

Research Paper

**EFFICACY OF PHONOPHORESIS THERAPY IN FREUND'S ADJUVANT
INDUCED ARTHRITIC RATS**

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Abstract

The effect of phonophoresis was assessed using Freund's complete adjuvant (FCA) induced arthritis in rats. *Plumbago zeylanica* (0.5g) root extract was prepared to obtain 0.5% gel. The animals were divided into four groups. Group I serves as normal. Group II to IV served as arthritic animals induced by FCA. Group III and IV treated with ultrasound and phonophoresis (Application of ultrasound along with *Plumbago zeylanica* root extract gel) respectively. Group II served as arthritic animals (Control). The phonophoresis has been conducted through Ultrasound device, one session per day for 3 weeks. A significant ($P < 0.05$) inhibition of paw edema volume was observed from day 4th to 21st in the treated groups. The biochemical parameters like erythrocyte sedimentation rate (ESR), alkaline phosphatase (ALP), acid phosphatase (ACP), malondialdehyde (MDA), reduced glutathione (GSH), rheumatoid factor (RF) and total WBC count was observed which are the major markers of arthritis. A significant increase in the level of all the markers was found in the arthritic rats. Ultrasound and phonophoresis (Application of ultrasound along with *Plumbago zeylanica* root extract gel) treated groups decreased in the level was observed. Phonophoresis treated groups significant decreased the above markers as compared with ultrasound treated rats. These results indicate that application of phonophoresis to arthritic rats acquire potential anti-arthritic therapy. Key words: *Plumbago zeylanica*, Phonophoresis, Ultrasound, Arthritis, Oxidative stress, Freund's complete adjuvant.

INTRODUCTION

Physiotherapy has great potential to play a vital role in ortho, sports, neuro as well as cardio and also the prevention of injury and developing a particular skill for an athlete in the specialized field. Proper diagnosis, choosing the appropriate modalities and applying the perfect methods are the pillars of the successful treatment [1,2]. So choosing the appropriate modality is the key to produce good results. Many drugs are poorly absorbed through the skin by passive diffusion alone. The use of topical agents often requires vehicle formulations or chemical penetration enhancers that are potential irritants or sensitizers. Phonophoresis, the use of ultrasound to enhance the percutaneous absorption of drugs, was first reported by [3]. They demonstrated successful treatment of polyarthritis of the hand by driving hydrocortisone ointment into the inflamed area with ultrasound. The term ultrasound refers to sound waves with frequencies beyond the human audible range of 20 kHz [4].

Typically arthritis is a common inflammatory joint disease characterized by inflammation of the synovial membrane, pain and restricted joint movement [5]. Rheumatoid arthritis (RA), one of the commonest autoimmune diseases, is a chronic, progressive, systemic inflammatory disorder affecting the synovial joints and typically producing symmetrical arthritis that leads to joint destruction, which is responsible for the deformity and disability. The consequent morbidity and mortality has a substantial socio-economic impact [6]. The prevalence of arthritis is approximately in the West [7]. The prevalence of RA in India subcontinent is 1.5-2 percent of population. The epidemiological ratio of arthritis in female and male is 3:1 and the prevalence is 1% of the world population. Adjuvant induced arthritis in rats is a chronic inflammatory disease characterized by infiltration of synovial membrane in association with destruction of joints resembles RA in humans [8]. Presently many non steroidal, steroidal and immunosuppressive drugs are used to control inflammatory symptoms and pain; they are associated with certain undesirable side effects. With these difficulties, the field of arthritis research has progressed exponentially towards herbal therapies that have been considered safe and effective in all elevating chronic pain associated with arthritis [9].

Natural products from plant, animal and minerals have been the basis of the treatment of human disease. Today estimate that about 80 % of people in developing countries still relays on traditional medicine based largely on species of plants and animals for their primary health care. The use of herbal medicine becoming popular due Medicinal plants play an important role in the development of potent therapeutic agents. There are over 1.5 million practitioners of traditional medicinal system using medicinal plants in preventive, promotional and curative applications [10]. Agents derived from plants that can modulate the expression of pro-inflammatory signals clearly have potential against arthritis. These include flavonoids, terpenes, quinones, catechins, alkaloids, anthocyanins and anthoxanthins, all of which are known to have anti-inflammatory effects. Some of these polyphenols, which have been tested for the treatment of arthritis [11]. The medicinal value of chosen plant *Plumbago zeylanica* root belonging to the family of Plumbaginaceae. Therefore, the present study was to investigate the anti-arthritic activity of ultrasound and phonophoresis therapy in Freund's adjuvant induced arthritic rats.

MATERIALS AND METHODS

Chemicals

Complete Freund's adjuvant was obtained from Sigma Aldrich (Saint Louis, Missouri, USA) and Trichloro acetic acid, Ethylenediamine tetra acetic acid (EDTA), Glutathione and Thiobarbitric acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Animals

Male rats were obtained from the Sri Venkateshwara Enterprises, Bangalore 560 021, India. The animals were housed in polypropylene cages. The cages were lined with paddy husk which was replaced every day. Rats were fed with pelleted food and water was provided through plastic bottles. All the rats used in the experiments were marked by tail marking growth of the animals was monitored regularly and rats showing poor growth rate were discarded from the experiments.

Collection of plant

The root of *Plumbago zeylanica* were collected from Thanjavur, December 2010, Tamil Nadu, South India. The collected leaves were identified and authenticated by a Botanist Dr. M. JEGADEESAN, Prof. and Head, Department of Environmental and Herbal Sciences, Tamil University, Thanjavur, Tamil Nadu. A Voucher specimen (TUH: 194) has been deposited at Tamil University Herbarium. The plants were cut into small pieces and shade dried and powdered finely then used for extraction.

Preparation of plant extract

The collected plant materials were washed, sliced and completely dried in a hot-air oven at 37°C. The dried materials was ground into make a fine powder and used for extraction. Three hundred grams (300g) of the powered plants were extracted with ethanol (70%) using "Soxhlet Apparatus" for 24 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The extract was stored in refrigerator until used.

Preparation of Gel base ointment

0.5g of *Plumbago zeylanica* root extract was weighed, dispersed in gel with mild stirring and allowed to swell for 5 minutes to obtain 0.5% gel.

Freund's Complete Adjuvant induced Arthritic Model

Adult Wistar male rat with an initial body weight of 180 to 220g were taken, and divided into four groups each containing six animals. Group I served as normal rats. On day zero, group II to IV rats were injected into the sub plantar region of the left hind paw with 0.1ml of Freund's complete adjuvant. This consists of *Mycobacterium butyricum* suspended in heavy paraffin oil by thorough grinding with motor and pestle to give a concentration of 5mg/ml (This dose confirmed in our lab followed by different concentrations (1 to 10mg/ml)). Application of ultrasound and phonophoresis based ointment treated to group III and Group IV rats respectively were started on the first day and continued for 21 days. Group II rats served as control rats (arthritis rats). The gel based plant extract of phonophoresis has been conducted through Ultrasound device, one session per day for 3 weeks. The rats were holding on comfortable position, then clean and hydrate the body part under treatment. The ultrasound device treated on paw edema sites. Adjust the US frequency to 1.5MHz, with intensity 1.5 W/cm² and the time of treatment was 5 min. For group III, the rats were applied the gel based ointment to the selected area once daily.

The degree of inflammation was measured by a mercury displacement method. The edema formation and the percentage of inhibition were calculated as follows.

$$\text{Percentage of inhibition} = \frac{V_c - V_t}{V_c} \times 100$$

Where V_c is the edema volume of the control group and V_t is the edema volume of the treated group.

Collection of blood and preparation of serum sample

At the end of the experimental period, the animals were anaesthetized using chloroform vapour prior to dissection. Blood samples were collected from the tail vein into a micro centrifuge tube containing 50mM ethylenediamine tetra acetic acid (EDTA) for the determinations of hematological profile. The blood was collected without EDTA to other test tubes. The blood was allowed to clot by standing at room temperature for 30 minutes and then refrigerated for another 30 minute. The resultant clear part was centrifuged at 3000rpm for 10minutes, and then the serum (supernatant) was isolated and stored at refrigerated until required for analysis.

Biochemical estimations

MDA released from endogenous lipoperoxides, reflecting the lipid peroxidation process, were assayed [12]. The levels of non-enzymatic antioxidant GSH was estimated by the method of Moron [13]. Acid phosphatase (ACP) and Alkaline phosphatase (ALP) activities were measured according to the method described by Annon [14] and King and King's [15]. ESR sedimentation rate and WBC counted by the method of Ochei and Kolhatkar [16].

Rheumatoid factor

The latex turbidimetry method was used in the present study using RF turbilatex kit of SPINREACT Company. Calibration was carried out for linear range up to 100 IU/ml the reading of RF factor of all the groups obtained was compared with the control animals. Values were expressed as IU/ml.

Statistical Analysis

Statistical analysis is performed using SPSS. Data are expressed as mean \pm SD and statistically assessed using one-way ANOVA followed by Tukey test; $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Ultrasound (US), which is a deep tissue heating modality, can elevate tissue temperature. The physiologic response due to ultrasound therapy includes increased collagen tissue extensibility, pain threshold and enzymatic activity, along with changes in nerve conduction velocity and contractile activity of skeletal muscle [17]. Recent evidence-based guidelines conclude that the therapeutic US was effective in the treatment of calcific tendonitis of the shoulder[18].

The purpose of the study is to screen and evaluate anti-arthritic activity using phonophoresis technique. Evaluation of anti-arthritic activity of phonophoresis therapy (Application of ultrasound along with *Plumbago zeylanica* root extract gel) and ultrasound was studied on Complete Freund's Adjuvant (CFA) induced arthritis in Wistar strain albino rats. The choice of the animal strain has been found to be very important for the performance of this test. Wistar-strain rats have been proven to be very suitable in contrast to other sub strains [19].

Inflammation and tissue injury related oxidative stress has been implicated in the pathogenesis of rheumatoid arthritis. Free radicals are enormously produced at the site of inflammation and tissue injuries [20]. Lipid peroxides that are generated at the site of

inflammation of tissue injury diffuses into blood and can be estimated in serum or plasma, which in turn reflect the severity of the tissue damage. Susceptibility of erythrocytes to peroxide stress is increased in several diseased conditions [21]. Thus, the elevated plasma lipid peroxidation observed in the present study in Freund's Adjuvant (FA) induced arthritis can be related to excessive lipid peroxidation observed in erythrocytes and erythrocyte membranes, with consequent leakage into plasma or as a result of excessive generation and diffusion of lipid peroxides from the inflamed or injured joints of rheumatoid arthritis. In the present study, we have observed a multidirectional change of non enzymatic antioxidants as compared to control rats (Table 2). Reduced glutathione is a well known antioxidants, play an important role in protecting the lipids of lipoproteins and other biomembranes against peroxidative damage by intercepting oxidants before they can attack the tissues [22]. Lower concentration of vitamin E has been reported in the joint fluid of Freund's adjuvant (CFA) induced arthritis. An inverse relationship between lipid peroxidation and non enzymatic antioxidants has been well documented[23]. Hence, the decrease in plasma non enzymatic antioxidants can be correlated to impairment in the antioxidant defence mechanism, due to excess utilization by the inflamed tissues to scavenge the excessive lipid peroxides that are generated at inflammatory sites, or to scavenge accumulated lipid peroxides in plasma. Phonophoresis therapy decreased lipid peroxidation and increased reduced glutathione content in Freund's adjuvant (FA) induced arthritis.

Table-1: Effect of ultrasound and phonophoresis on paw volume in Freund's adjuvant induced arthritis in experimental rats

Days	Group I	Group II	Group III	Group IV
Day 4	-	5.13±0.07	4.56±0.07 *	4.21±0.05*
Day 8	-	5.23±0.09	3.54±0.04 *	3.42±0.09*
Day 15	-	5.58±0.10	2.84±0.12 *	2.56±0.18*
Day 21	-	5.69±0.11	1.78±0.08 *	1.05±0.14*
% inhibition of paw swelling on 21 st day	-	-	60.96	75.05

Values were expressed as mean ± SD for six rats in each group.

* Significantly different from Group II *p< 0.05

The determination of paw swelling is apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and the therapeutic effects of drugs. The Freund's adjuvant model is chosen as it develop chronic swelling in multiple joints with influence of inflammatory cells with erosion of joint cartilage and bone destruction. Chronic inflammation involves the release of number of mediators like cytokines (IL-1B and TNF-alpha), GM-CSF, interferon's and PGDF. These mediators are responsible for the pain, destruction of bone and cartilage that can lead to severe disability [24]. On the 21st day, a significant decrease in edema volume was observed in phonophoresis therapy as compared to the FA injected control rats (Table 1).

Table-2: Effect of ultrasound and phonophoresis on Biochemical markers in Freund's adjuvant induced arthritis in experimental rats

Parameters	Group I	Group II	Group III	Group IV
MDA (nmol MDA formed/l)	11.81±0.80	20.00±1.36 [#]	15.63±0.92 [*]	13.63±0.92 [*]
GSH (mg/dl)	23.18±1.57	14.36±1.11 [#]	19.63±1.26 [*]	20±1.36 [*]
ACP (IU/L)	31±2.10	67±4.55 [#]	39±2.65 [*]	31±2.10 ^{**}
ALP (IU/L)	72±4.89	92±6.25 [#]	80±5.44 [*]	69±4.69 ^{**}
RF (IU/ml)	8.21±0.55	48.46±3.2 [#]	24.22±2.43 [*]	13.66±0.92 ^{**}
WBC (cu.mm)	3.1±0.21	4.6±0.31 [#]	3.8±0.25 [*]	3.3±0.29 [*]
ESR (mm)	15.30±1.04	22.30±1.51 [#]	19.12.4±1.38 [*]	18.4±1.31 [*]

Values were expressed as mean ± SD for six rats in each group.

[#] Significantly different from Group I, III and IV

^{*} Significantly different from Group II ^{*}p< 0.05

^{**} Significantly different from Group II and III ^{*}p< 0.05

Increased white blood cell counts are a common feature of inflammatory reactions, especially those induced by microbial infection. So in arthritic group an increase in total leukocyte number was found. A significant reduction in total leukocyte number was found in case of ultrasound and phonophoresis therapy groups (Table 2). In our study, it was found that phonophoresis therapy leads to inhibition of leukocyte migration which may have beneficial effect for joint preservation. The activity may be due to presence of steroidal glycoside of plant extract.

Erythrocyte sedimentation rate (ESR) in the FCA treated group several fold high compared to ultrasound and phonophoresis therapy groups (Table 2). This may be due to the flavonoid content of the *Plumbago zeylanica*. These flavonoids are having the surface charge neutralizing effects. ESR is strongly related with the ability of red cells to aggregate into orderly stacks or rouleaux. Proteins are thought to affect the repellent surface charges on red cells and cause them to aggregate into rouleaux and hence the sedimentation rate increases [25]. The rate of sedimentation was increased in arthritis control where as in case of treated groups, the ESR level was significantly decreased.

Serum rheumatoid factor (RF) is the immunological expression of an individual's immune system reaction to the presence of an immunoglobulin molecule that is recognized as "non-self." This response to the "non-self" immunoglobulin results in the presence of immune complexes. These, in turn, bind complement and may eventually lead to synovium, cartilage, and bone destruction. Higher the levels of serum rheumatoid factor, higher are the development of inflammation [26]. Phonophoresis treated animal showed significantly lesser serum RF when compared to disease control animals (Table 2).

Lysosomes are membrane enclosed cytoplasmic organelles, which possess an acidic interior that contain many hydrolytic enzymes. Lysosomal enzymes are widely distributed in tissue and circulating blood cells and are responsible for intracellular breakdown of complex macromolecules. They also degrade endothelial membrane glycol-conjugates. The altered enzyme activities in arthritis can be regarded as an index of lysosomal enzyme activation occurring in response to metabolic need of degrading various constituents of cells such as mucopolysaccharides and glycoproteins accumulated in tissue due to arthritis associated with vasculopathies [27].

Acid phosphatase (ACP) seem to be an important index for the examination of the integrity of the lysosomal membrane and are responsible for the tissue damage and necrosis of hepatic tissue. Cytoplasmic cellular enzymes, such as alkaline phosphatase (ALP) membrane bound indicator of type II cell secretory activity or the lysosomal enzyme β -glucuronidase, an indicator of phagocytic activity, can also be used as sensitive markers of cellular integrity and cellular toxicity induced by pathological conditions. A significant ($P < 0.01$) reduction of ALP level was observed after phonophoresis therapy (Table 2).

Increased activities of plasma ACP were observed in arthritic rats. This may be attributed towards persistent inflammation. These changes are in agreement with the decreased lysosomal stability in adjuvant induced arthritis [28]. In the present study, the activity of lysosomal enzymes in plasma was markedly increased in the adjuvant induced arthritic rats and significantly ($P < 0.05$) reduced after phonophoresis therapy (Table 2). An important mechanism of antiarthritic activity is the membrane stability modulating effect[29]. The phonophoresis therapy may exert its effects by modifying the lysosomal membrane in such a way that it is capable of fusing with the plasma membrane and thereby preventing the discharge of acid hydrolase or by inhibiting the release of lysosomal enzymes[30]. The activity is probable due to presence of flavonoids.

The result of the present experiment indicates that phonophoresis therapy possesses significant antiarthritic activity as compared with ultrasound application. The possible mode of action of anti-arthritic activity of phonophoresis therapy appears to be enhanced membrane permeability and inhibit the inflammatory reactions by scavenging of pro-oxidant and improving anti-oxidant parameters. The potential phonophoresis therapy might be due to various ingredients in *Plumbago zeylanica* extract acting synergistically and working in concert for overall antiarthritic activity.

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