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Role of phyto-stabilised silver nanoparticles in suppressing adjuvant induced arthritis in rats

Aparna Mani [a,*], C Vasanthi [a], V Gopal [b], Darling Chellathai [a]

[a] Department of Pharmacology, Sri Ramachandra Medical College and Research Institute, Porur, Chennai, India
[b] Department of Pharmacognosy, College of Pharmacy, Mother Theresa Post Graduate & Research Institute of Health Sciences, Puducherry, India

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ABSTRACT

Aim of the study: The present study was aimed to evaluate the anti-arthritic effects of silver nanoparticles synthesised using *Piper nigrum* extract and to further establish its mechanism of action in a rat model of adjuvant induced arthritis (AA).
Materials and methods: Adjuvant arthritis was induced by injecting complete Freund's adjuvant (0.1 mL) into the left hind paw of 36 albino Wistar rats (n = 6). Silver nanoparticles stabilised with *Piper nigrum* extract (25 and 50 mg/kg). Commercial silver nanoparticles (50 mg/kg) and methotrexate (0.1 mg/kg) were administered by intraperitoneal route from day 11 to day 22 on alternate days.
Results: It was found that treatment with silver nanoparticles stabilised with *Piper nigrum* (S-AgNPs) significantly reduced the paw edema and alleviated the histopathological changes of cell infiltration, synovial hyperplasia, bone and cartilage destruction. Furthermore, the phytostabilised silver nanoparticles (S-AgNPs) inhibited the protein expression of NF-kβ p65 and TNF-α as evidenced by immunohistochemistry analysis.
Conclusion: Our current findings suggest that silver nanoparticles stabilised with *Piper nigrum* extract (S-AgNPs) have potent anti-arthritic activity which is mediated by inhibition of TNF-α and suppression of pro-inflammatory cytokines that are secreted in response to activated transcription factors of NF-kβ.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder which leads to progressive destruction of the joints. The disease is characterized by inflammatory cellular infiltrates and synovial hyperplasia eventually leading to cartilage erosion and articular damage [1]. The pathogenesis of RA is contributed predominantly by pro-inflammatory cytokines like TNF-α and interleukins. TNF-α stimulates the production and release of chemokines and metalloproteinases from synoviocytes thereby inducing inflammation, hyperplasia and joint destruction [2]. NF-kβ p65 is a major transcription factor that regulates the genes that contribute to inflammation including TNF-α, IL-6, IL-8, inducible nitric oxidase synthase (iNOS) and cyclooxygenase-2 (COX-2). Furthermore, stimulation of NF-kβ suppressed apoptosis of synoviocytes thereby promoting synovial hyperplasia [3]. Thus, targeting TNF-α and NF-kβ can serve as an effective therapeutic modality.

The current treatment options for RA do not provide complete remission and are often associated with serious systemic side effects thereby compelling patients to opt for alternative medicine [4]. The use of modern technologies combined with natural products may pave way for better treatment strategies with enhanced potency and minimal toxicity. Nanomedicine is one such emerging field which offers targeted therapy for various inflammatory disorders including arthritis. Among the several types of nanoparticles, silver nanoparticles have been widely used for its potential medical benefits. Since ancient times, silver has been used for the treatment of inflammation [5] and previous studies have reported the anti-inflammatory effects of silver nanoparticles in other inflammatory models [6]. The synthesis of silver nanoparticles using plant extracts has been well established to have potential benefits as the phytochemicals are capable of reducing and stabilising the silver nanoparticles and also enhancing the therapeutic benefits [7].

Black pepper (*Piper nigrum*) is a commonly used spice in India, which is known to have anti-inflammatory, carminative and anti-flatulent properties. Botanically, the pepper corns are the unripe, dried fruits of *Piper nigrum* belonging to family of Piperaceae [8]. In our previous study we have reported the facile and eco friendly synthesis of phytostabilised silver nanoparticles using the aqueous extract of black pepper, and its in-vitro anti-inflammatory activity [9].

In the present study the anti-arthritic effect of silver nanoparticles synthesised using aqueous extract of unripe dried fruits of *Piper nigrum* (S-AgNPs) was evaluated using the adjuvant arthritis model in rats and the anti-arthritic activity of phytostabilised silver nanoparticles were compared with the commercial silver nanoparticles (C-AgNPs)

* Corresponding author at: Dept of Pharmacology, Sri Ramachandra Medical College and RI, Porur, Chennai 600116, India.
E-mail address: draparnacmc@gmail.com (A. Mani).

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synthesised using chemical methods and *Piper nigrum* aqueous extract (PN extract). Further, the present study evaluates the role of S-AgNP in ameliorating adjuvant induced arthritis by targeting TNF-α and NF-kβ p65. Perhaps, this is the first study to explore and elucidate the possible mechanism of the anti-arthritic effects of silver nanoparticles synthesised using *Piper nigrum* extract.

2. Materials and methods

2.1. Drugs and chemicals

Silver nanoparticles, methotrexate and complete Freund's adjuvant were purchased from Sigma-Aldrich Co.

2.2. Synthesis and characterization of silver nanoparticles

The aqueous extract of unripe dried fruits of *Piper nigrum* was prepared by decoction method [10]. The pH of the extract was adjusted to 9 using NaOH and 10 mL of the extract was added to 90 mL of 1 mM $AgNO_3$ solution. The solution was kept for 2 h on a magnetic stirrer, to allow for the complete reduction and stabilisation of silver nanoparticles. The solution was centrifuged and the pellet was used for characterization studies.

The silver nanoparticles synthesised using *Piper nigrum* extract (S-AgNPs) were characterized using UV-spectroscopy, HR-TEM, Zeta potential and DLS analysis.

2.3. Animals

The study was approved by the Institutional Animal Ethics Committee, Sri Ramachandra University (No-IAEC/XXXIV/SRU/290/2013). A total of 36 male Wistar rats weighing 150–200 g were purchased from Biogen, Bangalore. The animals were housed under standard laboratory conditions of temperature 24 ± 2 °C, relative humidity 50 ± 10% and 12 h dark-light cycles. The animals were fed with pellet diet and water ad libitum. All the animals were allowed to acclimatize for 5 days prior to initiation of the procedure.

2.4. Induction of adjuvant arthritis

Before starting the experiment, the animals were randomized into 6 groups, containing 6 animals each based on the body weights. Arthritis was induced by subcutaneous injection of 0.5 mL CFA into the left hind paw (day 0). Ten days after inoculation, the animals were randomized again into 7 groups based on the severity of the paw edema. Group 1 was control (no adjuvant, no treatment), Group 2-vehicle treated (2% CMC), Group 3-standard treated (methotrexate 0.1 mg/kg) [11], Group 4-commercial silver nanoparticles 50 mg/kg (C-AgNPs), Group 5-(PN extract 100 mg/kg) and Groups 6 & 7-phytostabilised silver nanoparticles at 25 mg/kg and 50 mg/kg (S-AgNP-25 & S-AgNP-50). The standard and test drugs were administered by ip route on alternate days starting from day 10 to day 22.

2.5. Arthritic assessment

The paw volumes were measured using a water plethysmometer on day 0 prior to CFA injection and also on days 13, 16, 19 and 22 following CFA injection. At each time point, the average of two measurements for each rat was used. The gait score was used to assess the severity of arthritic pain and each animal was given a score of 0 to 3, where 0-normal gait, 1-slight lameness, 2-lameness with weight bearing on toes only, and 3-non weight bearing animals [12]. The investigators performing the measurements were blinded to the assignment of treatment groups in order to avoid bias.

2.6. Detection of tissue TNF-α and NF-kβ p65 by immunohistochemistry

Paraffin sections of ipsilateral popliteal lymph nodes were deparaffinized in xylene and hydrated through descending grades of ethanol. The antigen retrieval was carried out by microwaving at HI-90 for 20 min using citrate buffer (pH 6.0) and endogenous peroxidase quenching was done by incubation with 3% H_2O_2 for 20 min. The slides were then blocked with 5% goat serum in 1% BSA for 30 min. The sections were stained with one of the primary antibodies: Mouse monoclonal IgG to rat NF-kβ p65 (Biovision-3012-100), or rabbit polyclonal IgG to rat TNF-α antibody (Biovision-3054-100) and was incubated at 4 °C overnight. The slides were washed with appropriate buffer and incubated with biotinylated secondary antibody for 30 min at room temperature. Sections were washed and stained with AEC (3-amino-9-ethylcarbazole, Sigma-Aldrich, U.S.A.) chromogen for TNF-α and DAB (3,3′-diaminobenzidine, Sigma-Aldrich, U.S.A.) for NF-kβ for 15 min. Counterstaining with hematoxylin was performed and the slides were mounted with aqueous mounting media and visualized under light microscope. The percentage of positive cells in 10 random high power fields was measured by two independent pathologists who were blinded to clinical data.

2.7. Histopathology of hind paw

Rats from each group were euthanized using anesthetic ether and the hind limb was amputated proximal to the ankle joint and fixed in 10% formalin, followed by decalcification in 5% nitric acid for 7 days. After sufficient decalcification, the limb was transected in a mid-sagittal plane. The tissues were processed and embedded in paraffin. About 5 μm thick sections were cut and stained with hematoxylin and eosin for microscopic evaluation. Histopathologic scoring of joint damage for evaluating inflammatory infiltration, synovial hyperplasia, and cartilage/bone erosion was done along a five level semi-quantitative scale (0-absent, 1-weak, 2-moderate, 3-high, 4-very high) by two independent and blinded observers [13].

2.8. Assessment of systemic toxicity

The biochemical parameters such as ALT, AST, ALP, urea, creatinine and total protein were estimated for the treatment groups. Tissues of liver were fixed in 10% formalin and the processed tissues were embedded in paraffin blocks to obtain 5 μm thick sections, which were further stained with hematoxylin and eosin and examined under light microscope for pathological changes.

2.9. Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). Significance of differences between the groups was determined using one way ANOVA followed by Tukey-Kramer test for multiple comparisons using SPSS software version 15.0 (Cary NC). P values < 0.05 were considered significant.

3. Results

3.1. Synthesis and characterization of phyto-stabilised silver nanoparticles

The phyto-stabilised silver nanoparticles (S-AgNPs) were synthesised using aqueous extract of *Piper nigrum* in an eco friendly and cost effective manner. The U–V spectroscopic analysis showed a Surface Plasmon Resonance peak at 415–420 nm which is characteristic of silver nanoparticle production as depicted in Fig. 1a. The average size of the nanoparticles was analyzed by Dynamic Light Scattering (Fig. 1b) and was around 26 nm, and the zeta potential was estimated to be −30.4 mV. S-AgNPs were also analyzed using Transmission electron microscopy (Fig. 1c), which showed spherical particles stabilised with phytochemicals that prevent the nanoparticles from aggregation.