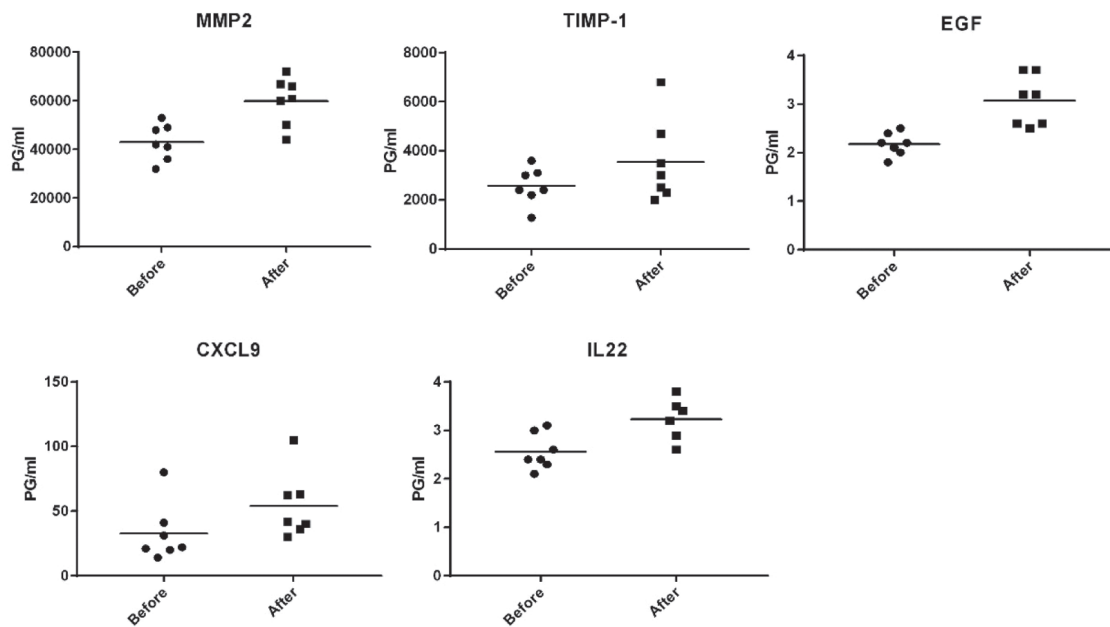


Fig. 3 Hypertonic dextrose prolotherapy treatment of knee osteoarthritis induce up-regulation of MMP2, TIMP-1, EGF, IL-10 and IL-22. Data shown as individual values, n = 12 synovial fluid, from the seven knee OA patients was collected *p < 0.05, comparing parent to pretreatment. EGF = epidermal growth factor.

synovial fluid. A number of immune cells such as macrophages, mast cells, leucocytes, and T cells, which have been found in the synovial tissues of OA patients, are involved in the pathogenesis of OA.²⁶⁻²⁹ RAGE, CA11, and GDF-15 control leucocyte adhesion, mast-cell-modulated inflammation, and macrophage activation, respectively.³⁰⁻³² IL-22, the proinflammatory cytokine, has been demonstrated to modulate inflammatory processes in inflamed and noninflamed synovium from OA patients.³³ We found that hypertonic dextrose increased many cytokines, such as RAGE, CA11, GDF-15, TREM-1, and IL-22, which contribute to the recruitment of inflammatory cells and inflammation.

The biology of OA is also influenced by the T cell-mediated immune response. Both CD4⁺ and CD8⁺ T cells induce inflammation and cartilage degradation.^{29,34,35} Regulatory T cells (Treg cells), suppressor T cells, inhibit the proliferation

of CD4⁺ and CD8⁺ T cells. The Treg cell response is decreased and is involved in the pathogenesis of OA.^{29,36} The accumulation of T cells and the chemoattraction of resting T cells are regulated by cytokines CXCL9,-16, and MPIF-1.³⁷⁻³⁹ Siglec-9 is a critical immunosuppressor that promotes Treg cell differentiation in the pathogenesis of rheumatoid arthritis.⁴⁰ The expected increased inflammation process is induced by hypertonic dextrose. Notably, we found that 25% dextrose not only diminishes the expression of CXCL16 and MPIF-1 but also increases the level of Siglec-9.

A number of growth factors, such as platelet-derived growth factor (PDGF), EGF, TGF-β1, basic fibroblast growth factor, insulin-like growth factor, and connective tissue growth factor, are essential for the growth and repair of ligaments, tendons, and cartilage.²⁴ TGF-β has been shown to trigger cartilage

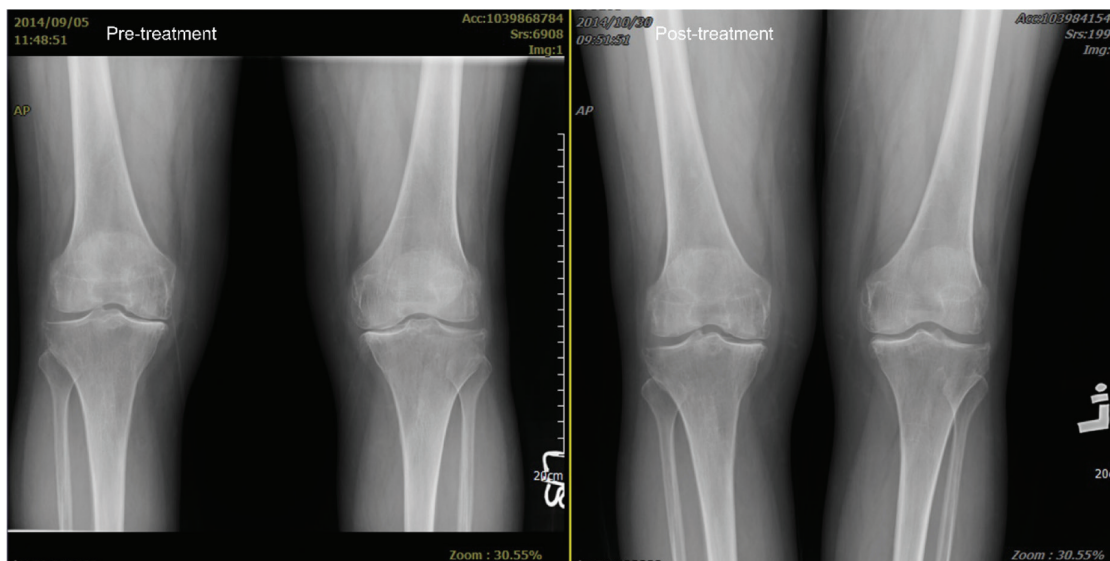


Fig. 4 Pretreatment (left) and posttreatment (right) knee joints X-ray of a 74-year old female.

matrix synthesis and chondrogenesis of bone-marrow-derived mesenchymal stem cells (MSCs) and to promote the repair of cartilage defects.^{41,42} BMP-6, a member of the TGF- β superfamily of cytokines, participates in the maintenance/repair of human articular cartilage.⁴³ EGF, a potent mitogen that augments MSCs and fibroblast proliferation, is involved in the development and healing of tendons and ligaments.⁴⁴ Thyroid hormone is the important regulator for remodeling and maintaining bone and cartilage repair. The concentration of thyroid hormone is modulated by the negative feedback regulation of thyrotropin-releasing hormone and thyroid-stimulating hormone (TSH).^{45,46} Some studies demonstrated that high glucose concentration induces the growth and repair of normal cells and tissues and the production of growth factors and hormones.^{24,47} The results showed that TGF- β , EGF, and TSH are overexpressed with 25% dextrose in synovial fluid.

MMPs, a large group of zinc-dependent endopeptidases, have been classified into six groups: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), matrilysins (MMP-7, -26), membrane-type MMPs (MMP-14, -15, 16, -17, -24, -25), and other nonclassified MMPs (MMP-12, -19, 20, -21, -23A/B, -27, -28). MMP-1, -2, -3, -9, and -13 cleave the components of the extracellular matrix (ECM) and serve as critical mediators of cartilage destruction in OA.^{48,49} The activities of MMPs are downregulated with tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3, and TIMP-4).⁵⁰ MMP-1, -3, -9, and -13-triggered degradation of ECM are suppressed with TIMP-1.⁵¹ Furin, proprotein convertase, has been shown to reduce MMP-13 expression in a TGF- β -dependent manner and to restrict OA in mice.⁵² Besides MMPs, serine proteases also degrade ECM and contribute to articular cartilage destruction in OA. Trappin-2, a small serine protease inhibitor, was found to bind to ECM, resulting in the inhibition of serine protease-mediated degradation of ECM.^{53,54} PAI-1, a serine protease inhibitor, suppresses the degradation of ECM and inhibits osteoclastic bone resorption and subchondral osteopenia after the induction of OA.^{55,56} We found that 25% dextrose enhanced MMP-2 expression, and the protein levels of TIMP-1, EGF, IL-10, and IL-22 were increased in synovial fluid.

Control group in studies for evaluating the clinical therapeutic effects of hypertonic dextrose prolotherapy is mandatory. For investigating the related biomarker mechanism, patients needed to receive multiple knee joint arthrocentesis to collect knee joint synovial fluid in our study. Considering the clinical ethics and the restriction of human study, single-arm without control group protocol was applied in this study. Nevertheless, our study is one of the paucity of researches provided human investigation of prolotherapy in both clinical and molecular biology aspects.⁵⁷ Further cell biology experiment or animal study may be conducted to verify the results.

Following the universal precaution for patient selection and guidance of aseptic injection, the adverse events can be avoided. However, postinjection pain, soreness or fullness sensation at injection sites are common in hypertonic dextrose prolotherapy.⁵⁸ These side effects are self-limited and relieved typically. Patients complain the pain flare post injection during the first 3 days after the injections is quite common.⁵⁹ It is understandable that hypertonic dextrose as a irritant agent will induce local tissue according to the preparation such as concentration, volume and injection protocol. During this treatment course, patients needed to receive five injections, and most of them felt short-term discomfort after each injection. Those factors decreased the willingness of the elderly with knee OA to participate in this research project. This may mean that the clinical application of this treatment protocol is not acceptable for every patient. Although the hypertonic dextrose prolotherapy is effective, multiple injections and postinjection discomfort are still clinical

issues that may lessen patient compliance. This was the reason for our interest, and why we tried to explore the key cytokines related to effective and side-effect mechanisms. For wide clinical application, further studies need to be designed.

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