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Abstract

Introduction

N-palmitoylethanolamine (PEA) is an endogenous fatty acid amide belonging to the family of the N-acylethanolamines (NAEs). Recently, several studies demonstrated that PEA is an important analgesic, anti-inflammatory and neuroprotective mediator. The aim of this study was to investigate the effect of co-ultramicronized PEA + luteolin formulation on the modulation of the inflammatory response in mice subjected to collagen-induced arthritis (CIA).

Methods

CIA was induced by an intradermally injection of 100 μl of the emulsion (containing 100 μg of bovine type II collagen (CII)) and complete Freund's adjuvant (CFA) at the base of the tail. On day 21, a second injection of CII in CFA was administered. Mice subjected to CIA were administered with PEA (10 mg/kg 10% ethanol, intraperitoneally (i.p.)) or with co-ultramicronized PEA + luteolin (1mg/kg, i.p.) every 24 hours, starting from day 25 to 35.

Results

Mice developed erosive hind paw arthritis when immunized with CII in CFA. Macroscopic clinical evidence of CIA first appeared as peri-articular erythema and edema in the hind paws. The incidence of CIA was 100% by day 28 in the CII challenged mice and the severity of CIA progressed over a 35-day period with a resorption of bone. The histopathology of CIA included erosion of the cartilage at the joint. Treatment with PEA or PEA + luteolin ameliorated the clinical signs at days 26 to 35 and improved histological status in the joint and paw. The degree of oxidative and nitrosative damage was significantly reduced in PEA + luteolin treated mice as indicated by nitrotyrosine and malondialdehyde (MDA) levels. Plasma levels of the pro-inflammatory cytokines and chemokines were significantly reduced by PEA + luteolin treatment.

Conclusions

We demonstrated PEA co-ultramicronized with luteolin exerts an anti-inflammatory effect during chronic inflammation and ameliorates CIA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that results in multiple joint inflammations with subsequent destruction of joint cartilage and erosion of bone. Type II collagen-induced arthritis (CIA) in the mouse is a useful model of RA, as it possesses many of the cell and humoral immunity characteristics found in human RA [1]. The pathogenesis of CIA is dependent upon the host's response to type II collagen challenge and the subsequent generation of antibodies that recognize collagen rich joint tissue [1]. Moreover, the recruitment and activation of neutrophils, macrophages, and lymphocytes into joint tissues and the formation of the pannus are hallmarks of the pathogenesis of both CIA and human RA. Recently, it has been demonstrated that interleukin (IL)-8, MIP-lα, MIP-1β, and

RANTES are differentially chemotactic for lymphocyte subsets [2]. The current treatments for delaying RA progression include several disease-modifying anti-rheumatic drugs (DMARDs) and biological agents that act as immunomodulatory drugs in RA [3], some also act by inhibiting cytokines and endothelial cell proliferation [4]. Moreover, all of these compounds have potentially serious side effects and there are substantial differences in toxicity among DMARDs [5].

N-palmitoylethanolamine (PEA) is an endogenous fatty acid amide belonging to the family of the N-acylethanolamines (NAEs). PEA is an important analgesic, anti-inflammatory and neuroprotective mediator, acting at several molecular targets in both central and sensory nervous systems as well as immune cells [6]. Several mechanisms have been proposed to explain the anti-inflammatory and anti-hyperalgesic effects of PEA, including: (i) the activation of a cell surface receptor (i.e. the "CBn" (or CB2-like) or, alternatively, the orphan GPR55 receptor) or otherwise a nuclear receptor of the peroxisome proliferator-activated receptors (PPARs) family [7]; (ii) the down-modulation of mast cell hyper-activity (ALIA mechanism) [8]; (iii) an action as "entourage" compound, i.e. the augmentation of eCBs activities at their receptors and/or the inhibition of eCBs degradation [9]. Although its presence in mammalian tissues has been known since the 1960s, PEA has emerged only recently among other bioactive N-acylethanolamines as an important endogenous lipid modulator which, due to its chemical stability, can be also administered exogenously as the active principle of current anti-inflammatory and analgesic preparations [10]. Moreover, several evidences indicate that superoxide anions (O_2^-) perpetuate the chronic inflammatory state associated with RA. Thus, it follows that one therapeutic approach to treat RA is to remove these reactive oxygen species (ROS). Osteoclasts, chondrocytes, synovial cells neutrophils/macrophages and fragmented particles of degraded extracellular matrix are excellent sources of superoxide [11] as suggested from studies performed in animals models of arthritis [12] as well as in pilot experiments carried out in patients with active RA [13].

Flavonoids are natural products widely distributed in the plant kingdom and currently consumed in large amounts in the daily diet. Dietary flavonoids possess multiple neuroprotective actions in central nervous pathophysiological conditions including depression [14] and it was reported that naringenin has potent antidepressant-like properties via central serotonergic and noradrenergic systems. It was further suggested that dietary flavonoids possess a therapeutic potential in disorders especially where the monoaminergic system is involved [14]. Luteolin is a common flavonoid found in many types of plants such as Apium graveolens L. var. dulce [15], Petroselium crispum [16], and Capsicum annuum L. var. 'grossum.'[17]. It has various pharmacological activities such as antioxidant and anticancer action [18].

The purpose of study was to determine whether chronic administration of co-ultramicronized PEA + luteolin (PEA-LUT) would ameliorate development of arthritis using a CIA model.

Methods

Animals

Male DBA/1J mice (9 weeks, Harlan Nossan, Italy) were used for these studies. Mice were housed in individual cages (2 for each group) and maintained under 12:12 light–dark cycle at 21 ± 1 °C and $50 \pm 5\%$ humidity. The animals were acclimated to their environment for 1

week and had *ad libitum* access to tap water and standard rodent standard diet. All animal experiments complied with regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L 358/1 12/18/1986) and USA (Animal Welfare Assurance No A5594-01, Department of Health and Human Services, USA). All behavioral testing was conducted in compliance with the NHI laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee (Council directive # 87–848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permission # 92–256 to SC). The study was approved by the University of Messina Review Board for the care of animals (PRIN ID 1042).

Experimental groups

Mice were divided into the following five experimental groups:

CIA-Control; mice were subjected to collagen-induced arthritis and administered 200 µl of 10% ethanol solution (i.p., vehicle for PEA) every 24 h, starting from day 25 to day 35 (n = 20).

CIA + LUT; mice subjected to collagen-induced arthritis were administered LUT (1 mg/kg, 10% ethanol i.p.) every 24 h, starting from day 25 to day 35 (n = 20).

CIA-PEA; mice subjected to collagen-induced arthritis were administered PEA (10 mg/kg, 10% ethanol i.p.) every 24 h, starting from day 25 to day 35 (n = 20).

CIA-PEA-LUT; mice subjected to collagen-induced arthritis were administered PEA and luteolin (single treatment combination) (1mg/kg, i.p.) every 24 h, starting from day 25 to day 35 (n = 20).

Sham-Control; mice subjected to an intradermal injection at the base of the tail of 100 µl of 0.01 M acetic acid instead of the emulsion containing 100 µg of CII, were treated with 200 µl of 10% ethanol solution (i.p., vehicle for PEA), every 24 h starting from day 25 to day 35 (n = 20).

Sham-LUT; mice subjected to an intradermal injection at the base of the tail of 100 µl of 0.01 M acetic acid instead of the emulsion containing 100 µg of CII, were administered LUT (1 mg/kg, 10% ethanol i.p.), every 24 h starting from day 25 to day 35 (n = 20).

Sham-PEA; mice subjected to an intradermal injection at the base of the tail of 100 µl of 0.01 M acetic acid instead of the emulsion containing 100 µg of CII, were administered PEA (10 mg/kg, 10% ethanol i.p.), every 24 h starting from day 25 to day 35 (n = 20).

Sham- PEA-LUT; mice subjected to an intradermal injection at the base of the tail of 100 µl of 0.01 M acetic acid instead of the emulsion containing 100 µg of CII, were administered PEA and luteolin (single treatment combination) (1mg/kg, i.p.), every 24 h starting from day 25 to day 35 (n = 20).

PEA-LUT preparation was formulated through a co-ultramicronization process by jet milling technology. The ratio between PEA and luteolin is 10:1 by mass. The doses of PEA and LUT were chosen based on our recent studies [19,20] to compare possible differences with PEA-LUT formulation.

Induction of CIA

The induction of CIA was performed as described in a previous our study [21]. Chicken type II collagen (CII) was dissolved in 0.01 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Dissolved CII was frozen at −70°C until use. Complete Freund's adjuvant (CFA) was prepared by the addition of *Mycobacterium tuberculosis* H37Ra at a concentration of 2 mg/ml. Before injection, CII was emulsified with an equal volume of CFA. On day 1, mice were injected intradermally at the base of the tail with 100 μl of the emulsion (containing 100 μg of CII). On day 21, a second injection of CII in CFA was administered.

Clinical assessment of CIA

The development of arthritis in mice in all experimental groups was evaluated daily starting from day 20 after the first intradermal injection by using a macroscopic scoring system: 0 = no signs of arthritis; 1 = swelling and/or redness of the paw or one digit; 2 = two joints involved; 3 = more than two joints involved; and 4 = severe arthritis of the entire paw and digits [22]. Arthritic index for each mouse was calculated by adding the four scores of individual paws. Clinical severity was also determined by quantitating the change in the paw volume using plethysmometry (model 7140; Ugo Basile).

Behavioral assays

Rotarod

Locomotor abilities were assessed using a protocol previously employed [23]. DBA/1J mice were given three days of training on the rotarod before disease induction. Trials were conducted, starting the 20 day after CIA induction, every five day until day 35. DBA/1J CIA mice treated with vehicle, or PEA (10 mg/kg, i.p.) or PEA-LUT (1 mg/kg, i.p.) were placed for 3 min on the rotating beam of a rotarod (Ugo Basile Italy) that was rotating at a fixed rate of 16 rpm. Each mouse was given three trials, after which the average time of a mouse remained on the rotating beam was calculated.

Pain sensitivity testing

Hotplate testing was used to evaluate pain sensitivity as previously described [24]. Briefly, mice were placed on a 55 °C hotplate and observed by two individuals masked to treatment. The latency to a behavioral response was recorded. Behaviors included rearing, paw licking, paw stamping or jumping. Mice were removed from the hotplate after 30s if no response was observed.

Thermal hyperalgesia

Hyperalgesic responses to heat were determined by the Hargreaves' Method using a Basile Plantar Test (Ugo Basile; Comeria, Italy) [25] with a cut-off latency of 20 s employed to prevent tissue damage. Animals were allowed to acclimate within a Plexiglas enclosure on a clear glass plate in a quiet testing room. A mobile infrared generator was positioned to deliver a thermal stimulus directly to an individual hind paw from beneath the chamber. The withdrawal latency period of inflamed paws was determined with an electronic clock circuit and thermocouple. Foot withdrawal latencies were taken on day 0 before CIA induction

(baseline) and subsequently on day 25, 30 and 35 of the experimental period to determine the analgesic effect of PEA or PEA-LUT treatments. A significant ($P < 0.05$) reduction in paw-withdrawal latency over time is characterized as thermal hyperalgesia. Data obtained were converted to percentage maximal possible antinociceptive effect (%MPE) as follows: (response latency − baseline latency)/(cut off latency − baseline latency) × 100.

Histological examination

On day 35, animals were sacrificed while they were under anesthesia (sodium pentobarbital, 45 mg/kg, i.p), and paws and knees were removed and fixed in 10% formalin. The paws were then trimmed, placed in decalcifying solution for 24 h, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin/eosin and studied using light microscopy (Dialux 22 Leitz). The following morphological criteria were considered: 0 = no damage; 1 = edema; 2 = inflammatory cell presence; 3 = bone resorption.

Staining of mast cells

Identification of mast cells was performed as described in previous studies [26]. Paw sections were cut 5 μm thick and stained with 0.25% Toluidine blue, pH 2.5, for 45 min at room temperature. The sections were then dehydrated and mounted in xylene-based medium for viewing. Three non-sequential sections were chosen from one random block from each spinal cord for examination. All sections were evaluated at 200x, while some sections were photographed at 400x using a Nikon inverted microscope.

Immunohistochemical localization of chymase, tryptase and nitrotyrosine

Immunohistological analysis was performed as described in previous studies [26]. On day 35, the joints were trimmed, placed in decalcifying solution for 24 h and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin. Sections were incubated overnight with 1) anti-chymase antibody (1:100 in PBS, v/v) (DBA, Milan, Italy) or 2) anti-tryptase antibody (1:500 in PBS, v/v) or 3) anti-nitrotyrosine rabbit polyclonal antibody (1:1000 in PBS, v/v). Controls included buffer alone or non-specific purified rabbit IgG. Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin- conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vector). To verify the binding specificity for chymase and tryptase some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In order to confirm that the immunoreaction for the nitrotyrosine was specific some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10mM) to verify the binding specificity. In these situations, no positive staining was found in the sections indicating that the immunoreaction was positive in all the experiments carried out. Immunocytochemistry photographs (N = 5) were assessed by densitometry by using Optilab Graftek software on a Macintosh personal computer [27].

Radiography

Radiography was performed as previously described [11]. The mice were anaesthetized with sodium pentobarbital (45 mg/kg, i.p.). Mice were placed on a radiographic box at a distance of 50 cm from the x-ray source. Radiographic analysis (Philips X12 Germany) of normal and arthritic mice hind paws was performed with a 40 kW exposure for 0.01 sec. An investigator blinded to the treatment regime scored the radiographs. The following radiographic criteria from hind limbs were considered: score 0, no bone damage; score 1, tissue swelling and edema; score 2, joint erosion; 3, bone erosion and osteophyte formation.

Measurement of cytokines

Tumor Necrosis Factor-α (TNF-α), interleukin (IL)-6 and IL-1β levels were evaluated in the plasma from CIA and sham mice as previously described [28]. The assay was carried out using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem Corporation, Milan, Italy) with a lower detection limit of 10 pg/ml.

Measurement of chemokines

Levels of chemokines MIP-1α and MIP-2 were measured in the aqueous joint extracts. Briefly, joint tissues were prepared by first removing the skin and separating the limb below the ankle joint. Joint tissues were homogenized on ice in 3 ml lysis buffer (PBS containing: 2 mM PMSF, and 0,1 mg/ml [final concentration], each of aprotinin, antipain, leupeptin, and pepstatin A) using Polytron (Brinkinarm Instruments, Westbury, NY). The homogenized tissues were then centrifuged at 2,000 g for 10 min. Supernatant were sterilized with a millipore filter (0.2 μm) and stored at −80°C until analyzed. The extracts usually contained 0.2-1.5 mg protein/ml, as measured by protein assay kit (Pierce Chemical Co., Rockford, IL). The levels of MIP-1α and MIP-2 were quantified using a modification of a double ligand method, as previously described [29]. Briefly, flat-bottomed 96-well microtiter plates were coated with 50 μl/well of rabbit anti-cytokine antibodies (1 μg/ml in 0.6 mol/liter NaCl, 0.26 mol/liter H_3BO_4 and 0.08 N NaOH, pH 9.6) for 16 h at 4°C, and then washed with PBS, pH 7.5, 0.05% Tween 20 (wash buffer). Nonspecific binding sites on microtiter plates were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer, and diluted aqueous joint samples (50 μl) were added, followed by incubation for 1 h at 37°C. After washing of plates, chromogen substrate was added. The plates were incubated at room temperature to the desired extinction, after which the reaction was terminated with 50 μl/well of 3 M H_2SO_4 solution. The plates were then read at 490 nm in an ELISA reader. This ELISA method consistently had a sensitivity limit of ~ 30 pg/ml.

Thiobarbituric acid-reactant substances measurement (MDA levels)

Thiobarbituric acid-reactant substances measurement, which is considered a good indicator of lipid peroxidation, was determined, as previously described [30]. Thiobarbituric acid-reactant substances were calculated by comparison with OD650 of standard solutions of 1,1,3,3-tetramethoxypropan 99% malondialdehyde bis (dymethyl acetal) 99% (MDA) (Sigma, Milan). The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

Myeloperoxidase (MPO) assay

Neutrophil infiltration to the inflamed joints was indirectly determinate using an MPO assay, as previously described for neutrophil elicitation [31]. Tissue was prepared as described above and placed in a 50 mM phosphate buffer (pH = 6.0) with 5% hexadecyltrimethyl ammonium bromide (Sigma Chemical Co.). Joint tissues were homogenized, sonicated, and centrifuged at 12,000 g for 15 min at 4°C. Supernatants were assayed for MPO activity using a spectrophotometric reaction with O-dianisidine hydrochloride (Sigma Chemical Co.) at 460 nm.

Materials

Unless otherwise stated, other compounds were obtained from Sigma-Aldrich Company (Milan, Italy). All chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, U.K.) or 10% ethanol (Sigma-Aldrich).

Data analysis

All values in the figures and text are expressed as mean ± standard error (s.e.m.) of the mean of n observations. For the *in vivo* studies n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissue sections collected from all the animals in each group. Data sets were examined by one- or two-way analysis of variance, and individual group means were then compared with Student's unpaired t test. For the arthritis studies, Mann–Whitney U test (two-tailed, independent) was used to compare medians of the arthritic indices [22]. A p-value of less than 0.05 was considered significant.

Results

Effect of PEA-LUT formulation therapy in the development of CIA

To imitate the clinical scenario of RA, mice were subjected to CIA. CIA developed rapidly in mice immunized with CII and clinical signs (periarticular erythema and edema) (Figure 1B) of the disease first appeared in hind paws between 24 and 26 days post-challenge leading to a 100% incidence of CIA at day 28 (Figure 2B). Hind paw erythema and swelling increased in frequency and severity in a time-dependent mode with maximum arthritis indices of approximately 12 observed between day 29 to 35 post immunization (Figure 1L) in CIA-control mice. The therapy with PEA-LUT significantly reduced the development of the inflammatory process (Figure 1D and L). A significant difference was found between the treatment with PEA-LUT and the higher dose of PEA alone (10 mg/kg) (Figure 1C and L) as well as between the PEA-LUT and LUT alone treatment (1mg/kg) (data not shown). Neither the clinical signs nor histopathological features of CIA were observed in sham controls mice four paws during the evaluation period (Figure 1A). PEA LUT significantly reduced the arthritis index (Figure 2B). A significant difference was found between the PEA-LUT therapy and the higher dose of PEA alone (10 mg/kg) (Figure 2B). No significant protection was found in the animal subjected to CIA treated with LUT (1mg/kg, Figure 2B).

Figure 1 Effect of PEA- LUT combination therapy on the clinical expression of CIA and on radiographic analysis. No clinical signs were observed in sham-mice **(A)**. CIA developed rapidly in mice immunized with CII and clinical signs like periarticular erythema and edema **(B)**. Hind paw erythema and swelling increased in frequency and severity in a time-dependent mode **(L)**. CIA-PEA treated mice demonstrated a significant reduction in the clinical signs of CIA **(C)**. Co-ultramicronized PEA + LUT formulation showed an enhanced reduction of clinical signs of CIA **(D)**. In addition, radiographic analysis was evaluated. There is no evidence of pathology in the femoral growth plate or in the tibiotarsal joints of normal mice **(E, I)**. Hind paws from CII-immunized (35 days) vehicle-treated mice showed bone resorption in the femoral growth plate as well as in the tibiotarsal joints **(F, I)**. PEA treated mice showed less bone erosion in the femoral growth plate as well as in the tibiotarsal joints of CIA mice **(G, I)**. A significant difference was showed between PEA and PEA-LUT combination therapy as well as between PEA LUT combination therapy and LUT alone treatment **(H, I)**. Figure is representative of at least 3 experiments performed on different days. Values are means ± SEM of 20 animals for each group. *$P < 0.01$ vs. sham-control. °$P < 0.01$ vs. CIA. [#]$P < 0.01$ vs. CIA-PEA. [§]$P < 0.01$ vs. CIA-LUT.

Figure 2 Effect of PEA- LUT combination therapy on paw edema and body weight. CIA developed rapidly in mice immunized with CII leading to a 100% incidence of CIA at day 28 **(B)**. Swelling of hind paws **(A)** over time was measured at 2-day intervals. Beginning on day 25, the CII-challenged mice gained significantly less weight and this trend continued through day 35 **(C)**. CIA-PEA mice demonstrated a significant reduced incidence of weight loss **(C)** as well as a less paw edema (A). CIA-LUT mice did not demonstrate a reduced incidence of weight loss **(C)** as well as a less paw edema (A) compared to PEA group. Furthermore, the combination therapy with PEA and LUT enhanced the reduction of incidence of body weight loss and paw edema **(A, C)**. Figure is representative of all the animals in each group. Values are means ± SEM of 20 animals for each group. *$P < 0.01$ vs. Sham-control. °$P < 0.01$ vs. CIA. [#]$P < 0.01$ vs. CIA-PEA. [§]$P < 0.01$ vs. CIA-LUT.

The data in Figure 2A demonstrated a time-dependent increase in hind paw (each value represents the mean values of both hind paws) volume (ml) in mice immunized with CII. Maximum paw volume occurred by day 35 in the CII-immunized mice. Treatment with PEA-LUT exhibited a continuously significant ($P < 0.01$) suppression of hind paw swelling from day 26 to 35 post-immunization, achieving a maximal response of 75% from day 28 to 35 (Figure 2A). A significant difference was found between the higher dose of PEA alone (10 mg/kg) and the combination therapy (Figure 2A) as well as between the PEA-LUT and LUT alone treatment (1mg/kg) (Figure 2A). No significant inhibition of the paw edema formation was found in the animal subjected to CIA treated with LUT (1mg/kg, Figure 2A). No increases in hind paw volume over time was observed with normal control (data not shown).

The rate and the absolute gain in body weight were comparable in sham-control and in CIA-control mice in the first week (data not shown). From day 25, the CII-challenged mice gained significantly less weight than the sham-control mice and this trend continued through day 35 (Figure 2C). PEA-LUT treatment determined a significant increase of the body weight compared with the vehicle-treatment in CIA-control mice (Figure 2C). A significant difference was found between the higher dose of PEA alone (10 mg/kg) and the combination therapy (Figure 2C) as well as between the PEA-LUT and LUT alone treatment (1mg/kg, Figure 2C). The treatment with LUT (1mg/kg) did not exert any significant effect on the body weight gain in the animal subjected to CIA (Figure 2C).

PEA-LUT formulation therapy increases motor activity in CIA

In a previous study we have demonstrated that PEA-LUT treatment is able to alleviate many of the clinical and neuropathological features of depression [32] but to our knowledge PEA-LUT has not been investigated in this regard in CIA. Therefore, we next assessed how chronic daily treatment with PEA-LUT affected the gross locomotor ability, assessed by performance on a non-accelerating rotarod, in CIA mice. As shown in Figure 3A PEA-LUT treatment significantly reduced the motor impairment in CIA mice. Moreover, a significant difference on the motor function impairment was found between the PEA-LUT therapy and the higher dose of PEA alone (10 mg/kg) and between PEA-LUT therapy and LUT at dose of 1mg/kg (Figure 3A). Treatment with LUT (1mg/kg) has not significantly ameliorated the locomotor ability in the animal subjected to CIA (Figure 3A).

Figure 3 Effect of PEA- LUT combination therapy on locomotor activity and pain evaluation. CIA subjected mice shortened time to stay on rotating rod compare to sham mice (**A**). Locomotor abilities on the rotarod are better maintained in CIA + PEA than CIA + Vehicle mice (**A**). Locomotor abilities on the rotarod are not better maintained in CIA + LUT than CIA + PEA mice (**A**). A significant difference in locomotor activity was found between PEA and PEA-LUT combination therapy as well as between PEA LUT combination therapy and LUT alone treatment (**A**). In addition, pain evaluation in CIA + vehicle, CIA-LUT, CIA + PEA, CIA + PEA-LUT and sham mice was measured by hotplate test and plantar test. Measurements were recorded in mice able to ambulate. CIA + vehicle mice exhibit increased pain sensitivity and thermal hyperalgesia compared to normal controls. (**B, C**). PEA treatment reduced significantly pain sensitivity and thermal hyperalgesia in CIA-PEA treated mice (**B, C**). LUT treatment did not significantly reduce pain sensitivity and thermal hyperalgesia compared to PEA group (**B, C**). The combination therapy with PEA-LUT enhanced the reduction of pain sensitivity and thermal hyperalgesia compared to higher dose of PEA (**B, C**). Figure is representative of all the animals in each group. Values are means ± SEM of 20 animals for each group. $*P < 0.01$ vs. Sham-control. $°P < 0.01$ vs. CIA. $^{\#}P < 0.01$ vs. CIA-PEA. $^{\S}P < 0.01$ vs. CIA-LUT.

Effect of PEA-LUT formulation therapy on pain sensitivity and thermal hyperalgesia in CIA

In the next step, the effect of PEA-LUT therapy on pain sensitivity was tested by subjecting mice to hotplate testing and recording latency to a response (Figure 3B). At days 25 after CIA induction, CIA + vehicle mice exhibit increased pain sensitivity compared to normal controls (Figure 3B). Moreover, between day 30 to 35 post immunization, PEA-LUT treated mice with CIA had response times that were comparable to normals (Figure 3B). Furthermore, at day 25 after CIA induction, mice became hypersensitive to noxious heat (thermal hyperalgesia) as evidenced by a significant reduction in hind paw withdrawal latency with a maximum hypersensitive response observed between day 30 to 35 post immunization in CIA-control mice (Figure 3C). This CIA-induced hyperalgesia was reduced by daily treatment with PEA-LUT (Figure 3C). Moreover, a significant difference on the pain sensitivity impairment was found between the PEA-LUT therapy and the higher dose of PEA alone (10 mg/kg) (Figure 3B, C) as well as between the PEA-LUT and LUT alone treatment (1mg/kg, Figure 3B, C).

Effect of PEA-LUT formulation therapy on histopathology and radiographic analysis of CIA

The histological evaluation (at day 35) of the paws from vehicle-treated mice revealed signs of severe arthritis, with bone erosion (Figure 4A, A1 and see histological score I). In addition, severe or moderate necrosis was observed (Figure 4A, A1 and see histological score I). The bone erosion and the necrosis were significantly reduced in the joint from PEA-LUT treated mice (Figure 4B, B1 see for histological score I). A significant difference was found between the higher dose of PEA alone (10 mg/kg) and the combination therapy as well as between the PEA-LUT and LUT alone treatment (1mg/kg, data not shown). Treatment with LUT (1mg/kg) did not reduce the histological alteration in the animal subjected to CIA (data not shown). No histological damage was found in sham animals (data not shown).

Figure 4 Morphologic changes of CIA. Representative hematoxylin/eosin-stained section of joint was examined by light microscopy. The histological evaluation of joint from CIA-control mice (**A**, A1 and **I**) revealed inflammatory cell infiltration and bone erosion. The histological alterations were significantly reduced in the tissues from CIA-PEA-LUT treated mice (**B**, B1 and **I**). Toluidine blue staining was also performed. A significant mast cell infiltration was observed in joint tissues of CIA subjected mice (**C**, C1) compared to sham animals (data not shown). PEA-LUT enhanced the reduction of mast cell infiltration (**D**, D1). In addition, a significant increase in chymase and tryptase expression was found mainly in the joint tissues collected after CIA induction (**E**, E1, **G**, G1 and **L**). Chymase and tryptase expression was significantly attenuated in the joint from CIA-PEA-LUT treated mice (**F**, F1, **H**, H1 and see **L**). Densitometry analysis of Immunocytochemistry photographs (n = 5) for chymase and tryptase from paw section was assessed (**L**). Data are expressed as % of total tissue area. $*P < 0.01$ vs. Sham-control. $°P < 0.01$ vs. CIA. $^{\#}P < 0.01$ vs. CIA-PEA. $^{\S}P < 0.01$ vs. CIA-LUT.

A radiographic examination of knee joint and femoral growth plate in the femur from vehicle-treated mice at 35 days post CII immunization revealed bone erosion (Figure 1F see radiograph score I). A significant less bone resorption was observed in the PEA-treated mice (Figure 1G and see radiograph score I). Treatment with LUT (1mg/kg) did not reduce significantly the bone resorption in the animal subjected to CIA (data not shown). A significant difference was found between the higher dose of PEA alone (10 mg/kg) and the combination therapy (Figure 1H and see radiograph score I) as well as between the PEA-LUT and LUT alone treatment (1mg/kg, data not shown). There was no evidence of pathology in sham mice (Figure 1E and see radiograph score I).

Effect of PEA-LUT formulation therapy on mast cells degranulation during CIA

In order to better study the mast cell infiltration during CIA, the joint tissues were stained by Toluidine Blue. In particular, a significant presence of mast cells was observed in the joint tissues collected at day 35 after CIA induction (Figure 4C) mainly localized in the articular space (see particles 4 C1). On the contrary, significant less mast cell infiltration was observed in the joint tissues from CIA subjected mice that have been treated with PEA-LUT (Figure 4D, D1). A significant difference was found between the higher dose of PEA alone (10 mg/kg) and the combination therapy as well as between the PEA-LUT and LUT alone

treatment (1mg/kg, data not shown). No resident mast cells were found in the joint tissues from sham-treated mice (data not shown).

Moreover, in order to test whether PEA-LUT treatment may modulate and direct the inflammatory response through the regulation of the serine peptidases, we analyzed by immunohistochemistry the joint expression of chymase and tryptase. There was no staining for chymase and tryptase in the joint tissues obtained from the sham-treated mice (data not shown). A substantial increase in chymase and tryptase expression was found mainly localized in mast cells in the joint tissues collected at 35 days after CIA induction (Figure 4E, E1, G, G1 and see L). Joint expression of chymase and tryptase were significantly attenuated in the joint from CIA mice that have received PEA-LUT treatment (Figure 4F, F1, H, H1 and see L). A significant difference was found between the higher dose of PEA alone (10 mg/kg) and the combination therapy as well as between the PEA-LUT and LUT alone treatment (1mg/kg, data not shown).

Effect of PEA-LUT formulation therapy on cytokines, chemokines expression and neutrophil infiltration

We initiated studies to assess the effect of PEA-LUT therapy on the expression of chemokines into the inflamed joints during the development of CIA. As shown in Figure 5A, B, the expression of MIP-lα and MIP-2, measured by ELISA, was significantly increased in the joint 35 days after CII immunization. MIP-1α and MIP-2 levels in CIA mice that have been treated with PEA-LUT on day 35 were significantly reduced in comparison with vehicle-treated CIA-mice (Figure 5A, B). Assessment of neutrophil infiltration into the inflamed joint tissue was also performed by measuring MPO activity. It was significantly elevated 35 days after CII immunization in vehicle-treated CIA mice (Figure 5F) while in the CIA mice treated with PEA-LUT therapy, MPO activity was markedly reduced (Figure 5F). A significant difference was found between the higher dose of PEA alone (10 mg/kg) and the combination therapy and as well as between the PEA-LUT and LUT alone treatment (1mg/kg, Figure 5A, B, F).

Figure 5 Effect of PEA-LUT combination therapy on cytokines, chemokines expression and neutrophil infiltration. A substantial increase in the expression of MIP-1α **(A),** MIP-2 **(B)**, IL-1β **(C)**, IL-6 **(D)**, TNF-α **(E)** and MPO activity **(F)** was found in CIA-control mice 35 days after CII immunization. CIA-PEA treated mice demonstrated a significant reduction in the expression of MIP-1α **(A)**, MIP-2 **(B)**, IL-1β **(C)**, IL-6 **(D)**, TNF-α **(E)** and MPO activity **(F)**. CIA-LUT treated mice did not significantly reduce the expression of MIP-1α **(A)**, MIP-2 **(B)**, IL-1β **(C)**, IL-6 **(D)**, TNF-α **(E)** and MPO activity **(F)**. The combination therapy with PEA-LUT significantly reduced the expression of MIP-1α **(A)**, MIP-2 **(B)**, IL-1β **(C)**, IL-6 **(D)**, TNF-α **(E)** and MPO activity **(F)**. Values are means ± SEM of 20 animals for each group. $*P < 0.01$ vs. Sham-control. $°P < 0.01$ vs. CIA-control. $^{\#}P < 0.01$ vs. CIA-PEA. $^{§}P < 0.01$ vs. CIA-LUT.

To test whether PEA-LUT modulates the inflammatory process through the regulation of cytokine secretion, we analyzed the plasma levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6. A substantial increase in TNF-α, IL-1β and IL-6 (Figure 5C, D, E) production was found in CIA-control mice 35 days after CII immunization. Levels of TNF-α, IL-1β and IL-6 (Figure 5C, D, E) were significantly reduced in CIA mice treated with PEA-LUT therapy. No significant difference was found between the higher dose of PEA alone (10

mg/kg) and the combination therapy and as well as between the PEA-LUT and LUT alone treatment (1mg/kg, Figure 5C, D, E).

Effect of PEA-LUT formulation therapy on nitrotyrosine formation and lipid peroxidation

The release of free radicals and oxidant molecules during chronic inflammation has been suggested to contribute significantly to the tissue injury [33]. On day 35, a positive staining for nitrotyrosine, a marker of nitrosative injury, was found in the joints of vehicle-treated CIA-control mice (Figure 6A, A1 and see C). The therapy with PEA-LUT significantly reduced the formation of nitrotyrosine (Figure 6B, B1 and see C). A significant difference was found between the higher dose of PEA alone (10 mg/kg) and the combination therapy and as well as between the PEA-LUT and LUT alone treatment (1mg/kg, data not shown).

Figure 6 Effect of PEA-LUT combination therapy on nitrotyrosine immunostaining and MDA levels. A marked increase in nitrotyrosine (**A**, see particular A1 and **C**), staining was evident in the paw 35 days after initiation of CIA. There was a marked reduction in the immunostaining for nitrotyrosine (**B**, see particular B1 and **C**) in the paw of CIA-PEA-LUT mice. Densitometry analysis of Immunocytochemistry photographs (n = 5) for nitrotyrosine from paw section was assessed **(C)**. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. *P < 0.01 *versus* Sham-control. °P < 0.01 *versus* CIA. In addition, MDA levels, a marker of lipid peroxidation, were evaluated. A substantial increase in MDA levels **(D)** was found in CIA-control mice 35 days after CII immunization. CIA-PEA treated mice demonstrated a significant reduction in MDA levels **(D)**. The combination therapy with PEA-LUT enhanced the reduction in MDA levels **(D)**. Values are means ± SEM of 20 animals for each group. *$P < 0.01$ vs. Sham-control. °$P < 0.01$ vs. CIA-control. #$P < 0.01$ vs. CIA-PEA. §$P < 0.01$ vs. CIA-LUT.

In addition, at 35 days after CIA induction, thiobarbituric acid-reactant substances levels were also measured in the plasma as an indicator of lipid peroxidation. A significant increase of thiobarbituric acid-reactant substances (Figure 6D) was observed in the plasma collected at 35 days after CIA induction from mice subjected to CIA when compared with sham-operated mice. Thiobarbituric acid-reactant substances (Figure 6D) were significantly attenuated in CIA-mice treated with PEA-LUT. Please note that a significant difference was found between the higher dose of PEA alone (10 mg/kg) and the combination therapy (Figure 6D) as well as between the PEA-LUT and LUT alone treatment (1mg/kg, Figure 6D).

Discussion

ROS have a crucial role in the pathogenesis of inflammatory diseases such as rheumatoid arthritis [34,35]. The flavonoid, apigenin, which has a structure similar to luteolin, showed an antidepressant-like effect [36] and a protective effect against endoplasmic stress-induced neuronal cell death [36]. These findings suggest the possibility that luteolin may be protective against oxidative stress-induced inflammation and cell damage. With this aim in mind, we applied the endocannabinoid congener PEA and the flavonoid luteolin to counteract the inflammatory process associated to RA. Our results demonstrated that co-ultramicronized PEA + luteolin formulation (PEA-LUT) is protective in a mice model of collagen-induced arthritis. The protective effects of PEA-LUT were not limited to an overall anti-inflammatory

effect but included significant protection of cartilage/bone compared to untreated collagen-immunized animals, as well as inhibition of pro-inflammatory cytokines known to be involved in the human disease. Through both histological and radiographical evaluations, we found that PEA-LUT was significantly protective on the cartilage and bone in tibiotarsal joints of mice immunized with CII.

PEA has been shown to be effective in several experimental models of inflammation, both of immunogenic and neurogenic origin [37,38]. We have recently demonstrated that PEA treatment significantly reduced spinal cord injury in mice [39]. Despite its various pharmacological properties, the cellular/receptor mechanism responsible for the actions of PEA is still debated. Mazzari et al. [37] demonstrated that in vivo anti-inflammatory effects of PEA were due to down-regulation of mast cell (MC) degranulation. The ability of MC to respond to a wide range of infectious and chemical stimuli facilitates their key functions in immunity and the response to tissue injury, by promoting a rapid release of pro-inflammatory mediators, mediators of hyperalgesia and itch mediators [40]. MCs were divided into two subtypes depending on the variable content of the neutral serine proteases, tryptase and chymase. We reported here that CIA caused a significant infiltration and activation of MC in the joint at 35 days after induction, whereas treatment with PEA-LUT significantly reduced both the infiltration and the activation. These observations are in agreement with other studies in which have been shown that PEA is an effective tool to control mast cell hyperactivity, which occurs in inflammation, inflammatory hyperalgesia [41], neuropathic hyperalgesia [42] and spinal and brain trauma [19,26]. It has been demonstrated that several cytokines also appear to direct cell to-cell communication in a cascade fashion during CIA such as: IL-1 [43], TNF-α [44], IL-6 [45]. TNF-α and IL-1β are initiators of the nuclear factor (NF-κB) activation cascade [34] and are under its transcriptional control, constituting a positive feedback loop. Recent studies observed that the Luteolin or PEA decreased the activation of NF-κB system in different experimental models [46,47].

In our paper, we confirmed that the pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) as well as the chemokines (MIP-Iα and MIP-2) are expressed at sites of inflamed joints and likely contribute to the progression of chronic joint inflammation. It has been demonstrated that MCP-1, MIP- Iα, MIP-Iβ are differentially chemotactic for lymphocyte subsets [2] and are expressed in tissue from the inflamed joints of patients with rheumatoid arthritis [48,49]. Interestingly, using PEA-LUT, we demonstrated a more pronounced inhibition of the release of pro-inflammatory cytokines and chemokines and a reduction of leukocyte infiltration measured by MPO activity in comparison to PEA alone.

The role of ROS and in particular of superoxide in degradation of cartilage and bone is well documented [11,50]. Cartilage is sensitive to degradation by superoxide and SOD strongly inhibits this degradation and evidence exists to support a link between chondrocyte lipid peroxidation and cartilage oxidation/degradation [11]. In this report, an intense immunostaining of nitrotyrosine formation and a significant lipid peroxidation also suggested that a structural alteration of joint had occurred, most probably due to the formation of highly reactive nitrogen-derivatives.

We demonstrated here that PEA-LUT reduced the nitrotyrosine and the lipid peroxidation formation during CIA. Several studies demonstrated that Luteolin also exerts antioxidant properties [51]. This effect on nitrotyrosine formation and lipid peroxidation by PEA-LUT was significantly more pronounced in comparison to PEA alone.

In previous studies, we demonstrated that the combination therapy using a potent M40403 SODm and clinical used drugs (e.g. Dexamethasone or Metotrexate) significantly exerts an important beneficial anti-inflammatory effect by blocking the possible progression of an emerging arthritis with the reduction of DMARS effective dose [11]. Similarly, in the present study we have demonstrated that PEA-LUT when given at the onset of the disease reduced paw swelling, clinical score, and the histological severity. Amelioration of joint disease was associated with inhibition of pain which is a key player in RA. Thus, these potent analgesic and anti-inflammatory effects observed with PEA-LUT were in contrast to that observed with the individual substances administered at lower doses (data not shown).

Conclusions

In conclusion, RA is a complex chronic inflammatory disease dependent on multiple interacting environmental and genetic factors, making it difficult to understand its pathogenesis and thereby to find effective therapies. This new pharmacological approach with PEA-LUT combination therapy may represent new and useful pharmacological tools for the therapy of chronic inflammation.

Abbreviations

CFA, Complete Freund's adjuvant; CIA, Type II collagen-induced arthritis; CII, Collagen type II; IL-1β, Interleukin-1β; IL-6, Interleukin-6; LUT, Luteolin; MC, Mast cell; MPO, Myeloperoxidase; NF-κB, Nuclear factor-κB; PEA, N-palmitoylethanolamine; RA, Rheumatoid arthritis; ROS, Reactive oxygen species; TNF-α, Tumor necrosis factor-α.

Competing interest

The authors declare that they have no competing interest.

Authors' contributions

DB, SC participated in research design and performed statistical analysis. AP and VM also participated in study design and coordination. DI, RD, carried out immunoassay and histological analysis and helped to draft the manuscript. AA, EE and MC also conducted experiments and helped to draft the manuscript. DI, EE and SC contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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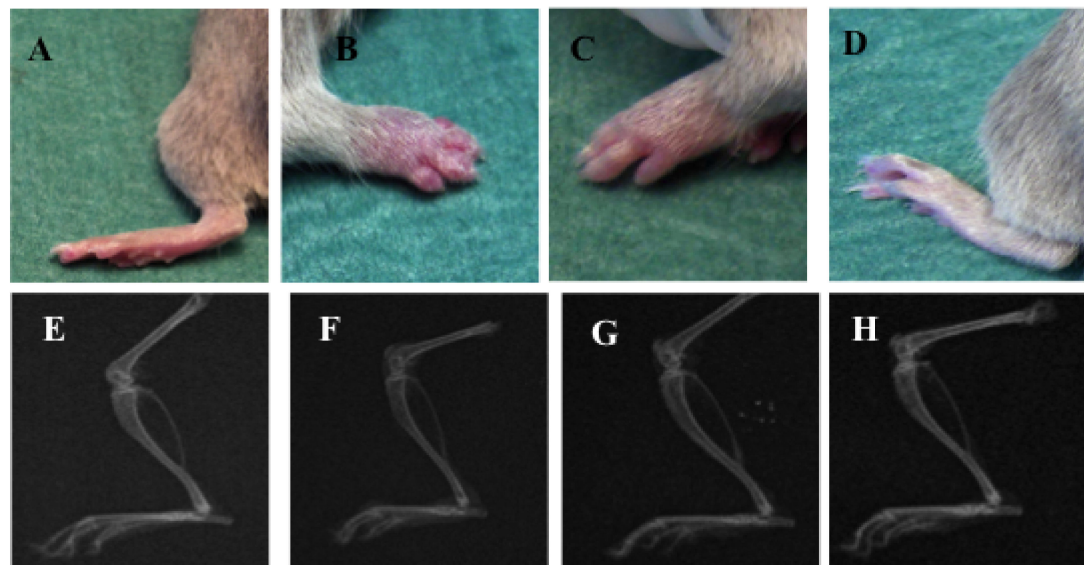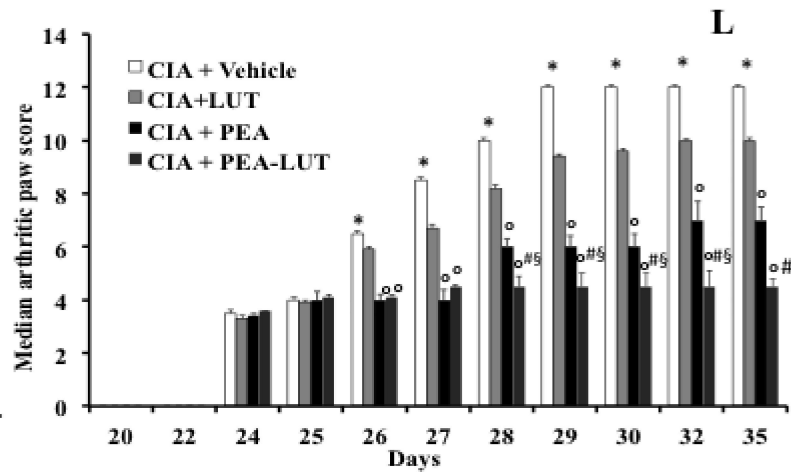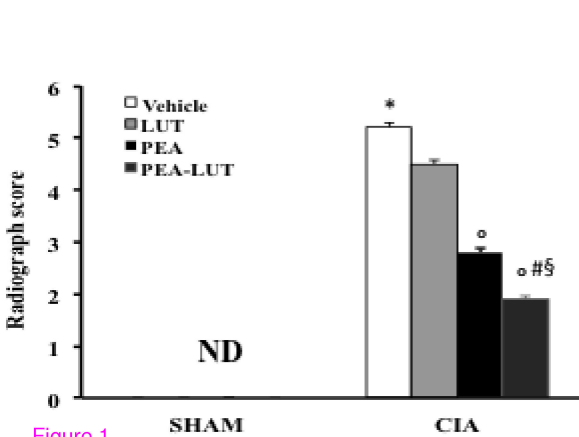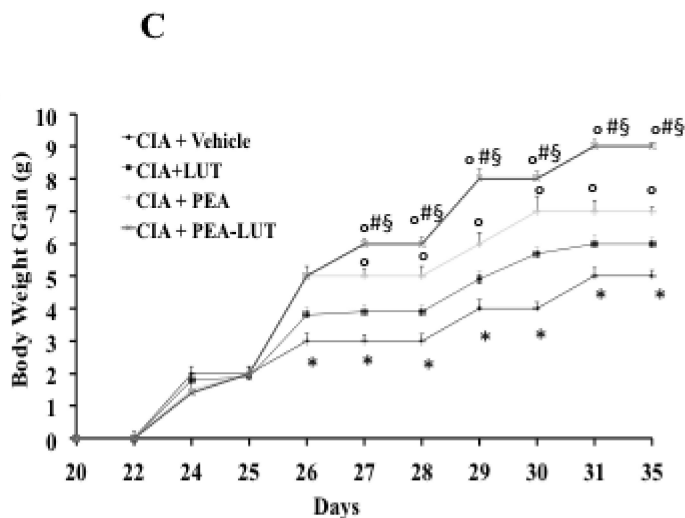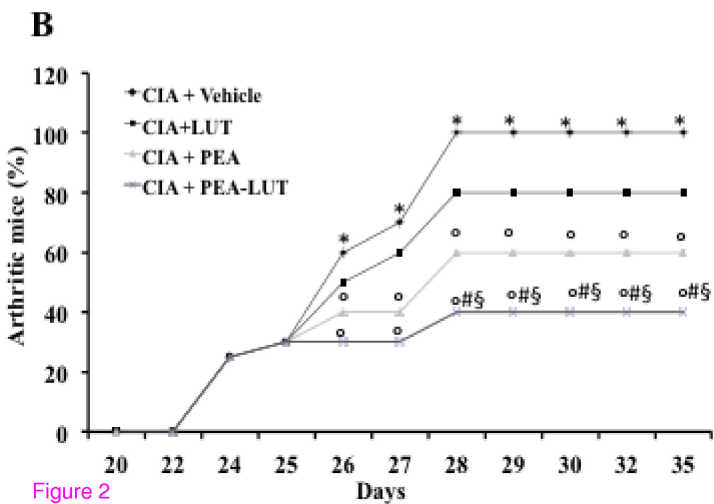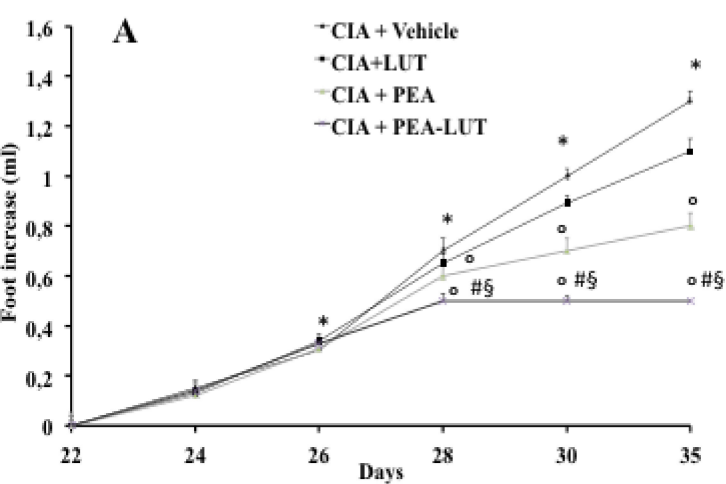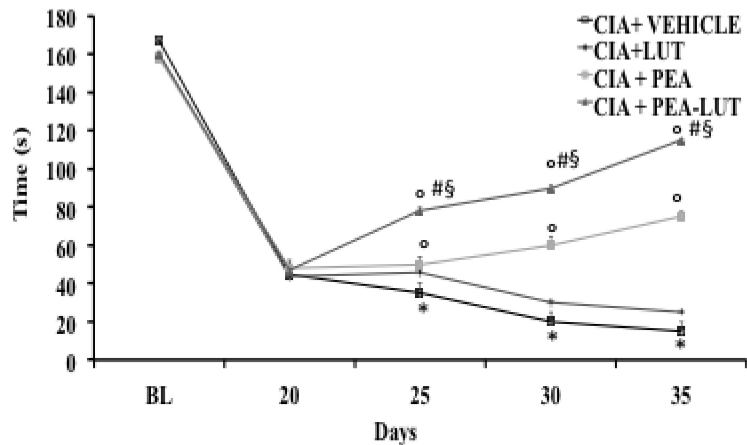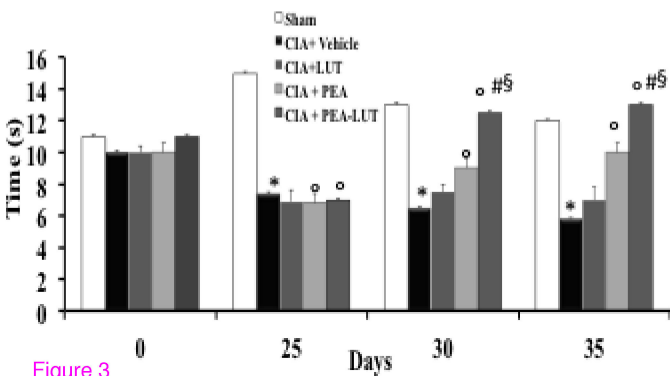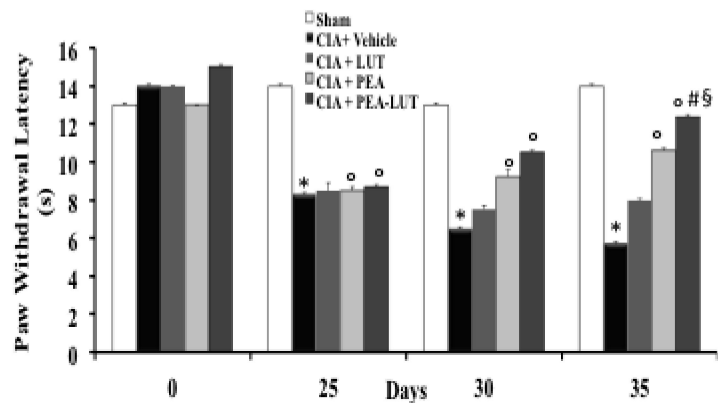
Figure 1
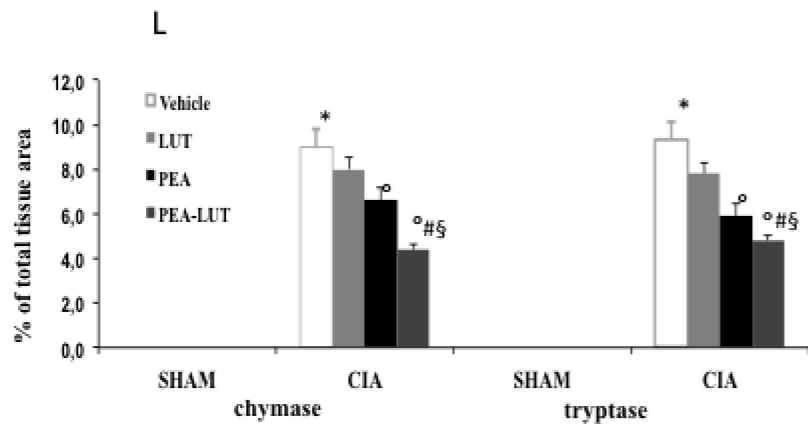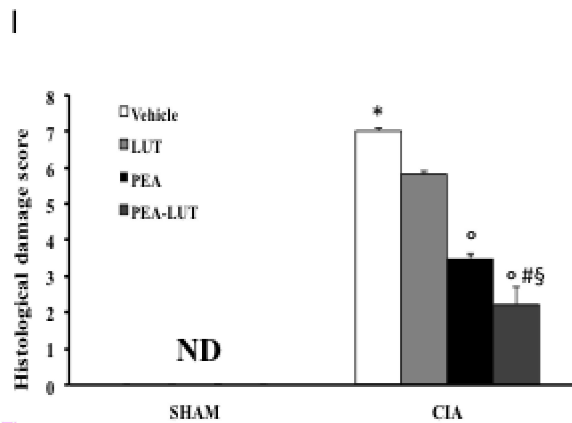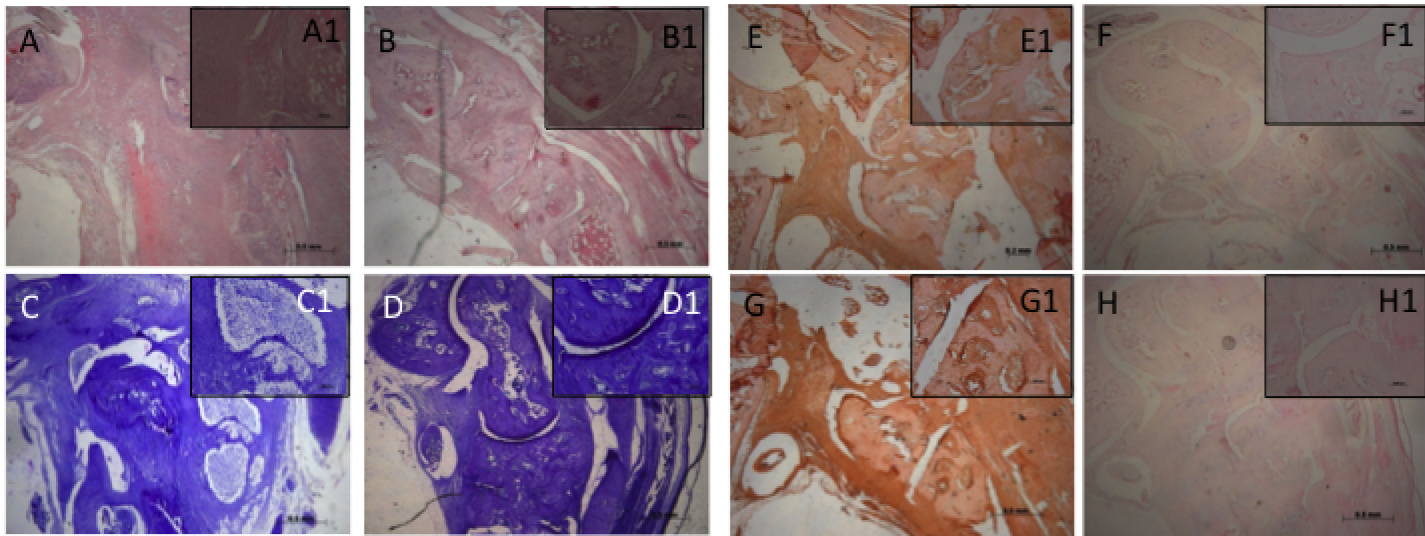
Figure 2

Figure 3

Figure 4

Figure 5

Figure 6