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6-Gingerol, an active ingredient of ginger suppresses monosodium ureate crystal-induced inflammation: An *in vivo* and *in vitro* evaluation

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ABSTRACT

Gouty arthritis is an extremely painful, intense, acute inflammatory disorder that erupts in response to articular deposits of monosodium urate (MSU) crystals. Apart from pain management, therapeutic approaches target at suppressing the inflammation. In the present study, we evaluated the efficacy of 6-Gingerol (25mg/kg b. wt) against monosodium urate crystal-induced inflammation in mice; an experimental model for gouty arthritis. The non-steroidal anti-inflammatory drug, indomethacin (3 mg/kg body weight) was used as a reference for comparison. Paw volume and levels/activities of lysosomal enzymes were assessed in control and monosodium urate crystal-induced mice. In addition, polymorphonuclear leucocytes (PMNL) incubated with monosodium urate crystals in vitro were also examined for the levels of acid phosphatase and lactate dehydrogenase released. Increase in levels of lysosomal enzymes, and paw volume were observed in monosodium urate crystal-induced mice. However, upon treatment with 6-Gingerol, these biochemical alterations were brought back to near normal levels as comparable to indomethacin treatment. 6-Gingerol also reduced the acid phosphate and lactate dehydrogenase release in monosodium urate crystal incubated PMNL cells in vitro. These results strongly support the possibility of 6-Gingerol to be employed as an anti-inflammatory agent against gouty arthritis.

Keywords: 6-Gingerol, Inflammation, Lysosomal enzymes, Polymorphonuclear leucocytes.

INTRODUCTION

Gout is a metabolic disorder manifested as an intense, extremely painful, inflammatory arthritis with a rapidly escalating inflammatory response resulting from deposition of monosodium urate crystals in the affected joint space, secondary to hyperuricemia [1]. The rise in incidence and prevalence of gout is attributed to factors like excessive alcohol intake and its association with metabolic components like insulin resistance, abdominal obesity, dyslipidemia and arterial

hypertension [2]. Clinically, gouty arthritis is associated with edema and erythema of joints, together with severe pain [3].

MSU crystals provoke an inflammatory response primarily through its interaction with synovial fibroblasts, resident synovial macrophages, infiltrating monocytes and neutrophils within the articulation, secreting proinflammatory mediators including TNF-α, IL (interleukin)-1β, chemokines like IL-8 [4] [5] [6]. Current therapeutic options for anti-inflammatory management of gout include non-steroidal anti inflammatory drugs (NSAIDs) like naproxen, indomethacin, corticosteroids and colchicine. However, these drugs are not devoid of adverse events like renal disease, gastro-intestinal bleeding and hepatic dysfunction.

Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) has been an important plant for the traditional Chinese and Indian pharmacopeia. In Asian traditional medicine, ginger has been used to relieve muscular aches, rheumatism, pains, coughs, sinusitis, sore throats, diarrhea, cramps, indigestion, loss of appetite, motion sickness, fever, flu, chills and infectious diseases. It is being used worldwide as a spice and a flavoring agent [7]. Ginger has been extensively studied for its broad spectral pharmacological properties in the form of dried powder, ginger juice and extracts of organic solvents. The prominent nonvolatile pungent components of ginger include gingerol, shogaol and zingerone. These active principles are known to have the ability to suppress the hyperproliferative, inflammatory and transformative processes of carcinogenesis [8]. 6-Gingerol [(S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone] is an aromatic polyphenol and the most pungent ingredient of fresh ginger. It has multifarious pharmacological activities including anti-oxidant, anti-inflammatory, anti-cancer [9], analgesic [10] and anti-platelet effects [11]. It also has an inhibitory effect on xanthine oxidase responsible for generation of ROS like superoxide anion [12]. In addition, 6-gingerol has also been found to inhibit the expression of COX-2, lipooxygenases and NF-κB which play pivotal roles in progression of inflammation and cancer [13]. To date, the effect of 6-Gingerol on gouty arthritis has not been studied to our knowledge. On account of mounting further evidence on the anti-inflammatory role of 6-Gingerol, the present study was carried out to investigate its anti-inflammatory effects over monosodium urate crystal-induced inflammation in mice, which is an experimental model for gouty arthritis. The standard NSAID, indomethacin, was used as a reference drug for comparison purposes.

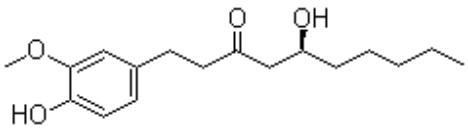

Fig.1 Structure of 6-Gingerol [(S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone)]

MATERIALS AND METHODS

Animals

Male Swiss albino mice, 25-30 g, were obtained from C. Abdul Hakeem College of Engineering and Technology, Vellore, India. They were acclimatized for a week in a light and temperature controlled room with a 12 h dark-light cycle and were fed with commercial pelleted feed from Hindustan Lever Ltd. (Mumbai) and water *ad libitum*.

Drug

The commercially available 6-Gingerol was purchased from Natural Remedies Ltd., Bangalore, India. Indomethacin was purchased from Tamil Nadu Dadha Pharmaceuticals Ltd., Chennai, India. A homogeneous suspension of 6-gingerol and indomethacin was made with 0.5% carboxymethyl cellulose in saline. The solution was freshly prepared before each experiment. All other reagents used were standard laboratory reagents of analytical grade and were purchased locally.

Dosage

Based on our preliminary studies with different dosages (12.5mg, 25mg, 50mg) of this 6-gingerol, it was found that 25mg/kg b.wt dosage produced significant anti-inflammatory effect by reducing paw swelling in monosodium urate crystal-induced animals. Hence 25mg/kg b.wt dosage was considered for this study. The dosage of standard drug indomethacin (3mg/kg b.wt) used in this study was selected based on our previous reports [14].

Synthesis of monosodium urate crystals

About 4 g of uric acid was dissolved and heated in 800 ml water with NaOH (9 ml/0.5N), adjusted to pH 8.9 at 60 °C; cooled overnight in a cold room, washed and dried. Needle-like crystals were recovered which were suspended in sterile saline (20 mg/ml) [14].

Monosodium urate crystal-induced inflammation in mice

The mice were divided into six groups-each comprising of six animals. Group I served as control. In Group II, inflammation was induced by intradermal injection of 0.2 ml (4 mg) of monosodium urate crystal suspension into the right foot pad [14]. Group III consisted of monosodium urate crystal-induced mice treated with 6-Gingerol (12.5 mg/kg body weight i.p.); Group IV comprised of monosodium urate crystal-induced mice treated with 6-Gingerol (25 mg/kg body weight i.p.) and Group V monosodium urate crystal-induced mice treated with indomethacin (3 mg/kg body weight i.p.). 6-Gingerol and indomethacin were administered intraperitoneally, 1 h before monosodium urate crystal injection (single dose) and then once daily for 3 days.

Assessment of inflammation

The inflammation was quantified by measuring the thickness of the paw with a vernier scale at different intervals for 3 days. At the end of the experimental period (72 h), the mice were killed by cervical decapitation. Blood from each animal was collected for serum separation. The liver and spleen were immediately dissected out and homogenized in ice-cold 0.01 M, Tris HCl buffer, pH 7.4 to give a 10% homogenate. The tissue homogenate of spleen, liver and serum were used for assaying the lysosomal enzymes, lipid peroxidation and anti-oxidant status.

Effect of 6-Gingerol and indomethacin on lysosomal enzymes

The activity of acid phosphatase was assayed by the method of King [15]. β- galactosidase was estimated by the method of Rosenblit et al [16] and N-acetyl glucosaminidase by the method of Marhun [17]. Cathepsin-D was estimated by the method of Saprosky et al (1963 modified) [18]. The protein content was measured by the method of Lowry et al [19].

In vitro studies (monosodium urate crystal – PMNL cell interaction)

Human PMNL cell suspension (3 x 10^6 ml^{-1}) was pre-incubated at 37 °C for 20 m with 6-Gingerol (50/100/150µg/ml) or indomethacin (10µg/ml) before addition of monosodium urate crystals (1 mg/ml). After incubation for a further 30 m at 37 °C, the cell suspension was removed and centrifuged at 1500 g at 4 °C for 20 m. The resulting cell free supernatant was assayed for the released activities of acid phosphate and lactate dehydrogenase. Appropriate control experiments were performed by measuring the release of enzymes tested in the untreated specimens and those incubated for 30 m at 37°C without drug. In all instances, the experiments were carried out in triplicate. Lactate dehydrogenase (LDH), a cytoplasmic enzyme was assayed by the method of King [20]. The activity of acid phosphatase was assayed by the method of King [15].Enzyme released was expressed as a percentage of maximal enzyme release after disruption of the cells with Triton X-100. Specific enzyme activity was expressed as units/mg of protein.

Statistical analysis

Results were expressed as mean ± S.D. and a statistical analysis was performed using ANOVA, to determine the significant differences between the groups, followed by Student's Newman–Keul's test. $p < 0.05$ implied significance.

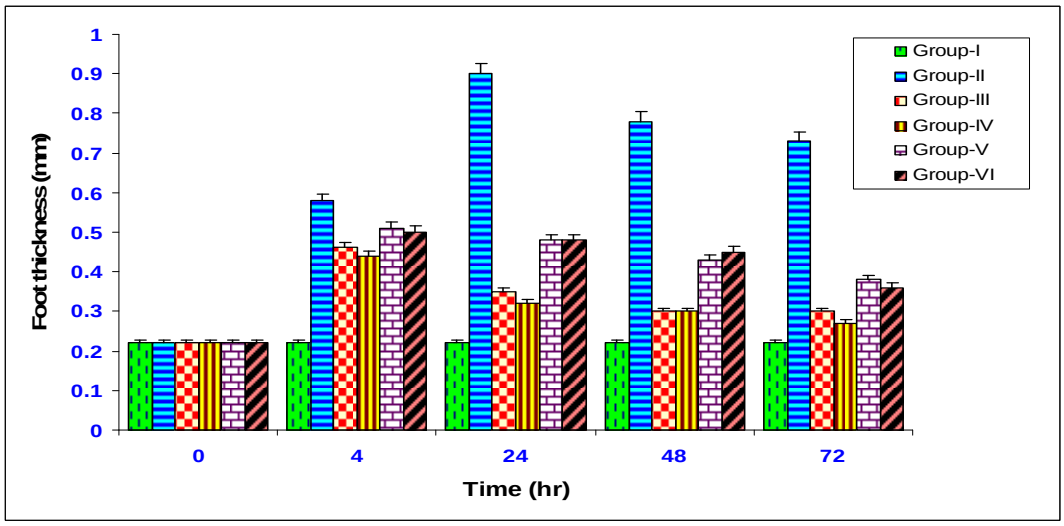

Fig. 2. Effect of 6-Gingerol (25mg/kg/b.wt) and indomethacin (3mg/kg/b.wt) on on monosodium urate crystal-induced paw oedema in mice

RESULTS

Effect of 6-Gingerol (25mg/kg b.wt.) and indomethacin (3 mg/kg b.wt.) on the activities of lysosomal enzymes in monosodium urate crystal-induced mice

Table 1 represents the effect of 6-Gingerol on acid phosphatase, β-galactosidase, N-acetyl glucosaminidase and Cathepsin-D in the serum, liver and spleen of control and experimental animals. Increased activity of acid phosphatase, β-galactosidase, N-acetyl glucosaminidase and Cathepsin-D were found in serum, liver and spleen of monosodium urate crystal-induced mice when compared to that of control mice. However, 6-Gingerol (25 mg/kg b.wt.) significantly reduced these enzyme activities near to that of control animals.

Table.1. Effect of 6-gingerol (25 mg/ kg/b.wt) and Indomethacin (3mg/kg/b.wt) on the activities of lysosomal enzymes In MSU crystal-induced mice

Parameter	Group –I (Control)	Group –II (MSU crystal-induced mice)	Group –III (MSU crystal-induced mice + 6-gingerol 25mg/kg b.wt)	Group –IV (MSU crystal-induced mice + indomethcin 3 mg/Kg b.wt)
Serum				
Acid phosphatase	2. 62± 0.50	5.78 ± 0.63 a*	3.0 ± 0.56 a* b*	3.67 ± 0.65a* b*
β-glucuronidase	1.60 ± 0.46	4.73 ± 0.43 a*	1.93 ± 0.33 a* b*	2.18 ± 0.29 a* b*
N-acetyl glucosaminidase	8.2 ± 0.51	18.70 ± 0.45 a*	9.48 ± 0.48 a* b*	9.98 ± 0.74 a* b*
β-galactosidase	4.72 ± 0.61	10.67 ± 1.03 a*	5.52 ± 0.44 a* b*	6.60 ± 0.39 a* b*
Liver				
Acid phosphatase	6.25± 0.69	19.63 ± 1.03a*	8.75 ± 0.52a* b*	9.08± 0.59a* b*
β-glucuronidase	8.65 ±0.55	18.17 ± 0.64 a*	9.92 ± 0.59 a* b*	9.92 ± 0.74 a* b*
N-acetyl glucosaminidase	31.17 ± 0.69	64.67± 1.64 a*	34.08 ± 0.59 a* b*	34.92 ± 0.97 a* b*
β-galactosidase	15.98 ± 0.75	30.67 ± 1.29 a*	18.15 ± 1.16 a* b*	21.58± 1.39 a* b*
Spleen				
Acid phosphatase	3.12 ± 0.71	8.90 ± 0.79 a*	3.78 ± 0.48 a* b*	6.58 ± 0. 59 a* b*
β-glucuronidase	13.42 ± 0.74	24.76 ± 0. 78 a*	13.75 ± 0.52 a* b*	14.42 ± 0.74 a* b*
N-acetyl glucosaminidase	45.87 ± 1.34	72.57 ± 1.24 a*	47.75 ± 0.96 a* b*	49.08 ± 1.88 a* b*
β-galactosidase	21.52 ± 0. 95	49.42 ± 1.39 a*	24.28 ± 2.04 a* b*	25.78 ± 1.81a* b*

*Values are expressed as mean±S.D. of six animals. Comparisons were made as follows: a- Group-1 vs. Groups-II, III, and IV, b-Group-IIvs. Group-III and IV. activities are expressed as: Acid phosphatase – μ moles x 10^{-2} of phenol; β-glucuronidase, N-acetyl glucosaminidase and β-Galactosidase- μ moles x 10^{-2} of p-nitro phenol liberated /h/mg protein. The symbols represent statistical significance at: *p < 0.05. Statistical analysis was calculated by one way ANOVA followed by Student's Newman-Keul's test.*

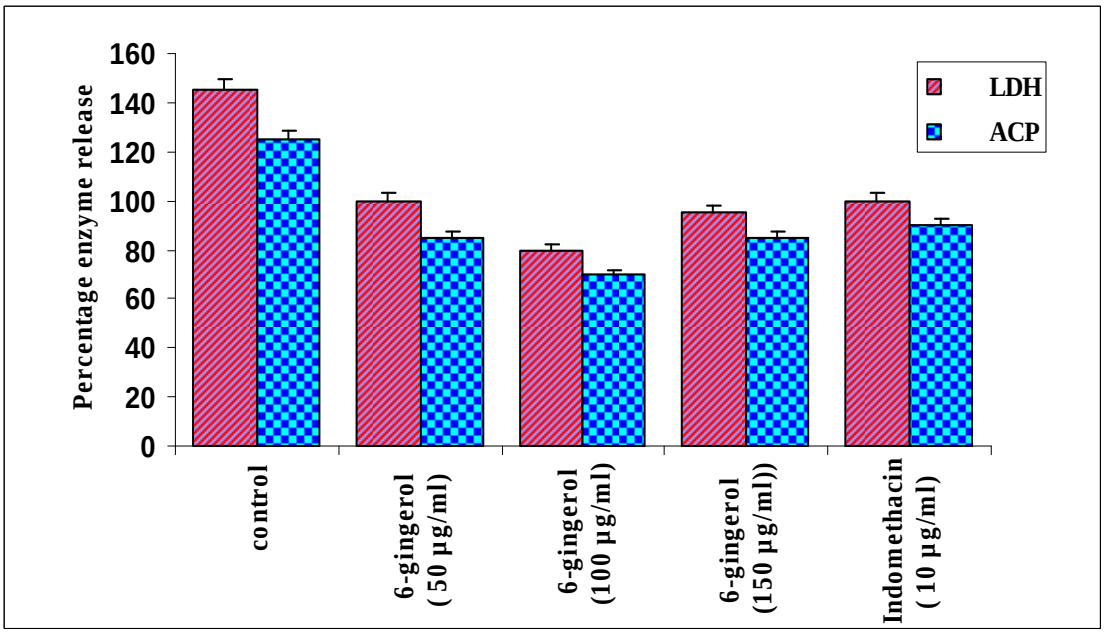

Fig 3.Effect of 6-Gingerol and indomethacin on enzyme leakage from the PMNL cells upon incubation with monosodium urate crystals

Effect of 6-Gingerol (25mg/kgb.wt.) and indomethacin (3mg/kgb.wt.) on monosodium urate crystal-induced paw oedema in mice

Paw oedema of the control and experimental animals are shown in Fig. 2. Measurement of the paw volume of monosodium urate crystal-induced mice showed an increase in ankle diameter. However, 6-Gingerol (25 mg/kg b.wt.) treatment caused a significant decrease in the paw diameter in monosodium urate crystal-induced mice.

Effect of 6-Gingerol and indomethacin on enzyme leakage from the PMNL cells upon incubation with monosodium urate crystals

Results presented in the Fig.3 show that the pretreatment of 6-Gingerol at 100 µg/ml of cells significantly suppresses the acid phosphatase (lysosomal) and lactate dehydrogenase (cytoplasmic) enzyme release from the PMNL cells incubated with monosodium urate crystals compared to controls, i.e. untreated PMNL cells incubated with monosodium urate crystals.

DISCUSSION

The precipitation of monosodium urate crystals within the joints triggers a strong inflammatory response, playing the etiological role in gouty arthritis. In our study, we have mimicked this pathological condition by injecting a suspension of monosodium urate crystals in mice joints. One standout feature of the inflammatory response to monosodium urate crystals is the recruitment of a large number of activated neutrophils into the joint. The neutrophils mobilizing into the synovium actively phagocytose monosodium urate crystals; undergo rupture of the phagolysosome leading to the generation of reactive oxygen species, release of tissue damaging lysosomal enzymes and other inflammatory mediators [21].

Lysosomes are cellular organelles containing various proteolytic enzymes that digest an array of biological polymers, including proteins, nucleic acids, carbohydrates and lipids [22]. Rupture of the lysosomal membrane, with the concurrent release of the constituent hydrolytic enzymes, stimulates the synthesis of several inflammatory mediators such as thromboxanes, prostaglandins and leukotrienes [23]. In our study, Group II (monosodium urate crystal-induced mice) showed an increase in paw edema and lysosomal enzyme activity. To have a reduction in the release of such enzymes would render beneficial against the inflammation [24]. Release of lysosomal enzymes by leucocytes could be due to intense endocytosis by the leucocytes [25] or ROS mediated lipid peroxidation of lysosomal membrane [26]. The significant reduction in the paw edema and lysosomal enzymes activity after 6-Gingerol treatment (25 mg/kg b. wt.) should have eventuated by its membrane stabilizing action by fusing with the plasma membrane and inhibiting the release of lysosomal enzymes [27]. The decrease in paw oedema of monosodium urate-induced mice after 6-Gingerol treatment convincingly signifies that the drug was involved in the reduction of neutrophil infiltration to the synovium and decreased release of the lysosomal enzymes. The anti-inflammatory property of 6-Gingerol is well determined earlier by the inhibition of transcriptional factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) and inflammatory mediators like TNF-α, IL-1, IL-12, iNOS and COX-2 [28; 29]. These evidences strongly support our claim for 6-Gingerol to be employed for anti-inflammatory action against gouty arthritis.

The responses of neutrophils to monosodium urate crystals represent an integral part of this innate response and a key component of the acute inflammatory response associated with gout. The contribution of neutrophils to the pathogenesis of acute gouty inflammation is believed to be partly caused by events that follow from the physical association of urate crystals and neutrophils [30]. Monosodium urate crystals can interact with the infiltrating cells by phagocytosis or direct interaction with cell surface receptors [31]. In response to monosodium urate crystals, these cells release lysosomal enzymes, chemotactic factors, and leukotrienes and generate reactive oxygen species [32]. In this study, PMNL cells incubated with monosodium urate crystals released substantial amounts of acid phosphatase and lactate dehydrogenase. It has been hypothesized that hydrogen donor sites on the crystal attach to acceptor sites on the outer surface of the lipid bilayer of cell membranes. Interaction between crystals and cell membrane can also alter cellular metabolism, and cause secretion of inflammatory mediators. Thus, urate crystals induce release of lysosomal enzymes from leucocytes in the absence of phagocytosis [33]. However, in our study, 6-Gingerol treatment (100 µg/ml) decreased the enzyme release, by its membrane protective action.

To conclude, our study has thrown light upon the potential of 6-Gingerol to strongly inhibit the inflammation in mice, induced by monosodium urate crystal injection. The decrease in paw volume and lysosomal enzymes leakage in monosodium urate crystal-induced mice after 6-Gingerol administration are indicative of its ability to suppress the inflammation. Treatment of monosodium urate crystal-induced mice with 6-Gingerol at a dosage of 25 mg/kg b.wt, has been found to be therapeutically more effective than the other dosages. Previous reports on the ability of 6-Gingerol to inhibit transcription factors NF-κB, AP-1 and expression of associated inflammatory mediators like TNF-α, COX-2, [9] [10], add thrust to our current study. With its membrane stabilizing ability, 6-Gingerol exerts an anti-inflammatory effect over monosodium

urate crystal-induced inflammation. Thus, 6-Gingerol treatment could prospectively be considered a therapeutic approach towards gouty arthritis.

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