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Clinical Experience

Phonopheresis Associated with Nanoparticle Gel from *Phyllanthus amarus* Relieves Pain by Reducing Oxidative Stress and Proinflammatory Markers in Adults with Knee Osteoarthritis*

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ABSTRACT **Objective:** To determine the changes in serum levels of inflammatory biomarkers and antioxidant levels among the knee osteoarthritis (OA) patients after treatment with *Phyllanthus amarus* (PP) by nanoparticle gel phonophoresis. **Methods:** This study was a randomized, double-blind, placebo-control, parallel-group, clinical trial involving 30 subjects with mild-to-moderate degree of knee OA. The patients were allocated to two groups using a computer-generated random numbers, and received conventional ultrasound therapy (control group, 15 cases) and PP (treatment group, 15 cases) once daily for 10 sessions. The pain was evaluated by visual analogue scale (VAS). Serum levels of tumor necrosis factor-α (TNF-α) were determined by enzyme-linked immunosorbnent assay (ELISA). Nitric oxide (NO) was determined by modified Griess reagent. The antioxidant effects, including superoxide dismutase (SOD) and total antioxidant capacity (TAC), were also measured by ELISA assay. **Results:** The VAS score was significantly decreased in the treatment group compared with the control group after treatment (P<0.01). The serum concentrations of TNF-α and NO were significantly reduced in the treatment group compared with the control group (P<0.01) after treatment. However, the serum concentrations of SOD and TAC in the treatment group were significantly higher after treatment compared with the control group (P<0.01). **Conclusion:** PP could alleviate knee pain and significantly reduce systemic anti-inflammatory effects in knee OA patients.

KEYWORDS phonopheresis associated with nanoparticle gel from *Phyllanthus amarus*, osteoarthritis, pro-inflammatory cytokines, antioxidants, tumor necrosis factor-α, total antioxidant capacity

Knee osteoarthritis (OA) is one of the most common musculoskeletal disorders.[1] It is characterized not only by cartilage destruction and joint degeneration, but also alteration of bone metabolism and synovial inflammation; affecting mobility and quality of life. There are many risk factors, including age, sex, race, obesity, joint trauma, and genetics.[2] Knee OA is particularly prevalent in elderly women more than men.[3] Mechanisms of systemic inflammation involve the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and IL-8; these are the major causes of pain.[4-6] TNF-α is the pro-inflammatory cytokine most responsible for shifting cartilage homeostasis towards catabolism and degradation. Pro-inflammatory cytokines are involved in the progression of OA pathology and symptoms; they stimulate production of reactive oxygen species and alter antioxidants, which are superoxide dismutase (SOD), and H_2O_2.

Knee OA is generally treated by drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs).[7] However, NSAIDs carry side effects; they affect the gastrointestinal system, including vomiting and peptic ulcers.[8] Other treatments include physical therapy modalities, such as short-wave ultrasound, which is non-invasive, easy to administer, and safe. Ultrasound can also be used in combination with drug therapeutics

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for absorption and permeating the skin, which is called phonophoresis. Many studies have investigated using phonophoresis in combination with NSAIDs to reduce pain in knee OA.[9-11] Kaur, et al[12] studied, both *in vitro* and *in vivo*, the antioxidant and anti-inflammatory properties of *Phyllanthus amarus*, a rich source of polyphenols. These studies suggested that *Phyllanthus amarus* could help alleviate symptoms in OA patients.

The herbal gel or cream is the topical drug applied directly to the skin for treating OA. The topicals drug is acting in part as fewer side effects or toxic when compared the administered orally.[13] The aim of this study was to evaluate the effect of *Phyllanthus amarus* gel *phonophoresis* (PP) on pain score, TNF-α, nitric oxide (NO) and antioxidant status in patients with knee OA.

METHODS

Indusion and Exdusion Criteria

The inclusion criteria were the subjects had knee pain of ⩾5 on the Visual Analogue Scale (VAS) and appeared the joint with at least one of the following features: age ⩾50 years, morning stiffness < 30 min, and crepitus on the motion. All patients presented with the chronic daily pain of the knee for at least 3 months. None of the patients were receiving treatment that might interfere with bone metabolisms, such as estrogen replacement therapy, thyroid replacement therapy, diuretics, or other drugs that might influence the serum biomarkers mentioned above. Patients were excluded if they had received immune suppressive drugs, had been treated with intraarticular sodium hyaluronate injections within the past 3 months, had prior surgery for OA or trauma, had other inflammatory or neurodegenerative disease, or were currently consuming antioxidants.

Patients

Adults with knee OA according to the American College of Rheumatology criteria[14] participated in this study. The sample size required for each group was 15 (power =0.9 and significant level =0.05), which was calculated from a previous study.[15] Thirty patients with knee OA provided informed consent and enrolled between August 2017 and December 2017.

Ethical Approval

This study was approved by the Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Thailand, with approval No. AMSEC-60EX-019.

Treatment Procedures

A randomized controlled trial study design was used. The participants were randomly assigned to groups using a computer-generated random numbers and examined by a physician double-blinded to the group assignment. Patients were divided into a control group and a treatment group. The control group received conventional ultrasound therapy (15 cases); the treatment group received PP (15 cases) from pharmacologist preparation. Both groups received ultrasound therapy (Primo Therasonic 460, EMS Phosio Ltd., United Kingdom) set to a continuous mode (1.0 W/cm^2 power and 1 MHz) once daily for 10 sessions.

The clinical efficacy outcomes were monitored at baseline and after 10 sessions of treatment. Patients need to undergo a skin irritation test before treatment. If skin irritation occurs, then they would refuse to receive the treatment. Moreover, the gel has been placed in different storage conditions at 4 ℃ and 45 ℃ as well as room temperature for 3 months and showed good stability.

Primary Outcome

VAS was evaluated before and after 10 sessions of ultrasound treatment. VAS was measured on a numeric rating scale of 0 to 100 (0, no pain/limitation; 100, very severe pain/limitation), anchored with the extremes of subjective pain.

Secondary Outcomes

Upon collection, blood samples were immediately centrifuged at 4,000 r/min for 10 min at 4 ℃. The serum was then stored at –80 ℃ until analysis.

Determination of TNF-α Level in Serum

TNF-α level was measured using the commercial enzyme-linked immunosorbnent assay (ELISA) kit (Sigma-Aldrich, USA) according to the manufacturer's instructions.

Measurements of NO Production in Serum

NO production was indirectly measured by NO derivatives nitrate and nitrite using NO assay kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. The absorbance was determined with a microplate reader at 540 nm.

Determination of SOD Activity in Serum

The serum SOD activity was measured by the SOD assay kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. The inhibition activity of SOD was determined by a colorimetric method using a microplate reader at 440 nm.

Serum Antioxidant Capacity in Serum

The serum total antioxidant capacity (TAC) was assayed using a colorimetric assay kit (Sigma-Aldrich, USA). The standard Trolox (nmol/well) *versus* optical density curve was used to obtain the sample antioxidant capacity expressed as nmol Trolox equivalent/μL serum.

Statistical Analysis

The statistical analysis was performed using SPSS statistical software (SPSS version 16.0). Results were expressed as mean ± standard deviation ($\bar{x} \pm s$). Data has been checked with Shapiro-Wilk test and showed normal distribution. Statistical analysis was carried out by 2 × 2 repeated measures ANOVA and was followed by Turkey's *post hoc* test. *P* values less than 0.05 were considered statistically significant. All analyses were performed as intention-to-treat (ITT) analysis principle.

RESULTS

Demographic Characteristics

The clinical and demographic characteristics of the studied patients with knee OA were summarized in Table 1. There was no significant difference in demographic characteristics between the control and treatment groups at baseline. The flow chat was shown in Figure 1.

Table 1. Baseline Characteristic of Enrolled Participants in Two Groups ($\bar{x} \pm s$)

Characteristic	Control group (15 cases)	Treatment group (15 cases)
Age (Year)	64.2 ± 8.1	67.1 ± 7.8
Gender (Case, Male/Female)	4/11	4/11
BMI (kg/m^2)	22.3 ± 7.0	23.7 ± 4.9
Duration of symptoms (Year)	2.1 ± 1.2	2.1 ± 1.3
VAS of pain	67.7 ± 8.4	70.3 ± 7.7

Effect of PP on VAS Scores in Patients with Knee OA

The VAS of pain at post-intervention in both groups was significantly reduced compared to pre-intervention (*P*<0.01). Moreover, VAS was significantly lower in the treatment group than the control group after treatment (*P*<0.01, Table 2).

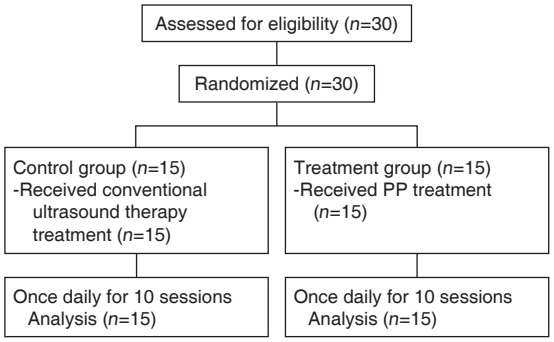

Figure 1. Flow Chat of PP for Treatment of Patients with Knee OA

Table 2. Comparison of VAS Scores between Two Groups ($\bar{x} \pm s$)

Group	Case	Time	VAS score
Control	15	Pre-treatment	67.7 ± 8.4
		Post-treatment	40.3 ± 5.7*
Treatment	15	Pre-treatment	70.7 ± 7.7
		Post-treatment	16.3 ± 6.9*△

Notes: *P<0.01 *vs*. pre-treatment in each group; △P<0.01 *vs*. control group at the same time

Effect of PP on Changes in TNF-α and NO Levels in Patients with Knee OA

Before treatment, the serum levels of inflammatory mediators TNF-α and NO did not differ significantly between two groups (*P*>0.05). After the treatment, the levels decreased significantly in both groups compared to baseline (both *P*<0.01). Moreover, the levels decreased significantly more in the treatment group than the control group (both *P*<0.01, Figures 2A and 2B).

Effect of PP on Antioxidant Status in Patients with Knee OA

The SOD activity and TAC of plasma significantly increased post-intervention in patients in both groups (both *P*<0.01, Figures 2C and 2D). Moreover, SOD and TAC activity after treatment significantly increased more in the treatment group than the control group (both *P*<0.01).

Adverse Effects

No adverse effects were shown during the application of PP.

DISCUSSION

The knee OA is a degenerative disease with chronic inflammation.[16] The systemic inflammation involves the biomarkers TNF-α and NO. Our study revealed that patients with OA had significantly higher

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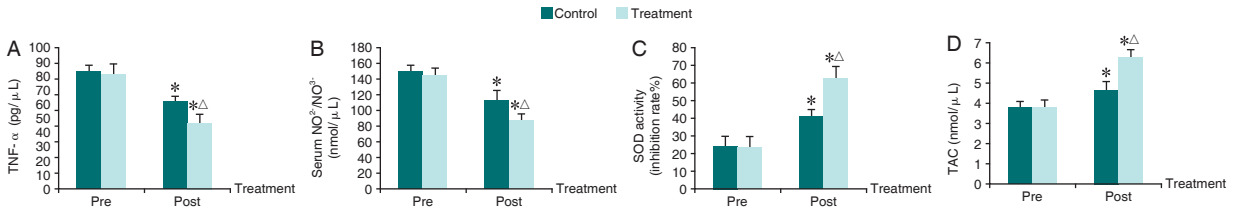

Figure 2. Comparison of Serum levels of TNF-α, Nitrite/Nitrate, SOD Activity and TAC between Two Groups (15 Cases in Each Group, $\bar{x} \pm s$)

Notes: *P<0.01 *vs*. pre-treatment in each group; △P<0.01 *vs*. control group at the same time

serum TNF-α level. We found that PP for 10 sessions had beneficial effects on VAS, TNF-α, NO, SOD, and TAC compared with conventional ultrasound treatments. The plasma NO level significantly decreased compared at baseline. Patients that received phonophoresis treatment showed significantly decreased levels of inflammatory cytokines, which was a result of decreased concentrations of TNF-α which in turn decreased NO production.

TNF-α is driving the inflammatory cascade. Serum TNF-α level is indicative of the degree of articular cartilage damage in the inflammatory phase of OA.[17] TNF-α is a pro-inflammatory cytokine associated with joint inflammation and induces the NO activity.[18] The level of serum NO and TNF-α amounts were increased, as well as OA disease activity. The NO mediates pro-inflammatory cytokines and is primarily a catabolic factor in OA.[19] Antioxidants play an important role in preventing reactive oxygen species formation and in scavenging free radicals. The chronic inflammation with knee OA releases cytokines into the serum, such as TNF-α, that induce NO production.[20] NO reacts with superoxides to generate the peroxynitrite that is found in inflammation[21] and toxic most highly reactive and toxic of the reactive nitrogen species (RNS). Pain relieve is according the change in inflammatory cytokines level. TNF-α is inflammatory which is related to pain symptom in arthithis.[22] This study showed post-treatment decreased inflammatory cytokines and pain score, indicating PP reduced pain and inflammatory cytokine.

The treatment group seems to increase protection against total antioxidant damage by normalizing TAC values. SOD is antioxidant enzyme and it is one of TAC. SOD activity could increase the potential for scavenging free radicals.[23] Our study showed that SOD activity significantly increased after treatment in both groups, with the increase in the treatment group significantly more than in the control group. SOD activity has been shown to reduce inflammation in collagen-induced arthritis.[24] Antioxidant enzyme activity might offer new targets for treating degenerative joint disease.[25]

Arthritis is a chronic inflammation of the joints generated by increased reactive oxygen species (ROS) and RNS levels in serum and synovial fluid.[26] The production of ROS leads to damage of the tissue and cartilage in joints that is associated with OA. Normally, free radicals are scavenged by antioxidants, such as SOD. There are many report that the topical NSAIDs and capsaicin is equally effective for pain relief in OA.[27,28] In the present study, we demonstrated that treatment with PP significantly decreased TNF-α and NO production and increased SOD and TAC in knee OA. This finding implied that NO was a potent mediator of cartilage damage in OA, whereas SOD was an antioxidant mediator of cartilage damage in OA.

This study suggested that PP in knee OA patients inhibited production of NO. OA was reducing the catabolic processes and increasing pain scale. PP may be used as a disease-modifying agent to suppress inflammatory cytokines by reducing production of TNF-α and NO, which are known to play a primary role in the pathophysiology of OA. Moreover, PP also activated SOD activity and TAC, thus inhibiting oxidative stress status in knee OA patients. This study supported the potential therapeutic application of PP in patients with OA knee. However, the limitation of this study should also be considered that it was a short treatment time which was only for 10 days. Further study would suggest for long term follow-up of the intervention to confirm the clinical efficacy. In addition, evaluation of the synovial fluid of joint related study should be involved in the future.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contribution

Conceptualization: P Decha, K Kanokwan. Investigation: P

Decha. Metodology: P Decha, K Kanokwan. Supervisor: T Jiraporn, A Pisittawoot. Writing: P Decha, T Jiraporn, and J Pichaya.

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