MATERIALS AND METHODS

Research design

This study was designed as a randomized, single-blind, placebo controlled trial. The treatment procedures consisted of placebo tablet plus placebo EA (placebo group), diclofenac tablet plus placebo EA (diclofenac group), placebo tablet plus true EA (EA group) and diclofenac tablet plus true EA (combined group). Patients considered to be non-responders in placebo or diclofenac groups were given the opportunity to receive true EA treatment after completion of the treatment previously assigned (partial cross-over design).

Subjects

Two hundred out-patients of either sex, age over 40, suffering from unilateral or bilateral OA of knee according to the criteria of the American College of Rheumatology [4] of more than 3 months duration were recruited. Lequesne's functional index evaluated at the screening visit had to be at least 6 points. Subjects had to be able to walk and were given both verbal and written information regarding the study and signed informed consent was obtained prior to entry. Exclusion criteria included an underlying inflammatory arthropathy, expectation of surgery in the future, recent injury in the area affected by OA of knee, intraarticular corticosteroid injections or EA within the last 3 months, hypersensitivity to NSAIDs or paracetamol, abnormal liver or kidney function test, evidence of leukopenia and coagulopathies screened by clinical laboratory, concomitantly receiving anticoagulants, history of peptic ulceration, anemia, uncontrolled hypertension, congestive heart failure, hyperkalemia, pregnancy, lactation and malignant tumors.

Treatment procedures

1. Drug administration

During a run-in period of one week, the patients refrained from using any NSIADs or analgesics except for "rescue analgesic" (two tablets of 500 mg paracetamol by mouth as needed, up to four times daily). The patients who had persistent pain and

Lequesne's functional index of at least 6 points at the end of the run-in period were randomized into four groups mentioned above. Diclofenac sodium, 25 mg film-coated tablets was a gift from Novartis (Thailand) Limited. Placebo tablets and diclofenac were prepared in identical appearance. Either placebo or diclofenac was prescribed 1 tablet, three times a day immediately after each meal for 4 weeks. In addition, two tablets of 500 mg paracetamol were still prescribed as "rescue analgesic" during this study.

2. True and placebo EA

The true EA treatment was standardized throughout the study. All patients possessed their own needles. The needles were steriled by using autoclave before re-use. The skin covering target points was cleaned with 70% ethyl alcohol before inserting the needles. In this study, true EA was performed by the physician acupuncturist who received acupuncture training in the Republic of China. Four fine stainless needles were inserted into acupuncture points around the affected knee [27] as presented in Table 1 and Figure 1.1. All needles used in order to conduct the electrical current through the points, were inserted superficially (approximately not more than 0.5 inch in depth). Thus, elicitation of needle sensation (so-called De Qi) during inserting needles was not intended. The electrical stimulation was slowly and simultaneously applied to each pair of needles until the maximal patient's tolerability was reached. The electrical stimulation used was biphasic pulses with frequency of 2 Hz (Figure 1.2) and was administered for 20 minutes in each treatment. The patients were treated three times a week (Monday, Wednesday, and Friday) for 4 weeks (12 times).

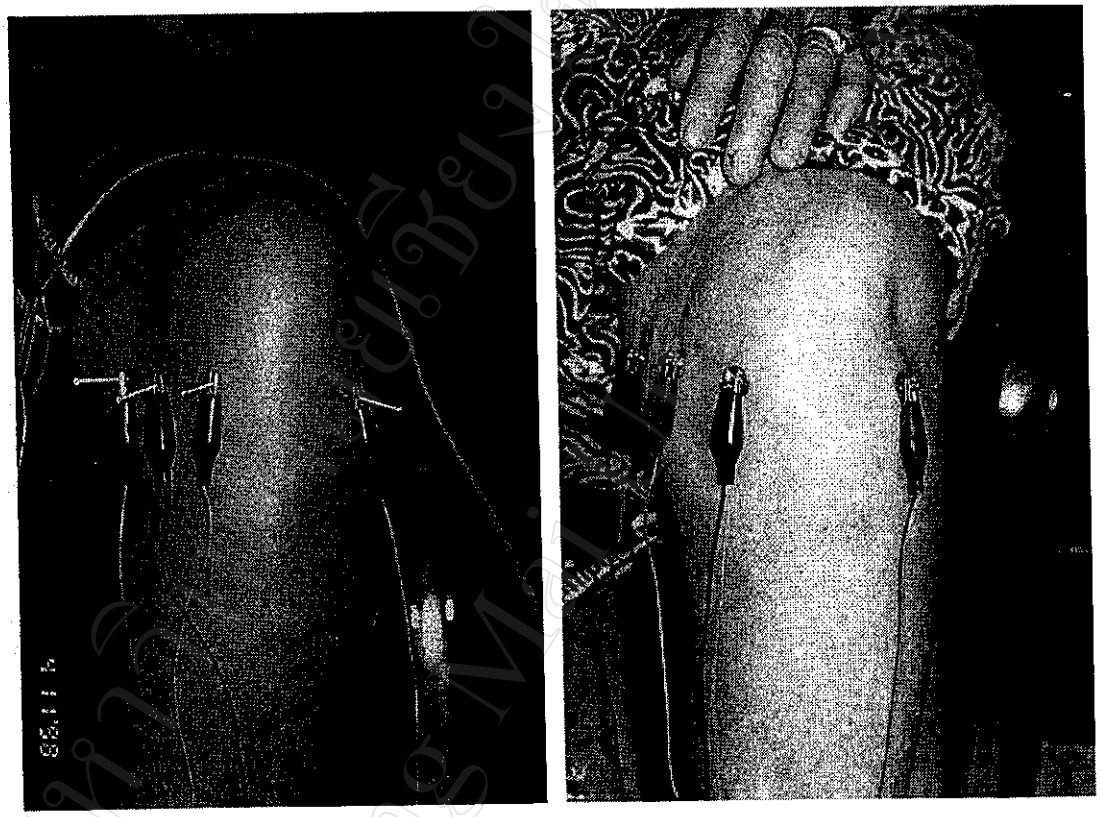
The placebo EA was performed by attaching the selected acupuncture points with the patch electrodes (Figure 1.1). Each electrode was connected to the sound producing dummy mode of the same apparatus as in the true EA treatment. The duration and frequency of treatment were the same as those in the true EA treatment. Both true and placebo EA were performed by the same physician. Thus, the physician acupunctuist was the only person in the research team who knew which patients recieved the true or placebo EA.

Table 1. The selected acupuncture points used in this study.

Acupuncture points	Location	Needling manipulation
Tupi (ST-35)	In the depression of the lateral part of the patella ligament, when the knee bent.	Slightly towards the medial side*.
Medial Hsiyen (Extra)	In the depression of the medial part of the patella ligament, when the knee bent.	Slightly towards the lateral side**.
Ah-shi (Trigger point)	The most painful point usually locates at the joint line between medial Hsiyen and Chuchuan.	Straight insertion*.
Chuchuan (Liv-8)	At the medial end of the knee crease, in front of the semi-membranous muscle behind the lower end of femur.	Straight insertion**.

* Stimulated with positive polarity at visit #1, 3, 5, 7, 9, 11 and negative polarity at visit #2, 4, 6, 8, 10, 12.

** Stimulated with negative polarity at visit #1, 3, 5, 7, 9, 11 and positive polarity at visit #2, 4, 6, 8, 10, 12.



A B

Figure 1.1. The selected acupuncture points around left knee, demonstrated in sitting position, during treatment with true (A), and placebo EA (B).

Figure 1.2. The pattern of electrical pulses used in EA treatment.

Compliance with treatments was assessed by counting the number of unused tablets (diclofenac or placebo) and the number of acupuncture treatment. During study period, all other additional therapies (e.g., oral or topical NSAIDs, intraarticular corticosteroids injection, other analgesics, chondro-protective agents, surgical procedures on knee joint, etc.) were not allowed. However, all other treatments for concomitant disorders could be continued, but had to be documented.

Assessments

Clinical assessments were evaluated at the end of run-in period (week 0) for base-line data and again weekly during treatment for four week (at the end of week 1, 2 ,3 and 4). These assessments included the amount of paracetamol tablets taken/week, a patient's global pain over the previous 3 days 100 mm visual analog scale (VAS), the Western Ontario and McMaster Universities OA Index (WOMAC: score ranging from 0-96) [71], Lequesne's functional index (score ranging from 0-24) [72], the orthopedist's and the patient's opinions of change (much better, better, same, worse, much worse), and 50 feet-walk time. In addition, the number of responders was evaluated each week. In this study, the subjects were considered to be responders if: (1) the number of paracetamol tablets taken in evaluated week was less than that at week 0, or less than 14 tablets/week, and (2) at least 4 of the following 5 items: VAS, WOMAC, Lequesne's functional index decreased by at least 50%, the orthopedist's or the patient's overall opinions of change were better or much better. Clinical assessments in each subject were evaluted by the same physician who was blinded. In addition to clinical assessments, the serum concentrations of cartilage markers were measured at the end of week 0, 1, 2, 3 and 4, respectively. Complete physical examination and non-directive questioning for adverse events were also performed weekly for four weeks for safety assessment.

Quantitative measurement of serum markers of cartilage metabolism

The serum markers of cartilage metabolism used in this study were HA and CS 3-B-3 epitope. In addition, the novel monoclonal antibody W-F-6 which recognizes a native epitope in CS was evaluated together with 3-B-3 monoclonal antibody which recognizes unsaturated terminal CS after chondroitinase ABC digestion.

An ELISA based assay for HA using biotinylated HA-binding proteins

A simple assay for determining HA in human serum was developed using biotinylated HA-binding proteins [73]. Human serum samples, or Healon®(standard HA) at various concentrations (19-10,000 ng/ml in 6% BSA-PBS, pH 7.4), were added to 1.5 ml plastic tubes containing biotinylated HA-binding proteins (1:200 in 0.05M Tris-HCl buffer, pH 8.6). The tubes were incubated at room temperature for 1 hour and samples (triplicate of 100 µl/well) were then added to the microplate which was previously coated with HA 10 µg/ml, 100 µl/well and blocked with 1% BSA (150 µl/well) and the plate was incubated at room temperature for an additional 1 hour. The wells were then washed 3 times with PBS-Tween 20 buffer and 1:2,000 dilution of peroxidase conjugated anti-biotin antibody (100 µl/well in PBS) was added. The plate was incubated at room temperature for a further 1 hour. The bound antibody was detected by adding O-PD substrate (100 µl/well in citrate buffer, pH 5.0). The reaction was stopped with 50 µl/well of 4M sulfuric acid and the absorbance was determined using a microplate reader at 492/690 nm. The amount of HA in samples were calculated against standard curve. This method showed a high correlation coefficient (r=0.856) with the commercially available kit for HA (Pharmacia Radioassay Kit) (data not shown). The intra- and inter-assay variations were 7% and 8%, respectively.

A competitive immunoassay technique using monoclonal antibody 3-B-3

A quantitative ELISA was modified from an assay in synovial fluid for the epitopes recognized by monoclonal antibody 3-B-3 [74]. Prior to competitive immunoassay, the serum samples were digested with chondroitinase ABC (equal volume of 0.1 U/ml in chondroitinase ABC buffer : 0.1M sodium acetate, Tris-HCl, pH 7.3), incubated at 37 $^{\circ}$C for 16 hours (overnight) followed by heating at 100°C for 10 minutes. The digested samples were centrifuged in microfuge for 10 minutes and the supernatants were collected and kept frozen until they were analysed. The standard used was porcine aggrecan core protein (chondroitinase ABC-digested porcine laryngeal cartilage aggrecan) [75] at various concentrations (4-2,000 ng/ml) in TE buffer (0.1M Tris HCl, pH 7.4, 0.15M sodium chloride, 0.1% Tween 20 and 0.1% BSA). The standard samples or supernatants from chondroitinase ABC digested human serum samples were

added to the 1.5 ml plastic tubes containing an equal volume of 1:10,000 (in TE buffer) of monoclonal antibody 3-B-3. Samples were incubated at 37°C for 1 hour, added to the microplate coated with porcine aggrecan standard, blocked with 1% BSA (triplicate of 100 µl/well) and incubated at 37°C for an additional 1 hour. The wells were washed 3 times with TE buffer and peroxidase conjugated anti-mouse IgM antibody was added (100 µl/well of 1:1,000 in TE buffer) and incubated at 37°C for a further 1 hour. The detection of bound antibody with O-PD substrate and plate reading was carried out as described above. The amount of 3-B-3 epitope in supernatant samples was calculated from the standard curve. The intra- and inter-assay variations were 8% and 10%, respectively. When a known amount of this standard inhibitor was added to a pooled human serum sample the detection of epitope in the assay was 92.3% of the expected amount.

A competitive immunoassay using monoclonal antibody W-F-6

A quantitative ELISA was modified from a previous study [76] for the epitopes recognized by monoclonal antibody W-F-6. The standard used was shark cartilage aggrecan (A1D1 fraction) at concentrations of 19-10,000 ng/ml in 6% BSA in TE buffer. The standard samples or diluted human serum samples (1:5 in 6% BSA-TE) were added to 1.5 ml plastic tubes containing an equal volume of monoclonal antibody W-F-6 (1:200 dilution in TE buffer). They were incubated at 37°C for 1 hour, then added to the microtitre plate which was previously coated with shark aggrecan, and blocked with BSA (triplicate with 100 µl/well). The plates were incubated at 37°C for an additional 1 hour and the wells were then washed 3 times with TE buffer and peroxidase conjugated anti-mouse IgM antibody (1:2,000) was added (100 µl/well; in TE buffer). The plate was incubated at 37°C for another 1 hour. The detection of bound antibody with O-PD substrate and plate reading was carried out as described above. The amount of W-F-6 epitope in samples was calculated from the standard curve. The intra- and inter-assay variations were 8% and 15%, respectively. The recovery of epitope was 104% when a known amount of standard was added.

Statistical analysis

Statistical analysis among the four groups

A non-parametric Kruskal Wallis test was used to determine whether the four treatment groups differed in their median values of the VAS, WOMAC, Lequesne's functional index. If there was any statistical significance among four groups, Dunn's multiple comparison was used to demonstrate statistical significance between each two groups. In contrast, one-way analysis of variances (ANOVA) was used to determine whether the four treatment groups differed in the magnitude of changes from baseline in paracetamol consumption, 50 feet-walk time and serum concentrations of HA and CS epitopes. If there was any statistical significance among the four groups, Scheffe method was used to demonstrate statistical significance between each two groups. Differences among the treatment groups in the overall opinions of change and the number of patients considered to be the responders were evaluated by chi-square or Fisher's exact test.

Statistical analysis within group

A non-parametric Wilcoxon's match paired test was used to determine the statistical significance of the differences in the median values of VAS, WOMAC, Lequesne's functional index between baseline and subsequent assessment points in each treatment group, whereas the differences in mean values of paracetamol tablets taken, 50 feet-walk time and serum concentrations of HA and CS epitopes between baseline and subsequent assessment points were evaluated by paired t-test.