Chapter VI

Piperine ameliorates functional and histological outcome, reduces oxidative stress and inflammation in experimental arthritis
6.1. Introduction:

Piperine is a nutritive food chemical present in black and long pepper (Piper nigrum and Piper longum). It has been widely used as a dietary spice by people in world and by many people as traditional medicine in Asia and Pacific islands especially in India (Selvendiran and Sakthi sekaran, 2004, Bae et al., 2010). Current literature reveals a wide spectrum of biological activities of piperine as it stimulates the digestive enzymes of pancreas, help in inhibiting oxidation reactions caused by free radicals and enhances the bioavailability of a number of therapeutic drugs. Its anti-inflammatory activities have been confirmed in diverse animal models (Bang et al., 2009). Piper species have shown to inhibit enzymes activity which is responsible for leukotriene and prostaglandin biosynthesis. It has also been reported to inhibits nitric oxide (NO), tumor necrosis factor-α (TNF-α), and pro-inflammatory gene expression in vitro, as well as in vivo (Pradeep and Kuttan, 2004).

Rheumatoid arthritis (RA) is a chronic inflammatory disease leading to joint destruction mediated in part by the migration of inflammatory cells into the synovial tissue, leading to progressive destruction of the joints (Lee and Weinblatt, 2001). In RA, the balance between pro-inflammatory and anti-inflammatory cytokines determines the degree and extent of inflammation (McInnes and Schett, 2007, Vierboom et al., 2007). Proinflammatory cytokines like interleukin (IL)-1β, tumor necrosis factor (TNF) α and IL-6 are highly expressed in the rheumatoid joint and play key role in the pathogenesis of RA (Kim et al., 2011). Fibroblast like synoviocytes (FLS), in response to these cytokines produce chemokines, metalloproteinases (MMPs), prostaglandin E₂ (PGE₂) and cyclooxygenase-2 (COX-2) which further promote inflammation, hyperplasia and cartilage destruction (Vaillancourt et al., 2011).

Other key modulators of inflammation in RA are reactive oxygen species (ROS) and reactive nitrogen species. They might perpetuate inflammation by facilitating the production of chemotactic factors at the local site (Merry et al., 1989, Woodruff et al., 1986). The hydroxyl radical, Superoxide anion radical, and the peroxynitrite anion (de Groot, 1994) are the major ROS generated during the disease condition. Nitric oxide (NO) is a free radical, serves as an important messenger molecule in inflammatory conditions (Guzzocrea, 2006). Decreased production of NO via suppressing or inhibiting iNOS reduces arthritic symptoms and affords protection (Rostoka et al).
Studies pertaining to identification of safe and active plant derived compounds for attenuation of inflammation, oxidative stress may be important in the management of rheumatoid arthritis. Pradeep and Kuttan reported that the nuclear translocation of p65, p50, c-Rel subunits of NF-kB and other transcription factors such as ATF-2, c-Fos and CREB were inhibited by the treatment of piperine in B16F-10 melanoma cells and also inhibit matrix metalloproteinase production. Bang et al recently showed that piperine inhibited the expression of IL-6 and MMP13 and reduced the production of PGE\(_2\) in human interleukin 1β-stimulated fibroblast-like synoviocytes and in rat arthritis models. Experiments were therefore, planned to investigate the action of piperine at various check points in collagen induced arthritis: (a) its anti-oxidative efficacy was determined by monitoring ROS and GSH, (b) anti-inflammatory effect by pro and anti inflammatory cytokines (c) elastase and myeloperoxidase activity which is directly proportional to the accumulation and activation of polymorphonuclear leukocytes in the inflamed tissue.

6.2. Materials and methods

6.2.1. Chemicals

Piperine, Freund’s adjuvant complete (CFA), N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide and Griess Reagent system, were purchased from Sigma Chemical Co. (St Louis, MO, USA). ELISA kits were purchased from eBioscience and Cayman Chemical USA, Collagen type II from bovine nasal septum was purchased from Elastin Products Co, ING, Owensville, Missouri, USA. Thiobarbituric acid (TBA), trichloroacetic acid (TCA), 5,5’dithio-bis-2-nitrobenzoic acid (DTNB), nitrobluetetrazolium (NBT), ethylene diamine tetra-acetic acid (EDTA), xanthine, xanthine oxidase, tris hydrochloride were purchased from SD Fine chemicals India. All other routine chemicals used in this investigation were of research grade.

6.2.2. Animals

Male Wistar rats weighing 150-170 g were used. They were kept in the Central Animal House of Hamdard University in colony cages at an ambient temperature of 25 ± 2 °C and relative humidity 45-55% with 12 h light /dark cycles after initial acclimatization for about 1 week. They had free access to standard rodent pellet diet and water ad libitum. The experimental study was conducted in accordance with the Institutional Animal Ethics Committee of the University, Jamia Hamdard, New Delhi, India.
6.2.3. Induction of collagen-induced arthritis (CIA) and experimental protocol

Arthritis was induced in rats as described previously (Haqqi et al., 1999). Collagen Type II from bovine nasal septum was dissolved in 0.05 M acetic acid at a concentration of 2 mg/ml was emulsified with an equal volume of Freund's adjuvant complete (CFA) containing 1 mg/ml Mycobacterium tuberculosis H37 RA and stored on ice before use. Rats were immunized intradermally at about 1.5 cm distal from the base of the tail. All rats were randomly assigned to three groups of six animals each. The first group served as control (C), the second was collagen induced arthritis (CIA), the third was administered with pipertine (100 mg kg⁻¹ body weight) (CIA + PIP₁₀₀) daily. The above-mentioned dose of pipertine was selected on the basis of previous studies and preliminary dose-escalation studies to determine the threshold dose producing a measurable response between arthritic and pipertine-treated rats.

6.2.4. Measurement of Clinical Severity of Arthritis

For macroscopic assessment of arthritis, the thickness of each affected hind paw was measured with digital calliper (YAMAYO, Japan) and the measurement was expressed as an average for inflamed hind paws per rats. The development of arthritis in rats was evaluated daily starting from day 0 after the intradermal injection using macroscopic scoring system (Larsson et al., 1990): 0 = No evidence of erythema and swelling, 1 = Erythema and mild swelling confined to the tarsals or ankle joint, 2 = Erythema and mild swelling extending from the ankle to the tarsals, 3 = Erythema and moderate swelling extending from the ankle to metatarsal joints, 4 = Erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb.

6.2.5. Preparation of cell-free extract of the knee joints

At the end of experiment, animals were sacrificed by cervical dislocation. Arthritic and nonarthritic joints were removed and cut into small pieces and homogenized in 5 vol. of 50 mM Tris HCl buffer, pH 7.4 containing 0.1 M NaCl and 0.1% Triton X-100 and 1 vol. of
fine glass powder by using a mortar and pestle. The crude extract was then sonicated for 20 sec. The homogenate was centrifuged at 3,000 x g for 5 min, and the resulting supernatant was stored at -80°C until further analysis.

6.2.6. Articular Elastase (ELA)
Articular elastase levels in the articular joints were evaluated as an index of polymorphonuclear leukocyte (PMNs) accumulation and activation in the inflamed tissue as described earlier (Yoshimura et al., 1994). The articular elastase activity was expressed as ng/g protein.

6.2.7. Myeloperoxidase (MPO) assay
Myeloperoxidase activity was analysed as an index of neutrophils infiltration in the synovial tissue, as it is closely correlated with the number of neutrophils present in the tissue. The assay was carried out by the method described earlier (Campo et al., 2003; Lefkowitz et al., 1999). Myeloperoxidase activity was expressed as U/g of protein.

6.2.8. Estimation of thiobarbituric acid reactive substances (TBARS)
The assay of TBARS was done according to the method mentioned earlier (Udley et al., 1967). The result was expressed in nmoles TBARS formed / h / g tissue.

6.2.9. Reduced glutathione (GSH)
GSH was measured in the groups following the method described earlier (Sedlak and Lindsay, 1968). Results were expressed as µg GSH/g tissue.

6.2.10. Total superoxide dismutase (SOD) activity
Total SOD were measured in joints as described earlier (Beauchamp and Fridovich, 1971). The SOD activity is expressed in Units /mg protein.

6.2.11. Catalase activity
Catalase activity in the joint tissues was assayed according to method described earlier (Sinha, 1972). The enzyme activity was expressed as µmol H2O2 consumed / min / mg protein.

Nitric oxide was determined with Griess method as described earlier (Sajad et al., 2009).

6.2.13. Measurement of cytokines level and PGE2 production
Levels of inflammatory cytokines IL-1β, TNF-α, IL-10 and PGE2 in the joints were determined by using commercially available cytokine ELISA kits (eBioscience and Cayman Chemical USA). Supernatants were removed and assayed in duplicate according to the
manufacturer's guidelines. Tissue cytokine concentrations were expressed as picograms of antigen per milligram of protein.

6.2.14. Histological examinations

Rats were sacrificed on the day 21 by cervical dislocation. Knee joints were removed and fixed in 4% formakdehyde. After decalcification in 5% formic acid, the samples were processed for paraffin embedding (Durie et al., 1993). Tissue sections (5 μm thick) were stained with haematoxylin–eosin for light microscope examination.

6.2.15. Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Tukey's test for all parameters. The p-value <0.05 was considered statistically significant.

6.3. Results

6.3.1. Clinical Severity of disease after piperine treatment

Arthritis was developed rapidly in rats immunized with collagen emulsified with CFA. Clinical signs of the disease were erythema of one or more ankle joints, followed by involvement of the metatarsal and interphalangeal joints, first appeared in the hind paws between 8 and 9 days after CIA immunization, with a 100% incidence by day 13 ± 1 (Fig. 6.1A). Piperine treatment suppressed the evolution of collagen induced arthritis. There was no macroscopic evidence of either hind paw erythema or oedema in the control group. Our data suggested that oral piperine administration to collagen-immunized rats reduced the progression of arthritis by inhibiting the increase in arthritis score (Fig. 6.1B) and paw swelling compared to RA rats.

![Fig. 6.1](image-url) Effect of piperine (PIP) on time course of change in hind paw diameter (mm) (1 A) and mean clinical severity score (1 B) rats immunized with collagen type II. Values are Mean ± SEM for six animals for each group.
6.3.2. Effect of piperine on articular elastase and myeloperoxidase activity

Articular elastase and myeloperoxidase activity assayed at the day 21st in the studied groups. Very low articular elastase and myeloperoxidase levels were measured in the joints of control rats. Instead, significant elevated activities of these were observed in CIA group. Administration of the piperine showed a significant decrease in articular elastase ($p < 0.05$) and myeloperoxidase activity ($p < 0.01$) resulting reduction in neutrophil activation and infiltration in the synovial tissue of the joints (Fig. 6.2).

![Graph showing the effect of piperine (PIP) treatment on articular elastase and myeloperoxidase activity](image)

**Fig. 6.2** Effect of piperine (PIP) treatment on (a) articular elastase activity (b) myeloperoxidase activity in joints of rats immunized with collagen type II. Data are expressed as Mean ± SEM of 6 rats.

**-** ($p < 0.01$), **-** ($p < 0.001$) vs. Control, # ($p < 0.05$), ## ($p < 0.01$), ### ($p < 0.01$) vs. CIA.

6.3.3. Piperine treatment decreased TBARS

The effect of piperine on TBARS level was measured to demonstrate the oxidative damage on lipid (Fig. 6.3 a). A significant increase ($p < 0.01$) in TBARS level was observed in CIA group when compared to the control group. Treatment with piperine decreased TBARS level significantly ($p < 0.05$) by inhibiting lipid peroxidation in the cartilage tissue.

6.3.4. Piperine restored the GSH and SOD

The concentration of GSH was evaluated to estimate endogenous defences against hydrogen peroxide formation and SOD activity to estimate endogenous defences against superoxide anions. Figure 6.3 b and c shows the changes in GSH and SOD levels evaluated in the joints (day 21) in the experimental groups. A marked decrease ($p < 0.05$) in GSH and SOD...
concentrations was found in the joint of CIA rats. Treatment with piperine significantly (p < 0.05) inhibited the decrease in GSH and SOD as compared to CIA group.

6.3.5. Effect of Piperine on catalase activity
The activity of catalase was decreased significantly in CIA group at the day 21st in the joints (Fig. 6.3 d) as compared to control. Also in this case the treatment with piperine was significantly effective (p <0.05) as compared to CIA group.

Fig. 6.3 Effect of piperine (PIP) treatment on (a) Lipid peroxidase (b) GSH level (c) SOD activity (d) catalase activity in joints of rats immunized with collagen type II. Data are expressed as Mean ± SEM of 6 rats.

* (p <0.05), **(p <0.01), vs. Control, # (p <0.05) vs.CIA.
6.3.6. Effect of Piperine on nitric oxide

Analysis of nitrite estimation is summarized in Fig. 6.4. A significant increase in nitrite was observed in CIA group as compared to control. The treatment with piperine declined the increase in the nitrite level significantly (p < 0.01) as compared to the CIA group.

6.3.7. Piperine suppresses IL-1β, TNF-α, PGE₂ and enhance production IL-10 in RA rats

Proinflammatory cytokines IL-1β, TNF-α and IL-10 as well as PGE₂ have central role in the perpetuation of chronic inflammation and tissue damage during progression of RA. Our result in Fig. 6.5 showed, there is significant (P < 0.05) increase in the level of TNF-α, PGE₂ and IL-1β (P<0.01) in RA rats compared to the controls while a significant (P<0.05) decrease in IL-10 level was observed. Oral administration of piperine suppressed the increase in the level of IL-1β (P<0.05), TNF-α (P<0.05) and PGE₂ (P<0.05) to significantly while an increase in IL-10 (P<0.05) was observed when compared to CIA group on day 21.
Fig. 6.5 Effects of piperine (PIP) on cytokine levels were measured in rat joints and concentration was expressed in pg/ml of a) Interleukin-1β (IL-1β), b) Tumor necrosis factor-α (TNF-α), c) PGE₂, d) Interleukin-10 (IL-10). Data are expressed as Mean ± SEM of 6 rats.

**p < 0.01, ***p < 0.001** vs. Control, # p < 0.05, ## p < 0.01, ### p < 0.01 vs. CIA group, NS - not significant

6.3.8. Histology

Persistent with the biochemical alterations, the histological findings (Fig. 6.6) revealed massive cell infiltration in the CIA group. Bone suffered resorption and pannus formation, while as synovial hyperplasia was consistent finding. The treatment with piperine ameliorated the changes at histological level and able to restore the changes to a greater extent.
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**Fig. 6.6** Histological findings. Massive and diffuse polymorphonuclear cellular flux in the collagen induced arthritic rats (B) in comparison to the control rats (A). Cellular infiltration leads to the cartilage erosion mainly by inflammatory necrosis (B). Reduction of cellular flux and cartilage erosion which was evidenced by the minimum necrotic lesions in the rats treated with piperine 100 mg/kg (C). Original magnification 40x.

6.4. Discussion

We have demonstrated the anti-oxidative and anti-arthritic activity of piperine in collagen induced arthritis, an experimental model of rheumatoid arthritis (RA). The present study was performed to elucidate the effects and the mechanisms of piperine in CIA model. It was found that piperine markedly inhibited clinical sign of joint swelling in RA rats; significantly decrease the free radical load, elastase and myeloperoxidase activity. It also enhanced antioxidant enzymes activity, modulates inflammatory mediators in RA rats.

A link between inflammation and bone homeostasis has been attributed to the effects of cytokines such as TNF-α, IL-1β, IL-6 and PGE₂ that are abundantly expressed in patients with RA and in the arthritic joints of rat with collagen-induced arthritis. Blockade of these molecules resulted in a reduction of disease severity and bone resorption (Schett et al., 2008, Williams, 2004), while IL-4 and IL-10 have potent anti-inflammatory effects and suppress cartilage and bone pathology in RA (Juaranz et al., 2005). (Duwejua et al., 1993, Safayhi et al., 1997, Mothana, 2011) Interestingly, the obtained results confirmed that piperine shift the balance of cytokines toward a bone protecting pattern that acts to both lower levels of TNF-α, IL-1β, and raise the levels of IL-10. Hence, it is plausible to suggest that part of the beneficial anti-inflammatory and cartilage / bone protective effects of piperine may be mediated through the inhibition of proinflammatory cytokines. Previous studies on piperine showed its anti-inflammatory effect by inhibiting proinflammatory cytokines and through inhibition of the adhesion of neutrophils to endothelial monolayer.
We evaluated elastase activity which is directly proportional to the accumulation and activation of polymorphonuclear leukocytes in the inflamed tissue as it is released from stimulated granulocytes at the site of injury. The inflammation so caused by the infiltrating cells leads to the release of reactive oxygen and nitrogen species (van der Vliet et al., 1997, Knight, 2000, Wills, 1969). We suggest that the decrease in elastase activity might be due to the inhibition of lipid peroxidation and the consequent decrease in the reduction of chemotactic peroxide (Wills, 1987). Lipid peroxidation is considered a critical mechanism of the injury that occurs during RA. The large amount of TBARS found is consistent with the occurrence of damage mediated by free radicals.

Oxidative stress is an imbalance between the prooxidants and antioxidant. In normal aerobic metabolism free radical production occurs in the body. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals (Knight, 2000). Cell damage can be prevented by detoxification of these free radicals, which eventually will prevent the progress of lipid peroxidation. In the present study, it is suggested that piperine which is a potent antioxidant (Tekeoglu et al., 2007, Budancamanak et al., 2006), reduced paw inflammation significantly in piperine treated animals by scavenging free radicals, which are thought to initiate cellular damage in cartilage in experimental animals. We found that QA caused a significant increase in lipid peroxides and depletion in GSH and SOD. These results are in agreement with other studies (Campo et al., 2003). The overproduction of reactive oxygen species can be detoxified by endogenous antioxidants, causing their cellular stores to be depleted (Khan et al.).

A reduction in GSH may impair $H_2O_2$ clearance and endorse $OH^-$ formation, thus increasing the free radical load that results in the disruption of homeostasis. As all antioxidant defences are interrelated (Sun, 1990), disruption of the microenvironment by a single factor, oxidative stress in this case, can shift the entire balance and lead to a catastrophe. On the other hand, piperine treatment increased the activities of antioxidant enzymes significantly in the CIA group treated with piperine. Our results clearly indicate that the protective role of piperine was mediated via its antioxidant effect through the suppression of lipid peroxidation and boosting the antioxidant defence system.

Nitric oxide (NO) is an important signalling molecule, produced as part of the inflammatory response from activated cells and macrophages (Seo et al., 2001). Compounds that hamper excessive NO production may have beneficial therapeutic effects in arthritis by
blocking degradation of cartilage (Shukla et al., 2008). In the present study, increased NO level have been detected in arthritic group similar with those previously reported in synovial fluids of patients with rheumatoid arthritis (van der Vliet et al., 1997). Treatment with piperine produced a significant decrease in nitric oxide level.

The biochemical alterations were further supported by histopathological observations of the joints. The higher number of infiltrating cells, extensive bone degradation and synovial hyperplasia which are hallmarks of RA was found in CIA. Treatment with piperine was able to reverse the histological findings to normal.

6.5. Conclusion

In conclusion, the major findings of the present study were that piperine suppressed the accumulation of lipid peroxidation products, nitric oxide, enhanced the activity of antioxidant enzymes and eliminated the accumulation and activation of polymorphonuclear cell. We believe that our results will contribute to the clinical applications in the treatment of rheumatoid arthritis. The hypotheses about the mechanism of action in CIA model, reported above, need further investigations for confirmation. More work directed toward understanding molecular and immunological aspects of the disease is required.