Nerolidol: a potential approach in rheumatoid arthritis through reduction of TNF-α, IL-1β, IL-6, NF-Kβ, COX-2 and anti-oxidant effect in CFA-induced arthritic model

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Abstract

Rheumatoid arthritis an autoimmune infectious disorder, is categorized by inflammation and increased level of pro-inflammatory cytokines which are released by immune cells, macrophages or activation of arachidonic acid metabolism. The expression of these cytokines, oxidative free radicals and the activation of COX-2 enzymes are crucial targets for chronic inflammation. On the basis of established anti-inflammatory efficacy of Nerolidol, the primary study was further appraised to determine its efficacy against Freund's complete adjuvant (CFA) rheumatoid model. Arthritis was persuaded by inoculation of 0.1mL CFA injection into left hind footpad of rats. Anti-arthritic potential of nerolidol (at 200, 400 and 800mg/kg doses) was assessed by measuring the paw volume, body weight, serum analysis, histopathological and radio-graphics of ankle joints. Expressions of cytokine's panels like IL-10, IL-4, COX-2, NF-$_K$β, TNF-α, IL-6, PGE-2 and IL-1β were determined by real time qPCR. Antioxidant enzyme analyses was calculated by measuring the SOD, POD and catalase activity from serum and equated with arthritic control group. Nerolidol prevented the body weight loss, stabilized the biochemical and haematological homeostasis and significantly reduced the paw volume. Furthermore, X-ray and histopathological assessment of ankle joints showed an improvement in the joint structure of rats treated with nerolidol. Besides that, over expression of gene pointers like TNF-α, IL-1β, IL-6, NF-$_K$β, PGE-2 and COX-2 in CFA treated control rats were also reversed with nerolidol. This anti-arthritic mechanism was further supported by the increased level of IL-10, IL-4 and serum anti-oxidant activity. The present findings demonstrate that nerolidol reduce the adjuvant arthritis by down-regulating the proinflammatory cytokines and up-regulating the aforementioned anti-inflammatory cytokines and may be used as a therapeutic substance for the management of human rheumatoid arthritis.

Introduction

Cytokines are involved in many biological processes including activation, differentiation and growth of cells, as well as inflammation (Feldmann *et al.*, 1996). Chronic inflammatory disorders are caused by dysfunctional effects of adoptive and innate immune responses that lead to the induction of cytokines and cause inflammation (Agonia *et al.*, 2020). Inflammatory reaction produces a protective appliance against pathogens and results in the release of chemokine's, cytokines and inflammatory mediators (Lin *et al.*, 2017). Arthritis is an autoimmune inflammatory syndrome that disturbs 1% of whole population and it is 2–3 times surplus abundant in womens than mens (Hegen *et al.*, 2008). The augmented level of proinflammatory cytokines like IL-1β, TNF-α, NF-$_K$β and interleukins-6 in acute phase ultimately leads towards bone deformity (Arya *et al.*, 2011). Apart from augmented level of proinflammatory cytokines, increased level of oxidative strain is also an important factor that damages the joints during pathogenesis of RA. These factors increase the production of inflammatory cells especially neutrophils and macrophages, accelerates the release of reactive oxygen sorts in synovial fluid, and cause further tissue destruction (Uttra *et al.*, 2018). Phagocytic macrophages and granulocytes produce hydrogen peroxide and superoxide in excessive amounts. Which degrade lipids by the process known as lipid peroxidation (Biemond *et al.*, 1984). Antioxidants defend the cells against oxidative stress on deoxyribonucleic acid, lipids and protein that has been associated with diseases like RA, diabetes, cancer and Alzheimer, and thus, involve in the improvement of treatment (De Carvalho *et al.*, 2018). Antioxidants act through the inhibition of reactive oxygen species formation in cells. Glutathione peroxidase, catalase and superoxide dismutase are the enzymes that catalysis the hydrogen peroxides and hydro peroxides into harmless molecules including metal ion binding proteins such as caeruloplasmin and transferrin (that chelates, sesquter iron) and prevent the production of free radicals (Ighodaro and Akinloye, 2018).

Conventional treatment of rheumatoid arthritis includes NSAIDs, corticosteroids, DMARDs that are aimed to decrease the joint inflammation and pain; are not pure anti-arthritic because they do not reduce the B cells and T cells mediated responses (Agarwal, 2010). So there is a need to introduce new novel herbal compound which is most effective and economical against B and T cells mediated response. Phytoconstituents derived from plants that inhibit the expression of cytokines have prospective against arthritis. Henceforth, efforts should be organized to seek out novel beneficial and efficient phytoconstituents which can be used for long standing treatment of RA (Arya *et al.*, 2011).

Nerolidol is derived from *Peperomia serpens* (Da Silva *et al.*, 2006). Despite that, *Peperomia serpens* were used to treat pain and inflammation and reduces the expression of IL-1β and TNF-α in mouse model for pain (Fonsêca *et al.*, 2016). Nano encapsulated nerolidol has also been evaluated for its anti-inflammatory role in zymogen induced arthritis in mice and they have also shown the gastro protective effect of nerolidol against ulcer (Trindade *et al.*, 2020). Anti-oxidant effect of nerolidol on hippocampus of mice shows that nerolidol is beneficial against oxidative stress (Neto *et al.*, 2013). Therefore, the aim of current investigation was to appraise the effect of nerolidol in standard model of arthritis (chronic immunological Freund's complete adjuvant rheumatoid arthritis).

The study was also intended to support the possible mechanism of action of nerolidol at molecular levels and to discover it's efficacy against joint infection.

Materials And Methods

Drugs and chemicals

Nerolidol (Sigma-Aldrich, USA), CFA (Sigma-Aldrich, USA), trizole solution, cDNA synthesis kit (Gene Direx, USA), forward / reverse primers (Gene Direx, USA), Cybergreen, deionized water, ethanol, chloroform.

Animals

Sprague Dawley rats (150-250g) of either sex were used for investigational procedure. Animals were housed at animal house of Department of Pharmacology, College of Pharmacy, University of Sargodha with recommended housing conditions. All animals fed on water and standard diet and they were controlled by following the guidance in accordance with National Research Council. All tests were approved by animal ethics and review committee at University of Sargodha (Approval NO. SU/Pharm/Animal Ethics Approval/2019/215).

Complete Freund's adjuvant induced rheumatoid arthritis model

Animals were separated randomly into six different groups (n=5). The 1st group designated as normal control group and 2nd group (arthritic control group) received 2% tween 80 (3mL/kg). The 3rd group (standard group) was given naproxen 20mg/kg while 4th, 5th and 6th group served as treatment groups and received 200, 400 and 800mg/kg of nerolidol respectively. Arthritis was persuaded by inoculation of 0.1mL of CFA injection (containing 1mg/mL of heat killed *M. tuberculosis* in 0.15mL mono-oleate and 0.85mL paraffin oil) into left hind footpad of each rats except normal control group. The day of CFA shot was served as 0 day. The oral administration of different doses to treatment groups was continued for 28 days consecutively. Arthritis in all groups were evaluated by recording various factors (Mahdi et al., 2018).

Evaluations of arthritis from body weights and paw volume of rats

During the period of treatments, body weight of each rat was observed at every 7th day. Paw size/oedema was measured using digital plathysmometer. Percent inhibition in paw oedema /size was calculated by following this equation.

$$\text{Percent inhibition} = \frac{vc - vt}{vc} \times 100$$

"VC" and "VT" are the paw volume of arthritic control and treatment group.

Assessment of arthritis from serum and blood

At 28th day, all rats were sacrificed and through cardiac puncture blood was collected for the assessment of biochemical and haematological markers comprising WBCs, RBCs, Hb, c-Reactive Proteins, RF, ESR, Platelets, ALP, SGOT, SGPT, Creatinine and urea. These tests were performed at diagnostic center, University of Sargodha (Hassan *et al.,* 2019).

Estimation of mRNA expression levels of TNF-α, IL-1β, IL-6, COX-2, IL-4, NF-Kβ and IL-10.

Collected blood samples were used for appraisal of expression of mRNA TNF-α, IL-1β, IL-6, COX-2, NF-Kβ, IL-4, and IL-10. TRIzol method was used for the extraction of total RNA from blood. According to this method, into 500uL of blood, 700uL of trizole solution was added. It was mixed gently and incubated it for 10 minutes. Then 200uL of chloroform was added into it. Shake thoroughly and placed it in the centrifuge machine for 15 minutes at 12000 rpm and 4ºC. The aqueous layer was taken and then added 500uL of isopropanol into it and again mixed vigorously. Samples were incubated for 10 minutes and retained it in the centrifuge machine at 12000 rpm and 4 ºC for 15 minutes. Discarded the supernatant solution and the RNA pellet was washed with absolute ethanol. Air dried the RNA pellet and added 30uL of purified water into it and then quantified the RNA from Nano drop reader. After this, cDNA was synthesized by following the kit manufacturer procedure (Gene- Direx). In brief, 1uL of RNA solution, 1uL of oligo (dT) 20, 1uL of dNTP Mix and then RNA free water was added into it. The reaction mixture was heated at 65°C for 3-5 minutes, spin it and placed promptly

on frost. Then added 4uL of 1st strand buffer, 1uL DTT, 1uL of Script RTase and finally made volume up to 20uL. Incubated it for 30-60 minutes at 50°C and the enzyme was inactivated at 70°C for 15 minutes.

Real time quantitative PCR was used to intensify and quantify the reaction by using Bio-Rad scheme in Pharmacology department, University of Health Sciences, Lahore. Afterwards, templates of cDNA were mixed with qPCR master mix and added the specific primers of genes, nuclease free water, and then placed it in a thermal cycler for 45 cycles with denaturation temperature at 95°C, annealing at 56°C, extension at 72°C and then terminated the reaction at 72°C. Various markers of genes were nominated from Ensemble Genome Browser for determination of specific gene primers physically by using Input primer 3 (v. 0.4.0.) which is available online software. The sequences of primers are provided in Table1 (Shabbir *et al.,* 2016, Lim *et al.,* 2017).

ELISA (Enzyme linked immuno-sorbent assay) for Prostaglandin E_2

ELISA test was performed for quantitative identification of rat Prostaglandin E_2 in serum samples according to kit manufacturer procedures (rat Prostaglandin E_2 ELISA kit, Bio-assay technology laboratory having Cat No. E0504Ra, standard curve range = 0.05ng/ml-15ng/ml, size = 96 wells, sensitivity= 0.026ng/ml). By adding acidic solution, reaction was terminated and absorbance was measured at 450nm (micro-plate reader with 450 ± 10nm).

Estimation of peroxidase antioxidant enzyme activity

Peroxidase activity was measured by determining its capability to decrease hydrogen peroxide at wavelength of 470nm (Zia et al., 2011). The 0.06mL of enzyme extract was added in 3mL of buffer substrate solution that comprised of 47mL of phosphate buffer (0.2M), 0.7mL of guaiacol and 0.32mL of H_2O_2. After three minutes of enzyme reaction, optical density was measured at 470 nm spectrophotometer against blank (phosphate buffer guaiacol). Peroxidase activity was measured by using the undermentioned formula.

$$\text{Peroxidase activity (U/mL)} = \frac{\Delta A}{26.6 \times 0.06 \times 3.0}$$

A = Absorbance at 470nm, 26.6= extinction coefficient of guaiacol ($Mm^{-1}cm^{-1}$, 0.06= volume of enzyme extract (mL), 3.0 = volume of phosphate buffer (mL)

Estimation of catalase antioxidant enzyme activity

Assay was performed for determining the catalase activity of antioxidant enzymes and its ability was checked to reduce H_2O_2 at 240nm. Reaction mixture was comprised of 3.0mL of K_2PO_4 buffer (50Mm, pH 7), 0.1mL of hydrogen peroxide (30Mm) and 0.1mL of enzyme extract. Absorbance was observed after 3 minutes of reaction time at 240nm (Chance and Maehly, 1955).Catalase activity was determined by given formula

$$\text{Catalase activity} = \frac{\Delta A\ 3}{0.04 \times 0.01} \times 3$$

A3= Absorbance at 240nm, 0.04= Extinction coefficient for H2O2 ($M^{-1}CM^{-1}$)

Evaluation of superoxide dismutase

The SOD bustle was performed to check its ability to inhibit the photo reduction of nitro-blue tetrazolium. This test was performed by adopting the protocol with slight modification as discussed by Worthington 1988. Assay mixture was contained 1mL of 0.0067M potassium phosphate buffer (7.8pH), 0.05mL extract of enzyme and 0.016mL of 0.012mM solution of riboflavin. The reaction mixture was incubated in a light box for 12 minutes. After that, 0.067mL of EDTA/NaCN solution and 0.033mL of nitroblue tetrazolium solution was added into the reaction mix. After 30 second of reaction time, the absorbance was observed against blank through spectrophotometer at wavelength of 560nm. The activity of SOD was calculated by under mentioned formula:

$$\text{Percent inhibition} = \frac{A\ (Blank)\ A\ (Sample)}{A(Blank)} \times 100$$

Histopathological assessment of ankle joints

At the end of the treatment, ankle joints of arthritic control and treated rat paws were collected and static in 10% solution of formalin for the assessment of histopathology of joints (Shabbir *et al.,* 2014).

Radio graphical assessment of joints

The legs were removed at knee joints and were subjected for radio graphical assessment with computerized radio graphical system (Toshiba 630 M) (Uttra and Hasan, 2017).

Results

Effect of nerolidol on morphological deviations and paw volume

The results specified in Fig. 1 describe that treated groups revealed a significant increase p<0.01) in paw oedema on day-1 but paw volume decreased significantly (p<0.001) throughout the period. A significant reduction (75.99, 79.97, and 81.54%) in paw volume was detected on 28 day of treatment for the dose of 200, 400 and 800mg/kg, respectively. Moreover, primary marks of chronic inflammation like arthralgia, redness, immobility and swelling of joints were substantially low in rats treated with nerolidol and naproxen sodium, respectively, compared to arthritic control. There was a noteworthy reduction in body weight from 207.00 ± 13.20 to 152.60±15.29g that was observed in arthritic rats during the treatment period. However, significant upsurge in weight was observed in rats treated with nerolidol at 200, 400 and 800mg/kg on 28^{th} day of treatment. Major difference was found in weight gain when dose doubled from 200mg/kg as described in Fig. 2 on day 7.

Effect of nerolidol on biochemical and haematological parameters

Haematologic alteration was observed in injected CFA arthritic control rats. Among them significant decline in hemoglobin RBCs count, increase in value of platelets, WBCs and ESR , ALP, SGPT, SGOT, creatinine, urea and C-reactive proteins had documented. Oral administration of nerolidol significantly increased the RBCs and hemoglobin while noticeably reduction in the liver enzymes, WBCs, C reactive proteins, ESR, Platelets, creatinine and urea was observed equated to the arthritic control rats as revealed in Table 2. These results showed protective effects against the signs of nephrotoxicity and hepatotoxicity at doses of 200, 400 and 800mg/kg. Moreover, nerolidol significantly decreased the RF values in CFA injected rats.

Nerolidol inhibits the mRNA expression level of TNF-α, IL-6, IL-1β, COX-2, NF-$_K$β, and induces the expression of IL-10 and IL-4

On 28^{th} day of treatment, blood was collected and RT-qPCR was performed for the evaluation of different inflammatory cytokines. Augmented manifestation of TNF-α was identified in diseased control rats (6.98 ± 0.26) and marked reduction in TNF-α was noticed in nerolidol treated rats at 200, 400 and 800mg/kg (3.09 ±0.28, 2.84 ±0.23 and 2.67 ± 0.16) respectively and overall percentage inhibition was 16.6%. Treated rats with nerolidol and naproxen revealed (p<0.001) significant decline in IL-1β (3.14±0.204, 2.93±0.22, 1.97±0.04 and 3.14±0.17) normalized with adjuvant control rats. Similarly, a noticeable decrease in IL-6 was 2.87 ± 0.08, 2.48 ± 0.18 and 1.92 ±0.12 in rats treated with nerolidol 200,400 and 800mg/kg respectively as compared to arthritic control rats (3.5 ± 0.32). We found a prominent decrease (p<0.001) in the expression of COX-2 in rats treated with nerolidol 200mg/kg (3.72±0.15), 400mg/kg (3.26±0.09) and 800mg/kg (3.12±0.097) as compared to expression of COX-2 in arthritic rats (7.58±0.408). Besides, significant reduction in NF-kβ (2.6 ± 0.17, 2.56 ± 0.08, 2.0 ± 0.07, 3.0 ±0.09) was found in nerolidol and naproxen treated rats as equated to arthritic rats (5.50 ± 0.17). The level of IL-4 and IL-10 was increased in rats treated with nerolidol (800mg/kg) and naproxen verses arthritic rats as shown in Figure 3 (A, B, C, D, E & F).

Effect of nerolidol on Prostaglandin E_2

The animals treated with nerolidol (p<0.001) revealed a significant decrease in level of PGE_2 (0.77±0.04, 0.57±0.02 and 0.74±0.037) as compared to diseased animals (1.18±0.03). Maximum effect was achieved at 400mg/kg (51.6%) of nerolidol in contrast to other doses (Figure 4). Nerolidol at medial dose, it was 14.6% more effective than naproxen sodium 20mg/kg (0.74±0.08).

Effect of nerolidol on antioxidant (SOD, POD and CAT) activities

The nerolidol by oral administration at the doses of 200, 400 and 800mg/kg, increased the antioxidant enzyme activity of SOD by 28.76, 33.66 and 44.76% respectively and induced the protective defense mechanism inside body. Catalase is also an alternative enzyme that work together with SOD and POD through antioxidant defense system of enzymes that breakdowns the H_2O_2 into O_2 and water that protects the cell from O_2 toxicity and lipid peroxidation. Nerolidol at 800mg/kg shows significant increase ($p<0.001$) in enzyme activity of catalase (47.41%). Similarly, peroxidase activity was 67.17% at 200mg dose of nerolidol for 28 days as compared to arthritic control. It was found that the highest concentration of nerolidol was 1.5% more effective than naproxen sodium.

Effects of nerolidol on histopathological assessment of rats ankle joints

Evaluation of histopathology of ankle joint was conceded at day 28 of treatment. Histopathology of normal control rats showed that they exhibited normal joint space with intact articular cartilage and synovial tissues having no signs of inflammation with compact arrangements of cells. Ankle joint of arthritic control rats showed noticeable synovial lining, distinct propagation of synoviocytes and incursion of inflammatory cells with granulous and pannus development. Similarly, the erosive changes in bone and cartilage as well as deposition of collagen fibers were also observed in arthritic control groups (Figure 5). However, naproxen (20mg/kg) showed mild incursion of inflammatory cells, cartilage destruction, and thickening of synovial intergalactic space and decreased pannus development. Also, oral administration of nerolidol at 800mg/kg showed considerable fortification against proliferation of vascular lesions, limited space between joints, cartilage destruction and small number of inflammatory cells with no pannus development that resemble to a very much normal architecture of joints. Whereas, the 400mg/kg dose of nerolidol showed a moderate cartilage destruction with little cellular invasion and pannus formation. Alternatively, nerolidol treated rats with 200mg/kg exhibited little reduction in cartilage with minor invasion of inflammatory cells and no formation of pannus.

Effect of nerolidol on radio graphical changes

The radio graphical examination is useful for the analysis of rheumatoid arthritis for estimation of severity and progression of disease. It has been found that reduction in joint space results in loss of cartilage which initiates the variety of irrational mechanism. X-rays of normal rats showed normal morphology and architecture of cartilage with no swelling around joints. While examination of x-rays of arthritic control rats showed that they possessed narrowing of joint space, severe swelling of tissues and bone erosion. In contrast, rats treated with nerolidol 800mg/kg exhibited good inhibition of tissue inflammation, bone injury and narrowing of joint space and joint distortion. Likewise, rats treated with nerolidol 400mg/kg showed moderate protection against swelling, bone erosion and joint deformity. Similarly, moderate changes occurred in soft tissues of joints with distinct reduction in joint space were also observed in naproxen treated rats as illustrated in Figure 6.

Discussion

In the present study we have piloted for the 1st time that nerolidol alleviated the CFA arthritis in rat's model of arthritis with pathological structures similar to those of human rheumatism (Lin *et al*., 2013). Rheumatism is a chronic demagogic autoimmune disorder that targets the cartilage, bones and synovial membrane (McInnes and Schett, 2007). However, exact etiopathagenesis is not yet known but significant validations have confirmed that liberal demolition of bone and cartilage in rheumatism results from synovial neovascularization, increased manifestation of proinflammatory cytokines, osteoclast interceded bone desorption and suspension of cartilage articular matrix mediated by proteinase (Liu *et al*., 2013). After CFA injection inoculation, inflammatory reactions begin within few days, as secondary lesions come after primary lesions within 2 weeks (Alamgeer *et al*., 2017). CFA comprised of temperature killed *M. tuberculosis* dissolved in liquefied paraffin that activates the cell arbitrated immunity, thus accelerates the creation of antibodies (Kim *et al*., 2016). *Mycobacterium* are associated in the formation of oedema that may be due to invasion of extracellular fluid and protein debris at the site of inflammation and it is comprised of three phases (induction phase, early synovitis and late synovitis) (Bose *et al*., 2014, Woode *et al*., 2009). So, nerolidol significantly reduced the paw volume as compared to arthritic paw volume of rats. It is possible that drug prevented the invasion of fluid into the joints or may be due to its anti-inflammatory effect together with immunomodulatory property by activating the MAPKs pathway (Valdivieso-Ugarte *et al*., 2019, de Cássia Da Silveira e Sá *et al*., 2015).

Rheumatoid cachexia was observed in rheumatism characterized by appetite and weight loss that is due to increased production of cytokines, which accelerates the proteolysis and resting metabolism (Adeneye *et al*., 2014). Proinflammatory cytokines TNF-α, IL-6 and IL-1β are key elements of synovitis and also stimulate the NF-kβ; causes the proteolysis of muscles by activating the proteasome pathways. Overexpression of these pro-inflammatory cytokines induces the anorexia and ultimately muscle loss. Besides that, consumption of glucocorticoids as pharmacological symptomatic treatment of RA might aggravate the rheumatoid cachexia (Masuko,

2014). The observed cachexia may also be due to muscle proteolysis induced by lysosomal protease that is mediated by decrease absorption of ^{14}C-glucose and ^{14} C-leucine and also prostaglandin-E_2 in the intestines of rats. Anti-inflammatory medications have the capacity to recover the damage caused by the disease (Alamgeer *et al*., 2017). Similarly they also decreased the expression of pro-inflammatory cytokines and also resolved the intestinal absorption of rats (Ahsan *et al*., 2021). In addition, in CFA persuaded arthritis, we also evaluated the biochemical and haematological tests. Anemia is the most common haematological abnormality in rheumatoid arthritis. From the findings of this study, it was apparent that decrease in haemoglobin level and RBCs count in arthritic rats, signify the anemia, that may be due to the destruction of premature RBCs, decreased level of erythropoietin and decreased iron loading in the synovial tissue of reticuloendothelial system and synovial tissues (Alamgeer *et al*., 2017). Nerolidol caused the notable increase in hemoglobin and RBCs possibly due to the decreased manifestation of NF-Kβ. So it normalized the invasion of inflammatory cells in the synovial fluid and ultimately inhibited the cascade of inflammation (Iqubal *et al*., 2019). Besides, the level of platelets and WBCs were augmented in arthritic rats due to the excretion of TNF-α and IL-6, (Fig. 5A, 5B) these cytokines initiates acute phase reaction in the rheumatoid arthritis. Though, nerolidol significantly decreased the level of platelets and WBCs in rats by preventing the manifestation of IL-6 and TNF-α, it is might be due to its immunomodulatory effect.

The overexpression of the pro-inflammatory cytokine causes the accumulation and release of ROS. When the production of reactive species is higher than natural antioxidant defense system, oxidative stress destroys normal physiological function of the DNA, proteins and lipids (Valdivieso-Ugarte *et al*., 2019). ESR and C-reactive protein are the primary markers for the early analysis of RA. The augmentation of CRP and ESR in the arthritic rats showed the presence of infective proteins in the blood while these levels were considerably reduced ($p < 0.001$) in rats treated with naproxen and nerolidol. Since its values relate with the radiological progression of disease (Babu *et al*., 2014). Treatment with nerolidol prevented the alleviated reactions of acute phase, when equaled with arthritic control rats. A striking decrease in RF values in treatment groups ensured the protective effect of nerolidol against arthritis. The factor RF is formed against Fc portion of autoantibody and initiates the immune cascade towards the progression of RA. Moreover, excessive concentration of serum ALP in diseased control group may be due to increased bone erosion and integrity of lysosomal enzymes (Chakraborty *et al*., 2010). However, administration of different doses of nerolidol to respective groups significantly reduced the ALP, inhibited the atypical rise in SGPT and SGOT that is might be due to reduction in bone loss and improved steadiness of lysosomes.

Proliferating synovial cells, T lymphocytes and macrophages play a crucial role in the progression of arthritis (Goronzy and Weyand, 2001). Proinflammatory cytokines are produced by macrophages and stimulated T cells that is involved in the initiation and progression of rheumatoid arthritis (Vandooren *et al*., 2009). T cell mediated immune reaction activates the discharge of pro-inflammatory cytokines and accelerates the formation of antibodies which lead to joint destruction. Over expression of these cytokines causes the irreparable proliferation of tissues, tissue eradication, bone loss and automatic cell death (Zou *et al*., 2013). Hence, potential antidotes against these cytokines has ability to protect the cells from inflammation (Shin *et al*., 2016). Therefore, in current investigation effect of nerolidol on expression level of proinflammatory (TNF-α, IL-6, IL-1β), inflammatory cytokines (NF-kβ, COX-2) and anti-inflammatory cytokines (IL-4, IL-10) were studied by using RT-qPCR. It has been attributed that IL-1β induces the fabrication of (MMPs) matrix metalloproteinase and instigation of osteoclast and ultimately bone erosions (Barksby *et al*., 2007). In addition, it is described that endogenous inhibitors of MMPs are tissue inhibitors of MMPs (TIMPs). In the manifestation of soluble IL-6R, IL-6 induces the fabrication of TIMPs in synovial fibroblast and cultured chondrocytes. The protective catabolic response produced in tissues are important for extracellular matrix turnover (Silacci *et al*., 1998). From present findings it was suggested that anticipatory effect of nerolidol on joint demolition was arbitrated to the prevention of induction of IL-6 induced RANKL and formation of MMPs via suppression of IL-6 followed by osteoclastogenesis. Therefore, therapeutic agents that reduced the IL-6, IL-1β and TNF-α categorized a major invention in the treatment of RA. Augmented level of these proinflammatory cytokines was observed in arthritic control rats, however, significant ($p < 0.001$) decrease in the expression of these cytokines were detected in rats treated with nerolidol at all doses as shown in figure (3B and 3C) which suggested its anti-arthritic and anti-inflammatory role. NF-kβ is involved in the activation and differentiation of osteoclasts which causes development of Th1 response and bone resorption. It has been already been shown that NF-kβ controls the manifestations of TNF-α, later, it works as an potent inducer for the stimulation of NF-kβ (Shabbir *et al*., 2016). The existing study elucidates that increased level of NF-kβ in diseased rats was significantly inhibited by nerolidol on last day of treatment.

The level of COX-2 is increased at the site of injection and they up-regulates the formation of prostaglandin-E2 that mediates the angiogenesis, vasodilation, extravasation of fluid and vascularization in the synovial membrane (Shabbir *et al*., 2016). Overproduction of inflammatory cytokines causes the increased expression of PGE_2. This augmented level of PGE_2 accelerates the differentiations of osteoclast and generating the degrading enzymes and hence stimulates the bone erosion, vasodilation and migration of leukocytes at site of inflammation. So, any substance that prevents the generations of aforementioned mediators carries great importance in the

management of rheumatism (Manan *et al.*, 2020). In current study, increased expression of COX-2 and PGE-2 were noted in diseased controlled rats, however, significant decrease in COX-2 and prostaglandin-E2 were observed in rats treated with nerolidol. Hence, we suggest that nerolidol protected the animals from joint inflammation, may be due to decreased production of prostaglandins and significant inhibition of COX-2 manifestations. Consequently, prevention of arachidonic acid metabolism might be another mechanism of action of nerolidol possessing anti-arthritic activity.

Moreover, IL-4 is an anti-inflammatory cytokine and maintains the production of Th2 cells and inhibits the autoimmune reaction mediated by Th1 cells (Schulze-Koops and Kalden, 2001). The IL-10 also has capability to inhibit the cellular defense mechanism by inactivating the macrophages in response and modifies the synovitis of rheumatoid arthritis (Bozkurt *et al.*, 2006). In the progression of RA, IL-10 not only inhibit the Th1 mediated immune response but also down regulate the function of antigen presenting cells and protects the reliability of joint (Uttra *et al.*, 2018) as same was observed with tested drug as shown in Fig. 3D.

In histopathological assessment, inflamed joints exhibited the continuous migration of polymorph -nuclear leukocytes, macrophages and lymphocytes into joints and synovium. They all produce the inflammatory cytokines in the synovial fluid and joints. Thus, inhibition of migration of macrophages, leukocytes decreased the accumulation of inflamed debris in joints and may have beneficial effect for the protection of joints (Gao and Issekutz, 1994, Wilder *et al.*, 1989). Our histopathological examination suggested that the nerolidol treated groups inhibited the migration of these leukocytes and protects the joint from hyperplasia, joint deformity, pannus formation and destruction of cartilage as shown in Fig. 5. Moreever, X-ray examination revealed that the nerolidol showed significant protection against expansion of subordinate lesions, development of bone desolation and from destruction of cartilage in contrast to arthritic control rats as shown in Fig. 6.

Free radicals and ROS have been stated as mediators in the pathogenesis of RA and cause tissue destruction. In rheumatism, affected articulations are penetrated by different cells like dendritic cells, macrophages and neutrophils. These cells generate the reactive oxygen species that liberate in greater amount at the site of inflammation and overcome the antioxidant enzyme defense system. So, they induce the annihilation of affected joints because during phagocytosis superoxide anions are produced that activate the NF-Kβ dependent manifestation of pro-inflammatory cytokines through second messenger system (Babu *et al.*, 2014). These reactive species cause the peroxidation of lipids, enzyme inactivation, oxidation of proteins, DNA damage and other changes in the cell organelles (Sghaier *et al.*, 2011, Valdivieso-Ugarte *et al.*, 2019). Endogenous enzymes of antioxidant like catalase protect them from tissue damage caused by the reactive species. Catalase involved in lessening the level of hydrogen peroxide in synovial tissues. SOD is the antioxidant enzyme that catalyzes the superoxide into hydrogen peroxide and oxygen free radical which is further catalyzed by catalase enzymes into water and oxygen molecules. In current study, nerolidol was able to up regulate the antioxidant enzymes activity of POD, SOD and CAT and considerably ($p < 0.001$) decreased the lipid peroxidation in the synovial fluid persuaded by release of ROS. This increased the concentration of antioxidant enzymes in the synovial fluid that diminished the destruction of cartilage and tissue deformity in the ankle joint of treated rats. Lipid peroxidation is the process in which poly unsaturated fatty acids undergo the oxidation process and cause functional abnormality in the cell membrane. However, nerolidol decreased the lipid peroxidation level, so they act as an *in-vivo* antioxidant agents and reduce oxidative stress.

Conclusion

In a nutshell, oral administration of nerolidol in arthritic rats significantly reduced the paw volume, regained body weight, normalized the altered biochemical and haematological parameters and also inhibited mRNA expression level of the above-mentioned cytokines. So anti-arthritic effect of nerolidol may be due to its capability to decrease the level of NF-$_{K}$β, COX-2, PGE2, TNF-α, IL-1β, IL-6 and by increasing the level of IL-4 and IL-10 and antioxidant enzyme activity of SOD, POD and CAT. Hence, nerolidol may be recommended as a therapeutic substance for the treatment and management of severity of rheumatism for clinical trials.

Declarations

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Declaration of competing interest

All authors declare that they do not have any known competing interest.

Availability of data and material

All the data of this study is transparent.

Code availability

Not applicable

Author contributions

The study conceptualization was designed by Dr. Hafiz Muhammad Irfan and Dr. Alamgeer; methodology and research work was developed by Miss Shanila Akhter, manuscript draft was prepared by Dr. Muhammad Bilal Latif, PCR analysis was performed by Dr. Shah Jahan and Dr. Muhammad Shahzad.

Ethics approval

The experiments conducted on animals were performed according to animal ethics guidelines with approval NO. SU/Pharm/Animal Ethics Approval/2019/215

Consent to participate

We the authors give consent as a participant in this study.

Consent for publication

All the contributing authors provide consent for the publication of this study in your journal

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Tables

Table 1: Forward-reverse primers sequences with respect to gene markers used in real time - PCR

Gene Markers	Forward Reverse	Sequences	Base pair (amplified band)
IL-1β	Forward Reverse	5′-GCTGTCCAGATGAGAGCATC-3′ 5′-GTCAGACAGCACGAGGCATT-3′	293
IL-6	Forward Reverse	5′-AGACTTCCAGCCAGTTGCCT-3′ 5′-CTGACAGTGCATCATCGCTG-3′	233
COX-2	Forward Reverse	5′-GCATTCTTTGCCCAGCACTT-3′ 5′-GTCTTTGACTGTGGGAGGAT-3′	210
TNF-α	Forward Reverse	5′-AGGACACCATGAGCACGGAA-3′ 5′-GGGCCATGGAACTGATGAGA-3′	234
NF-Kβ	Forward Reverse	5′-GCAACTCTGTCCTGCACCTA-3′ 5′-CTGCTCCTGAGCGTTGACTT-3′	203
IL-4	Forward Reverse	5′-CACCTTGCTGTCACCCTGTT-3′ 5′-CCTGCAGATGAGCTCGTTCT-3′	195
IL-10	Forward Reverse	5′-GCCCAGAAATCAAGGAGCAT-3′ 5′-CGTAGGCTTCTATGCAGTTG-3′	210

Table 2: Effect of oral administration of nerolidol on biochemical and haematological parameter in CFA induced model

Biochemical and hematological parameters	Arthritic control	Normal Control	Naproxen (20mg/kg)	Nerolidol (200mg/kg)	Nerolidol (400mg/kg)	Nerolidol (800mg/kg)
SGOT (U/L)	149.61±0.26	110.83±0.09***	125.24±0.36***	134.62±0.32***	127.49±0.26***	120.84±0.10***
SGPT (U/L)	49.95±0.04	21.05±0.02***	35.03±0.02***	37.33±0.33***	33.72±0.31***	32.29±0.28***
Urea (mg/dL)	37.66±0.32	18.58±0.26***	27.89±0.04***	28.36±0.31***	26.42±0.21***	22.95±0.42***
ALP (U/L)	312.32±0.27	158.58±0.25***	203.00±1.00***	201.65±0.88***	195.44±2.35***	192.07±0.70***
CREAT (mg/dL)	0.99±0.00	0.46±0.00***	0.58±0.00***	0.63±0.02***	0.57±0.00***	0.51±0.00***
CRP (mg/dL)	39.69±0.12	3.91±0.07***	12.48±0.49***	13.94±0.03***	10.33±0.32***	8.98±0.54***
RF (IU/L)	35.75±1.2	6.38±0.41***	9.78±0.11***	11.32±0.65***	9.62±0.03***	8.61±0.31***
ESR (mm/h)	9.58±0.29	3.55±0.04***	4.88±0.00***	4.98±0.00***	4.82±0.01***	3.96±0.03***
PLAT (10^3/UL)	1321.08±0.30	941.21±0.33***	963.22±3.18***	983.66±3.18***	963.67±1.20***	955.36±0.31***
WBCs ($10^{3/}$µL)	14.03±0.22	8.08±0.48***	12.27±0.64*	10.97±0.45***	8.33±0.21***	6.96±0.29***
RBCs ($10^{6/}$µL)	5.25±0.25	9.30±0.23***	7.10±0.06***	7.58±0.22***	8.10±0.07***	8.48±0.33***
Hb (g/dL)	9.13±0.09	14.76±0.06***	11.69±0.30***	11.05±0.03***	12.90±0.03***	14.26±0.25***

Note: Values are expressed as mean ± *SEM* (n=5) followed by one-way ANOVA with Dunnet posttest. *** $p<0.001$, the values were significant as compared to arthritic control.

Table 3: Effect of nerolidol and other treatments given for 28 days on antioxidant enzyme activities

Treatment groups	POD (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
Arthritic rats	3.45±0.14	9.91±0.21	42.18±1.68
Normal rats	15.26±0.20***	20.00±0.00***	95.33±1.76***
Naproxen (20mg/kg)	13.83±0.02***	14.76±0.10***	67.07±0.41***
Nerolidol (200mg/kg)	10.51±0.07***	13.91±0.06***	63.73±0.96***
Nerolidol (400mg/kg)	12.95±0.03***	14.94±0.03***	73.91±1.03***
Nerolidol (800mg/kg)	14.05±0.03***	17.94±0.03***	80.21±0.28***

Values are articulated as mean ± SEM followed by one-way ANOVA with Dunnett posttest (***p< 0.001)

Figures

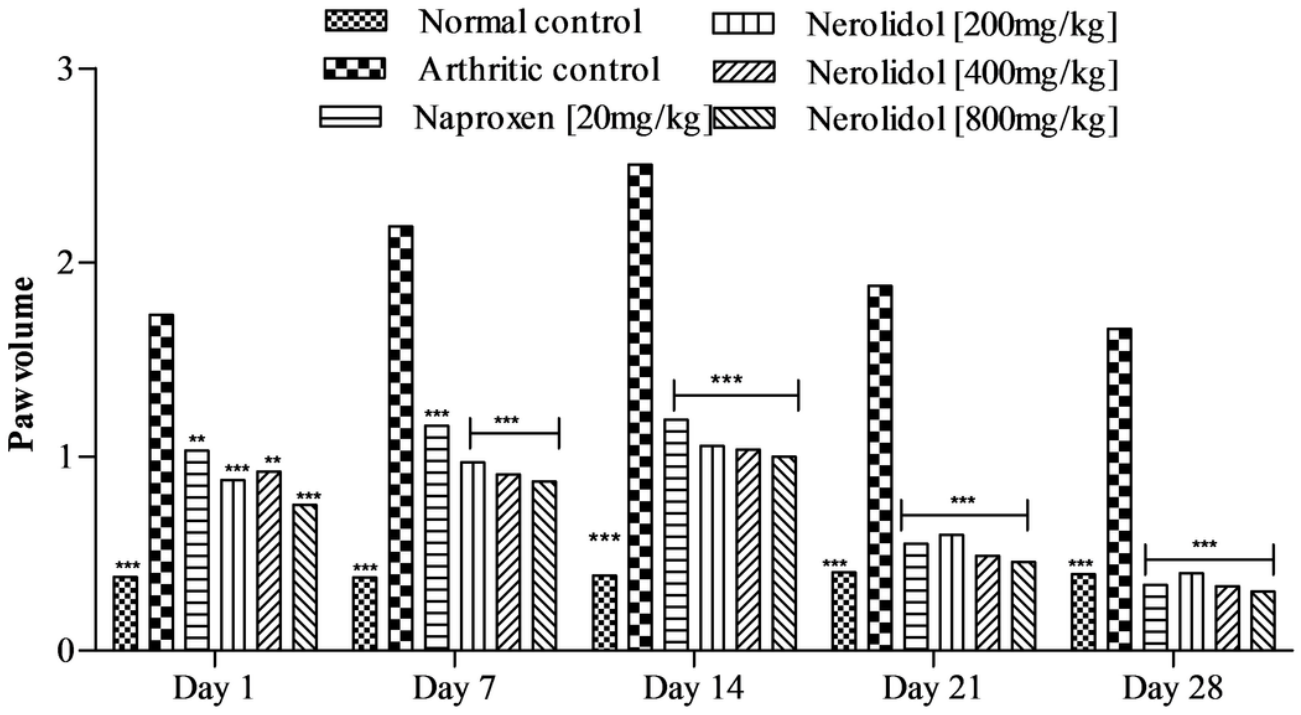

Figure 1

Effect of different doses of nerolidol given for 28-days on paw volume induced by CFA. Result was followed by Two way ANOVA using graph pad prism with Bonferroni posttest whereas ***p< 0.001, **p< 0.01

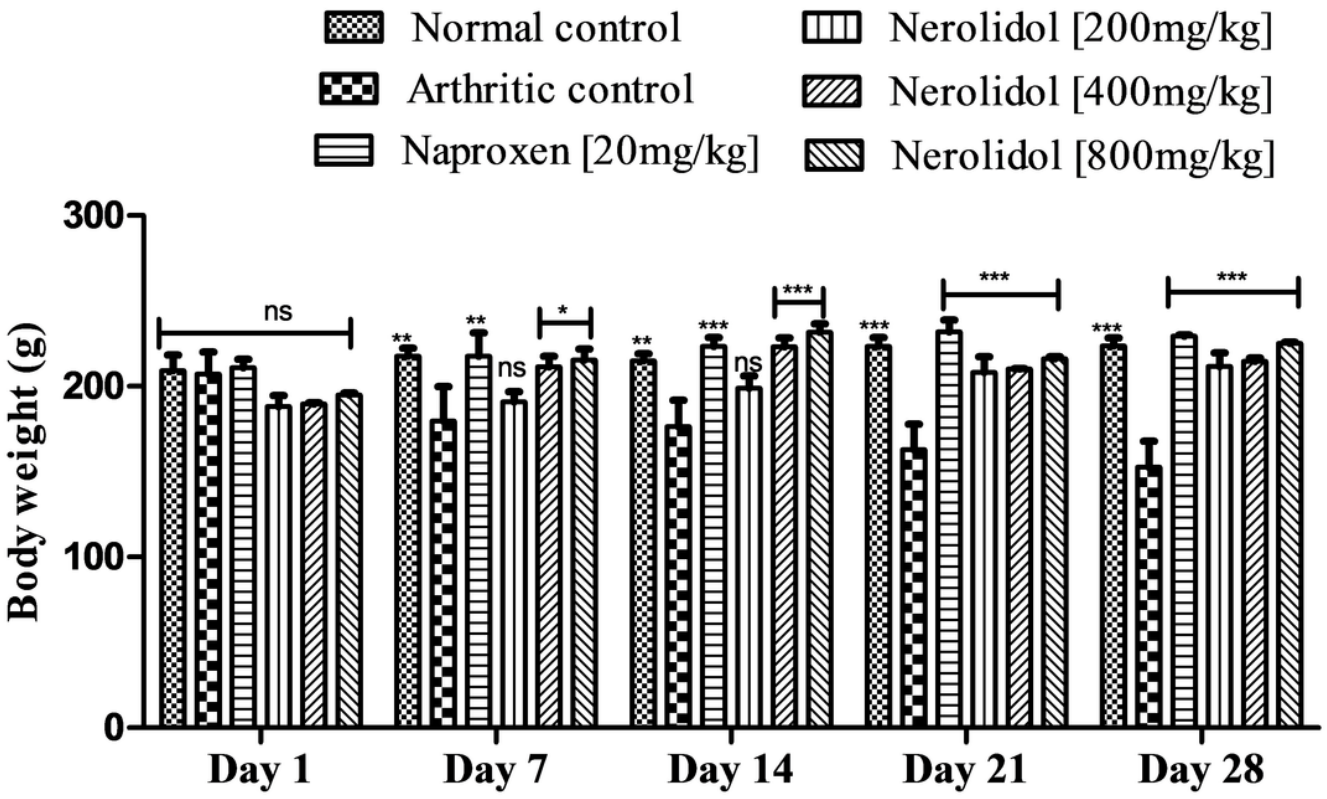

Figure 2

Effect of different doses of nerolidol given for 28-days on body weight of CFA-induced rats. Result was followed Two way ANOVA using graph pad prism with Bonferroni posttest whereas ***p< 0.001, **p< 0.01 and * p< 0.05.

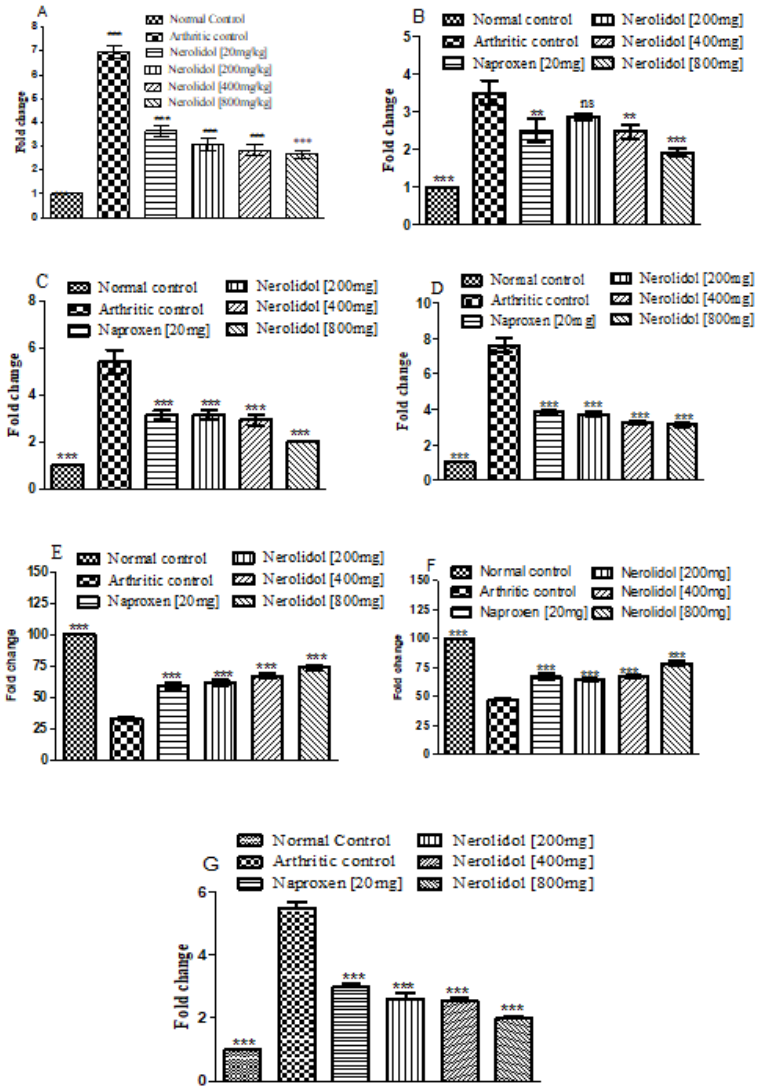

Figure 3

Expression of cytokines assessed by RT-qPCR treated with nerolidol for 28 days in CFA-induced model. Analyzed by one-way ANOVA with Dunnett posttest. ***p<0.001, **p<0.01 and ns is non-significant. Key: A (TNF-α); B (IL-6); C (IL-1β); D (COX-2); E (IL-4); F (IL-10); G (NF-Kβ)

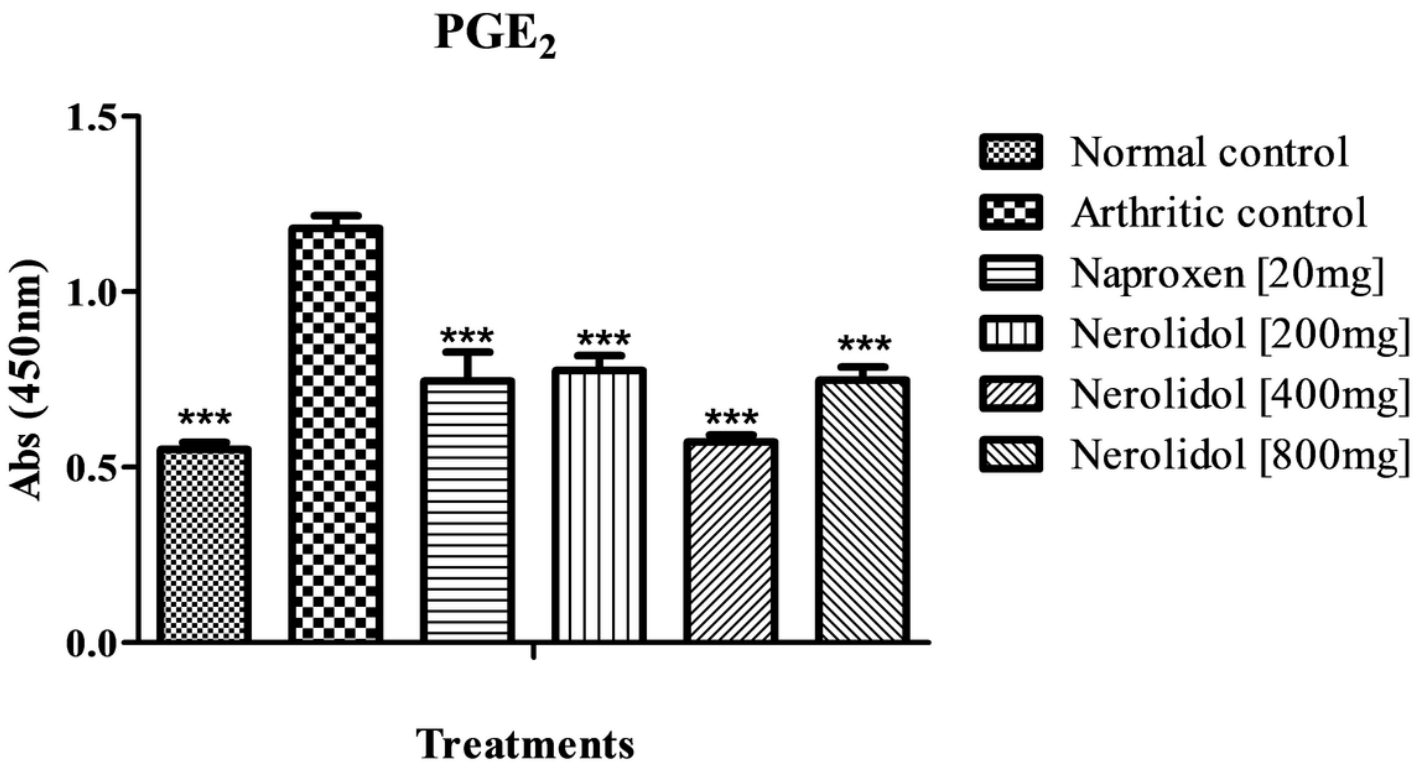

Figure 4

Effect of daily oral administration of nerolidol (200, 400 and 800mg/kg) and Naproxen for 28 days on Prostaglandin E2. The results were analyzed by using Mean±SEM followed by One-way ANOVA was performed (n=5), where ***p<0.001.

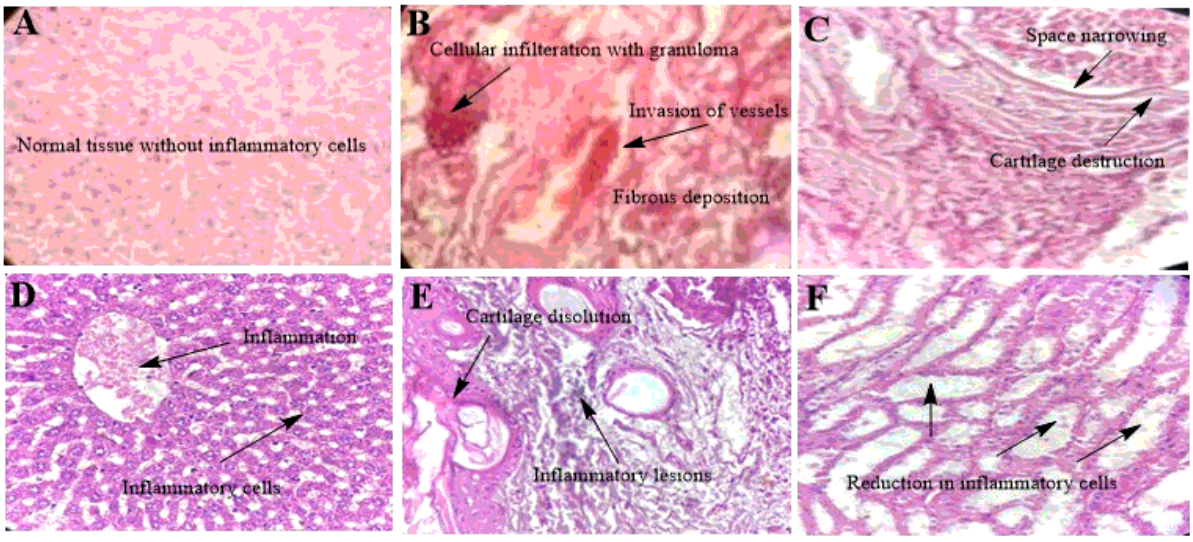

Figure 5

Effect of oral administration of nerolidol for 28 days on histological variations in frend's induced arthritis. Whereas A represents slide of normal rat possessing no inflammatory cells, B represent histology of arthritic control rat showing vessels invasions and fibrous deposition, while C, D, E and F represents slides of treated rats with naproxen and different doses of nerolidol (200, 400 and 800mg/kg) showing reduction in inflammatory cells.

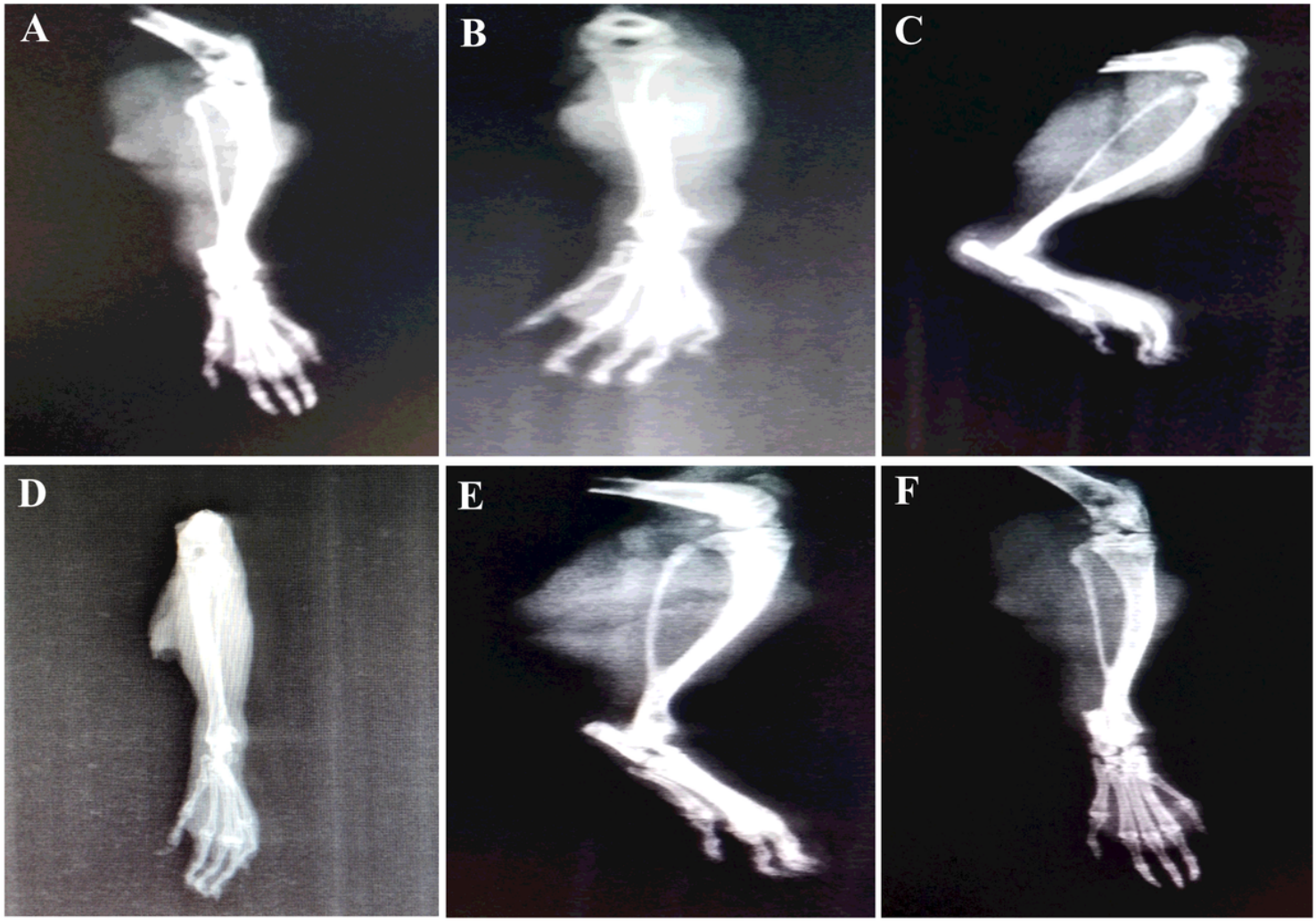

Figure 6

Pictorial representation of x-ray examination of treated rats versus arthritic control rat. Whereas A represents ankle joint of normal rat with normal morphology and architecture of cartilage, B represents ankle joint of arthritic control rat showed bone erosion and narrowing of joint space, C represents ankle joint of rat treated with naproxen while D, E and F represents ankle joints of treated rats with different doses of nerolidol (200, 400 and 800mg/kg) exhibited inhibition of space narrowing and tissue swellings.