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Anti-inflammatory effect of ATB-352, a H_2S -releasing ketoprofen derivative, on lipopolysaccharide-induced periodontitis in rats

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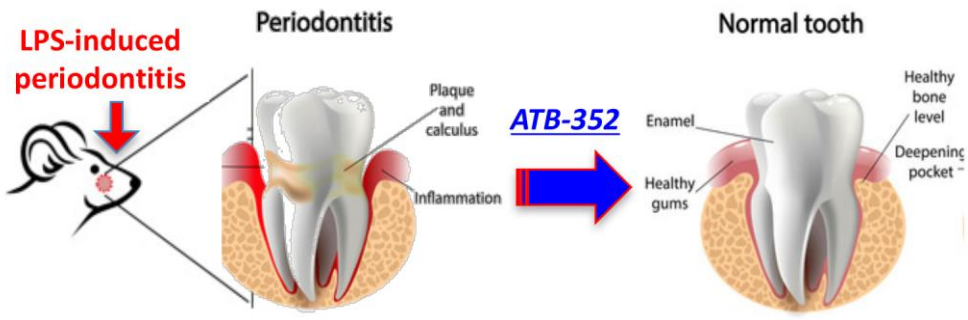
Graphical abstract



Abstract

Periodontal disease is the most common cause of tooth loss in humans, is an inflammatory disease initiated by oral microbial biofilm. Given the involvement of the inflammatory pathway in this type of pathology, the main pharmacological strategy for the treatment of periodontitis, is the inhibition of the inflammatory process in order to prevent tissue destruction and bone resorption, a condition associated with a painful state. To do this, the best class of drugs are Non-steroidal anti-inflammatory drugs (NSAIDs), however, the presence of side effects, especially at the gastrointestinal tract, limits their use for long-term therapy. Recently, some evidence shows that derivatives of NSAIDs capable of releasing hydrogen sulphide exhibit lower collateral effects, particularly at the gastric level. In fact, H_2S is an endogenous gaseous mediator with a cytoprotective role at the gastric level. In this study, we have compared the protective effects of ketoprofen with ATB-352, a hydrogen sulfide-releasing derivative of ketoprofen, in an experimental model of periodontitis in rat. Periodontitis was induced by a single intragingival injection of 1 µl LPS (10 µg/µl), Our results show that 14h after intragingival injection of LPS, there was a high tissue damage associated with bone resorption, and in gingivomucosal tissues there was a significant expression of NF-kb p65 and pro-inflammatory cytokine as well as a higher expression of COX-2 and iNOS, activation of the apoptotic process, and also increased levels of NGF expression, often associated with a higher nociceptive perception. Treatment with ATB-352 at

the dose of 20mg\kg, was able to reduce the inflammatory process associated with intragingival LPS injection and also had a positive effect on bone resorption and tissue damage.

Keywords: Periodontitis, inflammation, LPS, ketoprophen,

INTRODUCTION

Periodontitis is one of the most common infectious diseases and is the leading cause of tooth loss in the world (1). This pathology is characterized by accumulation of bacteria called plaque, these bacteria with their components, present in the dental plaque induce an host inflammatory response. subsequently the presence of the chronic inflammatory process in the gingivomucosal tissue is the cause of structural damage to the area between the tooth and the supporting bone and may result ultimately in tooth loss (2). Inflammatory process regulation occurs through the release of pro-inflammatory mediators, in particular arachidonic acid metabolites and cytokines play a key role in host modulation of periodontal disease (3). therefore, the presence of these mediators in gingivomucosal tissues supports the inflammatory process, clinically visible as periodontal pocketing and alveolar bone loss in patients. Based on this knowledge it is evident that pharmaceutical inhibition of host response pathways is an important strategy for treating periodontal disease. Non-steroidal anti-inflammatory drugs (NSAIDs) such as ketoprofen are among the most commonly used anti-inflammatory drugs the common feature of inhibiting activity of the cyclooxygenase (COX) enzymes. NSAIDs reduce the production of prostaglandins and thereby reduce inflammation, but their use is associated with significant, sometimes life-threatening, adverse effects, particularly in the gastrointestinal (GI) tract (4). The presence of these side effects often causes a limitation in the use of this class of drugs. Therefore, in the design of new NSAIDs, attempts have been made to reduce the occurrence of these undesirable effects, especially at the GI level. In particular, the hydrogen sulfide (H_2S)-releasing derivatives exhibit excellent gastrointestinal safety, despite producing suppression of prostaglandin synthesis and

reduction of inflammation at least as effective as the parent NSAID (5) (6). The interest in the new analogous H_2S -releasing derivatives of NSAIDs, derives from the fact that H_2S is known to be an endogenous gaseous mediator that is produced through different pathways in many tissues. Especially at the level of GI tract H_2S is known to have a cytoprotective and anti-inflammatory action (Gemici et al., 2015), it has also been seen as H_2S endogenous production is rapidly increased as a result of GI mucosal damage, as a factor that promotes inflammation and damage repair (7), Finally, it has been seen that inhibition of H_2S generation inhibits gastric damage induced by NSAIDs (8) . Furthermore H_2S is increasingly being recognized as an important signaling molecule in the regulation of the cellular metabolism, the immunological and inflammatory responses, and several important transcription factors, though its precise role in inflammation is still in part unknown but gained increasing recognition. Therefore, in this study we have evaluated the effects of ketoprofen and ATB-352 in a rat experimental model of periodontitis. ATB-352 is an H_2S -releasing derivative of ketoprofen which did not produce GI damage, but did suppress COX activity as effectively as the parent NSAID (9).

MATERIALS AND METHODS

Animals

Adult male Rats (Sprague–Dawley; 200–230 g, Envigo, Italy) were kept in a well-organized environment with standard rodent chow and water. Animals were accommodated into a room at 22 ± 1 ◦C with a 12-h light, 12-h dark cycle. The study was permitted by the University of Messina Review Board for the care of animals. All animal experiments were performed following the regulations in Italy (D.M. 116192) and Europe (O.J. of E.C. L 358/1 12/18/1986).

Surgical Procedure

Male Sprague-Dawley rats (200– 230 g) were lightly anaesthetized with pentobarbitone (35 mg/kg), and periodontitis was induced by a single intragingival injection of 1 µl LPS (10 µg/µl) derived from Salmonella typhimurium (Sigma, Poole, Dorset, UK) in sterile saline solution. The injection was made in the mesolateral side at the interdental papilla between the first and the second molars. It was performed slowly and the needle kept in place for some seconds after the injection to guarantee that LPS was not lost through needle extraction. To evaluate periodontitis lesions, rats were sacrificed at 14 days after LPS injection (10).

Exsperimental Groups

Rats were casually divided into the following experimental groups (n=10 for each group)

- LPS + vehicle group: rats were subjected to LPS-induced periodontitis as described above (N=10).

- LPS + KETOPROFEN: same as the LPS + saline group, and Ketoprofen (20mg/Kg) was administred by oral gavage every 24h (1h after LPS-injection) for 14 days (N=10).

- LPS + ATB-352: same as the LPS + saline group, and ATB-352 (20mg/Kg) was administred by oral by gavage every 24h (1h after LPS-injection) for 14 days (N=10).

- Sham + vehicle group: animals received a single intragingival injection of saline solution with an identical surgical procedure described for LPS group (N=10)

- Sham + KETOPROFEN group: same as the Sham + saline group, and KETOPROFEN (20 mg/kg) was administered by oral gavage every 24 h for 14 days (N=10).

- Sham + ATB-352: group: same as the Sham + saline group, and ATB-352: (20 mg/kg) was administered by oral gavage every 24 h for 14 days (N=10).

Radiography

Mandibles Radiography were performed by an X-ray machine (Bruker MS FX Pro, Billerica, Massachusetts, USA) with a 30 kW exposure for 0.01 s. Fourteen days after LPS injection we evaluated the dental alveolar bone level, defined as the distance from the cemento–enamel junction (CEJ) to the maximum coronal level of the alveolar bone crest (CEJ–bone distance) as previously described (11).

Histological Examination

Biopsies of gingivomucosal tissue and stomach tissue samples were taken 14 days after periodontitis induction. Samples were fixed in 10% (w/v) PBS-buffered formaldehyde solution at 25°C for 24 h, then dehydrated by graded ethanol solutions and subsequently embedded in

Paraplast (Sherwood Medical, Mahwah, NJ, USA). Hence, samples were deparaffinised with xylene and coloured with hematoxylin and eosin. All sections were investigated using Axiovision Zeiss (Milan,Italy) microscope. Histological observations were performed in a blinded fashion. A histological injury score for gingivomucosal tissue was determined using a semiquantitative scale that measures the following morphological criteria: 0, normal gingivomucosal tissue (0-2+) ; grade 1, minimal oedema or infiltration (0-2+); grade 2, moderate oedema and inflammatory cell infiltration without obvious damage to gingivomucosal architecture (0-2+); grade 3, severe inflammatory cell infiltration with obvious damage to gingivomucosal architecture (0-2+) (maximum score =6). For the assessment of gastric damage was performed according to the following criteria, briefly, the damage scores were classified as follows: 0) normal mucosa; 1) edema and/or vacuolation but minimal changes in crypt architecture; 2) epithelial disruption; 3) erosion extending to the muscularis mucosa (12). In order to evaluate fibrosis degree, gingivomucosal sections were positioned longitudinally from the teeth crowns and stained with the Masson trichrome stain, according to the manufacturer's instructions (Bio-Optica, Italy, Milan).

Mast cell staining

Identification of mast cells was performed as described previously (13). Gingivomucosal sections were cut at 5-µm thickness and stained with 0.25% toluidine blue, pH 2.5, for 45 min at room temperature. The sections were then dehydrated and mounted for observation. The density of mast cells is expressed as the number of mast cells per unit area of gingivomucosal tissue.

Myeloperoxidase (MPO) Activity

Myeloperoxidase activity, an index of polymorphonuclear cell accumulation, was determined in the gingivomucosal tissues from each rats, as previously described (14). The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was measured as the quantity of enzyme degrading 1 mM of peroxide min^{-1} at 37°C, and was expressed in units per gram weight of wet tissue.

Immunofluorescence staining for TRAP

14 days after LPS injection, mandibles were collected and post-fixed in 10% formalin and decalcified in EDTA for 24 h. The specimens were then embedded in paraffin. After deparaffinization and rehydration, detection of TRAP was carried put after boiling 0.1M citrate buffer for 1min.Non-specific adsorption was diminished by incubating the section in 2% (vol/vol) normal goat serum in PBS for 20 min. Sections were incubated with mouse monoclonal anti-TRAP (sc-376875; 1:200 in PBS, v/v), in a humidified chamber O/N at 37 °C. Sections were washed with PBS and were incubated with FITC-conjugated anti-mouse Alexa Fluor-488 antibody (1:2000 v/v Molecular Probes, UK) for 1 h at 37 °C. Sections were laved and for nuclear staining 4′,6′-diamidino- 2-phenylindole (DAPI; Hoechst, Frankfurt; Germany) 2 μg/ml in PBS was added. Sections were seen and photographed using a Leica DM2000 microscope (Leica, Milan Italy). Contrast and illumination were established by examining the most intensely labeled pixels and applying backgrounds that allowed clear image of structural details while keeping the highest pixel intensities close to 200. The same backgrounds were used for all images acquired from the other samples that had been managed in parallel. Digital images were collected and figure montages arranged using Adobe Photoshop CS6 (Adobe Systems; Milan Italy).

Immunohistochemical Localization of TNF-α, IL-1β, COX-2 and INOS

14 days after LPS-injection, the tissues were collected and fixed for 24 h in formaldehyde solution (10% in PBS) at room temperature, dehydrated through a graded series of ethanol and xylene and embedded in BioPlast Plus (Bio Optica, Milan, Italy). Thereafter, 7 μm sections were cut from the paraffin-embedded tissue. Following deparaffinization with xylene and graded ethanol as above, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Slices were permeablized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was diminished by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin and avidin binding sites were blocked by progressive incubation for 15 min with biotin and avidin (Vector Laboratories, Burlingame, CA, USA), respectively. Subsequently, slices were incubated overnight with: anti-TNF-α antibody (1/100 in PBS, v/v, sc-52746), anti- IL-1β antibody (1/100 in PBS, v/v, sc-7884), anti-i-NOS antibody (1/100 in PBS,v/v, sc-8310), anti-COX-2 (1/100 in PBS,v/v, sc-376861). Sections were rinsed with PBS and incubated with peroxidase-conjugated bovine anti-mouse immunoglobulin G (IgG) secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2,000 Jackson Immuno Research, West Grove, PA, USA). Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG or biotin conjugated goat anti-mouse IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Immunohistochemical images were collected using a Zeiss microscope and Axio Vision software. For graphic display of densitometric analyses, the intensity of positive staining (brown staining) was measured by computer-assisted color image analysis (Leica QWin V3, UK). The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as percent of total tissue area (red staining), as described above (15).

Western Blot Analysis for IkB-α, NF-kB , NGF, BAX and Bcl2

Gingivomucosal tissues from each rats were suspended in extraction Buffer A containing 0.2 mM PMSF, 0.15 mM pepstatin A, 20 mM leupeptin, 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 12,000 rpm for 4 min at 4°C. Super-natants represented the cytosolic fraction. The pellets, containing enriched nuclei, were resuspended in Buffer B containing1%Triton X-100, 150 mMNaCl, 10 mM Tris–HCl pH 7.4, 1mM EGTA, 1mM EDTA, 0.2 mM PMSF, 20-mm leupeptin, and 0.2-mM sodium orthovanadate. After centrifugation for 10 min at 12,000 rpm at 48C, the supernatants contain the nuclear protein. Protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Briefly, samples were heated to 100 °C for 5 min, and equal amounts of protein were separated on 18% SDS-PAGE gel and transferred to nitrocellulose membrane. Specific primary antibody, anti-IkB-α (1:1000; sc-371), or anti-NF-kB p65 (1:1000; sc-109), anti-NGF (1:1000; sc-365844), anti-BAX (1:1000; sc-526), anti-Bcl2 (1:1000; sc-492), were mixed in 1PBS, 5% w/v nonfat dried milk, 0.1% Tween-20 (PMT), and incubated at 4°C, overnight. After, membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2,000, Jackson Immuno Research) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against laminin (1:1,000; Santa CruzBiotechnology) and β-actin (1:1,000; Santa Cruz Biotechnol-ogy). Signals were detected with enhanced chemiluminescence detection system reagent according to manufacturer's instructions (SuperSignalWest, Pico Chemiluminescent Substrate, Pierce). The relative expression of the protein bands was quantified by densitometry with Bio-Rad ChemiDoc XRS software, and standardized to bactin levels. Images of blot signals (8-bit/600-dpi resolution) were imported to analysis software (Image Quant TL, v2003). A preparation of commercially available molecular weight markers made of proteins of molecular weight 10 to 250 kDa was used to define molecular weight positions and as reference concentrations for each molecular weight.

Materials

All compounds were purchased from Sigma Aldrich (Milan, Italy). All chemicals were of the maximum commercial grade available. All stock mixes were made in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

Statistical Evaluation

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) of N observations. The figures are representative of the three experiments performed on different experimental days. The western blots analyses are representative of 3 different gels made by dividing the number of samples obtained from 10 animals for each experimental group in different days. For *in vivo* studies, N represents the number of animals studied. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered important. The minimum number of animals or samples for each technique was calculated with the statistical test *a priori* power analyzes of the G-power software, this statistical test provides an efficient method to determine the sample size necessary to perform the experiment.

Results

Effect of ATB-352 on tissue damage, mast cells density and myeloperoxidase activity

The histological examination of gingivomucosal tissues showed that, 14 days after LPS injection, the vehicle group exhibited evident presence of edema, tissue injury as well as infiltration of the tissue with inflammatory cells (figure 1 B see histological score) compared to the sham group (figure 1 A see histological score). ATB-352 treatment (figure1 D see histological score) showed an important reduction of tissue damage compared to the LPS group. The effect of ATB-352 was higher compared to ketoprofen group (figure1 D see histological score). No significant changes were seen by administration of ATB-352 and ketoprofen in sham (data not shown). Moreover Masson's trichrome stain, presented increase in the concentration of collagen fibers in gingivomucosal tissues in vehicle group when compared with sham group (figure 1G see fibrosis score panel L). Treatment with ATB-352 (fig 1 I see fibrosis score) significantly attenuated collagen formation as before, the ATB-352 effect was higher compared with ketoprofen (figure 1 H see fibrosis score). No significant changes were seen by administration of ATB-352 and ketoprofen in sham group (data not shown). As show in figure 1 N, 14 days after intragingival injection of LPS, we observed an increase of mast cells infiltration. Treatment with ATB-352 reduce the number of mast cells infiltration, The effect of ATB-352 (figure 1 P) was higher compared with ketoprofen (figure 1 O). In the gingivomucosal tissues collect from LPS-injected rats we observed an increased of MPO activity compared with rats from sham group (figure 1 R), daily treatment with ATB-352 and ketoprofen for 14 days significantly reduced the MPO's activity compared to the vehicle group.

Effect of ATB-352 on bone resorption

A radiographic examination of the mandibles, at day 14 after LPS-injection, indicated a significant increase in the distance between cemento-enamel junction to the maximum coronal level of the alveolar bone crest (CEJ–bone distance) (figure 2 B, see distance CEJ–bone E) in the vehicle group. Treatment with ATB-352 significantly reduced the increase in the distance between cemento-enamel junction and alveolar crest (figure 2 B, see distance CEJ–bone). The protective effect of Ketoprofen was less compared to ATB-352. No significant changes were seen by administration of ATB-352 and ketoprofen in sham group (data not shown). Then we evaluated the expression of the osteoclastic markers such as tartrate-resistant acid phosphatase (TRAP). The immunofluorescence staining for TRAP show, that TRAP 14 days after LPS injection we observed a higher number of TRAP positive cells at the alveolar bone surface (figure 2 G) compared to rats from sham group (figure 2 F), while daily treatment with ATB-352 significantly reduce the number of TRAP positive cells (figure 2 I). the ATB-352 effect was higher compared with ketoprofen (figure H).

Effect of ATB-352 on IκB-α degradation and NF-κB p65 traslocation

By western blot analysis we evaluated IκB-α degradation and nuclear NF-κB p65 expression. A basal level of IκB-α was detected in the gingivomucosal tissue sections taken from the sham group, at 14 days following LPS injection. IκB-α levels were substantially reduced in the gingivomucosal tissues from vehicle-treated rats, ATB-352 treatment prevented IκB-α degradation (figure 3 A, A1. Moreover, LPS increased NF-κB p65 level in the nuclear fractions of gingivomucosal tissues from vehicle group rats, compared to rats of sham group. Treatment with ATB-352 significantly prevented the LPS-mediated NF-kB expression (figure 3 B, B1).

Effect of ATB-352 on IL-1β and TNF-α expressions

immunohistochemical analysis revealed a important increase in the expression of IL-1β and TNF-α in the gingivomucosal tissue of the vehicle group (figure 4 B and G respectively) when compared with sham group (figure 4 A and F respectively). Daily treatment with ATB-352 (figure 4 D and I) and ketoprofen (figure 4 C and H) significantly reduced the expression of these proinflammatory cytokines in gingivomucous tissues. As show by the densitometric analysis (figure 4 E and L) the effect of ATB-352 treatment was higher compared with ketoprofen.

Effect of ATB-352 on iNOS and COX-2 expressions

Sections of gingivomucosal tissue from sham-treated rats did not reveal any immunoreactivity for COX-2 and iNOS within the normal architecture (figure 5 A and F respectively). 14 days after injection of LPS, a positive staining for COX-2 and iNOS was found in the gingivomucosal tissues of the vehicle group (figure 5 B and G respectively). After treatment with ATB-352 (figure 5 D and I) and ketoprofen (figure 5 C and H), a significant reduction of positive coloration was observed for COX-2 and iNOS respectively. The densitometric analisis show that the effect of ATB-352 treatment was higher compared with ketoprofen (figure 5 E and L respectively). No significant changes were seen by administration of ATB-352 and ketoprofen in sham group (data not shown).

Effect of ATB-352 NGF expressions

Western blot analysis shows that 14 days after intragingival LPS injection, in gingivomucosal tissues from vehicle group rats there is a significant expression of NGF. Daily treatment for 14 days

with ATB 352, was able to significantly reduce the NGF expression into gingivomucosal tissues (figure 6 A, A1).

Effect of ATB-352 on apoptosis pathway

gingivomucosal tissue samples were collected 14 days after periodontitis induction in order to determine the expression of Bax and Bcl-2 by western blot analysis. A basal expression of Bax was found in the gingivomucosal tissue of the sham group, a significant increase in Bax expression was found 14 days after intragingival injection of LPS in vehicle group as show in figure 7 (A, A 1). Treatment with ATB-352 significantly reduced the increased of BAX expression induced by LPS (figure 7 A and A1). Moreover western blot analysis detected Bcl-2 expression in gingivomucosal homogenates tissue collected from sham rats, The expression of Bcl-2 was significantly lower in the vehicle group, but treatment with ATB-352 was able to increase the expression of Bcl-2 at levels similar to the sham (figure 7 B and B1).

Effect of ATB-352 on gastric damage

At the end of the 14-day after the periodontitis induction, the histological examinations of sham group indicated there was no alteration of the surface epithelium, while the samples taken from vehicle group showed a mild disruption of the surface epithelium as show in figure 8 respectively panels A and B . The histological examination of the ketoprofen treated rats showed the presence of extensive damage to the gastric mucosa and also the presence of edema and infiltration of the submucosal layer as show in figure 8 panel C. The samples taken from ATB-352 treated rats showed moderate disruption of the surface epithelium and a mild presence of edema and infiltration into submucosal layer (figure 8 D).

Discussion

Periodontal disease is the most common cause of tooth loss in humans, the process of periodontal disease is initiated by bacteria. It has been suggested that bacterial stimulation induces an inflammatory response of the host leading to loss of attachment of the teeth and to damage the surrounding cells and connective tissue structures. The chronic inflammatory response that occurs within the periodontal tissue is a complex process that involves innate and adaptive immune cells and their molecules secreting. It is currently accepted that pro-inflammatory cytokines produced locally by periodontal tissue and inflammatory immune cells contribute to the progression of the disease (16)**.** Thus it is evident that inhibition of chronic inflammatory process is a useful therapeutic strategy in the treatment of periodontitis (17). NSAID therapy is one of the most common pharmacological strategies to inhibit an inflammatory response, antagonizing pro-inflammatory pathways and /or signaling, mainly dependent on COX derived eicosanoids. Even previous reports shown that NSAID treatment is effective to reducing inflammation and bone loss in periodontitis (18). NSAID therapy drugs is not applicable to the treatment of clinical periodontitis, because it need to prolonged therapy. it has been demonstrated that a long-term use of NSAIDs produces severe cardiovascular, renal and gastric side effects (Harirforoosh, Asghar, & Jamali, 2013). So, the interest to obtain a valid and safe anti-inflammatory strategy has led to the synthesis of new NSAID derivatives, in order to minimizing their side effects. New analogous H2S-releasing derivatives of NSAIDs, increase interest for the ability to provide anti-inflammatory effects and significantly reduce gastric adverse effects. In fact it was seen as another analogous H2S-releasing derivative of naproxen is more effective than its parent naproxene, in an experimental model of periodontitis. Thus combining the positive effects of its NSAIDs parens and H_2S. Also, this H2S-releasing derivative of naproxen does not produce harmful gastric effects such as naproxen (Herrera et al., 2015). Furthermore, it has also been seen as H2S plays a key role as an endogenous mediator in several inflammatory states(12) (19), and it has been shown that

exogenous H2S has protective effects in pathological conditions, such as in a rat model of regional myocardial I/R(20), or in a rat model of airway inflammation (21). so the interest in synthesis of new NSAIDs capable of releasing H2S can enhance its anti-inflammatory effects as well as improve gastric tolerability(22).Our results demonstrated that ATB-352, a new H_2S -releasing ketoprofen derivative, treatment provided significantly reduction in LPS-induced inflammatory process in this experimental periodontitis models. Ketoprofen treatment showed a minor protective effect as clearly demonstrated by the histological framework of gingivomucosal tissues. Mast cells play an important role both in physiological and pathological conditions, since they are able to release different proinflammatory mediators and thus increase inflammation of leukocytes in inflammatory states. Infact experimental studies have been associated mast cells associated with oral affections, such as periapical lesions, gingivitis, odontogenic cysts. (23). Moreover Huang et al. (24) demonstrated a correlation between MCs degranulation and periodontitis severity. In accord with precedent studies our results show a significant increase in the number of mast cells in the gingivomucosal tissues of rats injected with LPS compared to the sham group. The daily treatment with ATB-352 reduced the number of mast cells in gingivomucous tissues. Ketoprofen treatment was also effective in reducing the number of mast cells but less efficiently than ATB-352. Also, as shown in the radiographic analysis of the mesial root surface displaying the ability of ATB-352 to reduce periodontal bone-supporting ratio caused by LPS, ketoprofen treatment show a minor protective effect. In addition, the number of positive TRAP cells, a marker for the presence of mature osteoclasts, was significantly increased in the alveolar bone of the rats from vehicle group, indicating an important activation of osteoclasts and an accelerated bone resorption, probably due to the presence of the state inflammatory. treatment with ATB-352 significantly reduced the number of positive TRAP cells indicating a lower activation of osteoclasts. treatment with ketoprofen showed a lower protective effect, in preventing the increase of TRAP positive cells, than the ATB-352. Furthermore, various experimental evidence has clearly

suggested that NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation (25). NF-κB is normally found in cytoplasm linked to regulatory proteins IκBs. In response to different stimuli, such as infection, oxidative stress hypoxia, extracellular signals and inflammation, IκBs regulatory proteins are phosphorylated by the enzyme IκB kinase. So the result is the release of NF-κB dimer that is free to move within the nucleus. In this study, western blot analysis results, shown a remarkable increase of NF-κB p65 expression in gingivomucosal tissues from LPS-injected rats, and the corresponding degradation of IκB-α. Treatment with ATB-352 for 14 days after LPS-injection significantly reduced the degradation of IκB-α and the consequent increase in expression of NF-κB p65 in gingivomucosal tissues. This effect is common to many NSAIDS drugs (26), moreover some evidence suggests that even H_2S exerts an anti-inflammatory action through the inhibition of the expression of NF-κB p65 (27), thus enhancing the anti-inflammatory action of ketoprofen (28). It has been extensively established that NF-kb activation produces regulation of various inflammatory proteins and mediators such as TNF-α and IL-1β. In this study, we confirmed an increase in the expression of proinflammatory cytokines TNF-α and IL-1β 14 days after the intragingival injection of LPS (29). Therefore, according to what seen for NF-κB, our results show that treatment with ATB-352 is able to reduce the expression of TNF-α and IL-1β in gingivomucosal tissues, the protective effect of ketoprofen was less when compared with ATB-352. Another aspect we have evaluated is the increase in iNOS expression in gingivomucosal tissues, after the LPS injection. Since, it has been shown that nitric oxide (NO) produced by iNOS, may contribuite to the inflammatory process and tissue damage associated with periodontitis (30). Therefore, it has also been shown that inhibition in iNOS expression is associated with an improvement in tissue damage and inflammatory process associated with periodontitis disease (31). In this study it has been seen as there is a significant increase in iNOS expression in gingivomucosal tissue, 14 days after intragingival LPS injection. Our results show that, daily treatment with ATB-352 significantly reduced the expression of iNOS,

contributing with the protective effects of ATB-352 in this periodontitis model. This may be due not only to the well-known anti-inflammatory action of ketoprofen, but also to the effects of H_2S which seems to be able to reduce the expression of iNOS (32), in fact ketoprofen treatment showed a minor protective effect. In addition, it has been shown that in inflammatory tissues there is an increase in expression of COX-2 (33), and consequent increases in prostaglandin E2 (PGE2) derived from COX-2. PGE2 in particular play a critical role, as inflammatory mediator and in alveolar bone resorption (34). PGE2 has also been associated with the aggression of the disease, it can be used as a clinical marker of the pathology in progress (35). Therefore, the inhibition of COX-2, and the consequent decrease in PGE-2 is a target of drug therapy, to decrease inflammation and tissue destruction (36). In fact, NSAID treatment reduces bone resorption and inflammation induced by periodontitis (37), but because the presence of side effects, especially at the GI level, restricts the chronic use of NSAIDs (38). Our results show that the ATB-352 significantly reduces the COX-2 expressions in gingivomucosal tissues from LPS-injected rats. Another important aspect of inflammatory process and bone resorption associated with periodontitis is the release of proinflammatory neuropeptides from sensory nerve, inflamed tissues have been shown to over-express the nerve growth factor (NGF) (39). NGF is a pain mediator in the inflammation process, it has been seen that pharmacological inhibition of NGF blocks the state of hyperalgesia associated with inflammation (40). Moreover, NGF is able to up-regulate the release of neuropeptides in sensory neurons and it has been seen that systemic NGF inhibition during periodontitis significantly reduces interleukin 1 beta levels and alveolar bone resorption (39), suggesting that NGF inhibition is useful not only to improve painful but also to reduce inflammation and bone resorption In this study, we found that after 14 days of intragingival injection of LPS, there was a significant increase in the expression of NGF. Daily treatment with ATB-352 significantly reduced the expression of NGF, suggesting also a possible positive effect on nociceptive perception, in this model of LPS-induced periodontitis. Programmed cell death or apoptosis, is a form of physiological cell death. is a

complicated and carefully regulated process, which can be increased or decreased depending on the different physiological and pathological conditions. by western blot analysis we evaluated the expression of two main components, one with proapoptotic role Bax and one with antiapoptotic role Bcl-2. Our results show that treatment with ATB-352 in periodontitis inhibits and prevents the activation of the proapoptotic pathway. Finally, we evaluated the gastric effects of ketoprofen and ATB-352 treatment. our results show how, the rats treated daily with ketoprofen, showed extensive damage to the gastric mucosa with edema and abundant presence of infiltrate. instead the rats, that after the induction of periodontitis were treated with ATB-352 at a dose of 20mg / Kg, showed a better condition of the gastric mucosa than with ketoprofen treatment. Therefore, data from this study show that, ATB-352 treatment is able to, reduce tissue damage and bone resorption by inhibiting the inflammatory process induced by intragingival LPS injection. Therefore the lower presence of side effects, especially at the GI level for this new analogous H2S-releasing derivatives of ketoprofen, suggests that it could be a useful pharmacological strategy for treating the periodontitis.

Conflict of interest
The authors declared no conflict of interest.

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Figure 1 Effect of ATB-352 on tissue damage, mast cells density and myeloperossidase activity

Inflammatory cells infiltration and edema were observed in gingivomucosal section from LPS injected rats (B) when compared with gingivomucosal tissue section taken from sham rats (A). Significantly decrease in edema and inflammatory cell infiltration was observed in gingivomucosal sections from LPS-injected rats treated with ATB-352 (D), ketoprofen treatment showed a minor protective effect (C). Moreover Masson's trichrome stain shown an increase of collagen formation in gingivomucosal tissues sections of LPS injected rats (G) when compared with tissue samples collected from sham rats (F). ATB-352 treatment reduced the increase of collagen (I). the effect of ketoprofen (H) was minor respect ATB-352. Mast cell number was increased in vehicle-treated rats (N) compared to the sham group (M). Treatment with ATB-352 reduced periodontitis–induced increase in mast cell number (P), ketoprofen treatment showed a minor protective effect (O). ATB-352 and ketoprofen treatment significantly reduced myeloperoxidase activity levels, respect vehicle group (R). Figures are representative of at least 3 experiments performed on different experimental days. Data represent the mean ± S.E.M. for 20 counts obtained from the gingivomucosal tissue of each treatment group. Values are expressed as mean ± SEM (N =10 rats in each group). $^{*}P < 0.05$ *vs* sham group. $^{\circ}P < 0.05$ *vs* LPS group.

Figure 2 Effect of ATB-352 on bone resorption

14 days after intragingival LPS injection, radiographic picture of mandible from LPS-injected rats (B) showed a bigger distance from the CEJ to the bone (E), compared to the one of sham group rats (A), while mandible from ATB-352-treated animals showed a shorter distance (D). the protective effect of ketoprofen was minor when compared with ATB-352 (C). Pannels F show immunofluorescence staining for TRAP in alveolar bone from sham group, (G) the vehicle group shows a significant increase in TRAP positive cells. Daily treatment with ATB-352 show a

significantly reductionin TRAP positive cells (I). Ketoprofen treatment (H) show minor protective effect compared to ATB-352 in TRAP positive cells expressions (L). Values reported are expressed as mean ± SEM of 10 rats for each group. $^{*}P < 0.05$ *vs* sham group. $^{\circ}P < 0.05$ *vs* LPS group.

Figure 3 Effect of ATB-352 on IκB-α degradation and NF-κB p-65 traslocation

We evaluated IκB-α degradation and nuclear NF-κB p-65 by Western Blot analysis. A basal level of IκB-α was detected in the gingivomucosal tissue sections taken from sham rats (A, A1). IκB-α levels were substantially reduced (A, A1.) in the gingivomucosal tissues from LPS-injected rats. ATB-352 treatment prevented IκB-α degradation, (A, A1). Periodontitis caused a significant increase in the NF-kB p-65 levels in the gingivomucosal tissues from vehicle group (B, B1). ATB-352 treatment significantly prevented the periodontitis-mediated NF-kB p-65 expression (B, B1). Data reported are presented as mean ± SEM (N =10 rats for each group). $^{*}P < 0.05$ *vs* sham group. $^{\circ}P < 0.05$ *vs* LPS group.

Figure 4 Effect of ATB-352 on IL-1β and TNF-α expressions

Positive staining for IL-1β (B) and TNF-α (G) was observed in gingivomucosal tissue 14 days after intragingival injection of LPS, compared to the sham group (A, F respectively). In gingivomucosal tissue of ATB-352 treated rats no positive staining was observed for IL-1β (D) and TNF-α (I). ketoprofen treatment showed a minor protective effect against the expression of IL-1β (C) and TNF-α (H). Data are mean ± SEM of 10 rats for each group). $^{*}P < 0.05$ *vs* sham group. $^{\circ}P < 0.05$ *vs* LPS group.

Figure 5 Effect of ATB-352 on iNOS and COX-2 expressions

Sections obtained from LPS-injected rats showed intense positive staining COX-2 (B) and iNOS (G), that were found significantly reduced in ATB-352 treated rats (D and I respectively). Treatmen with ketoprofen significantly reduces the COX-2 expression (C) and less the exspession of iNOS (H). No positive immunostaining for COX-2 (A) and iNOS (F) was observed in gingivomucosal tissues from rat belonging to sham-group. Values in graphs are expressd as mean ± SEM (N =10 rats per each group). $^{*}P < 0.05$ *vs* sham group. $^{\circ}P < 0.05$ *vs* LPS.

Figure 6 Effect of ATB-352 NGF expressions

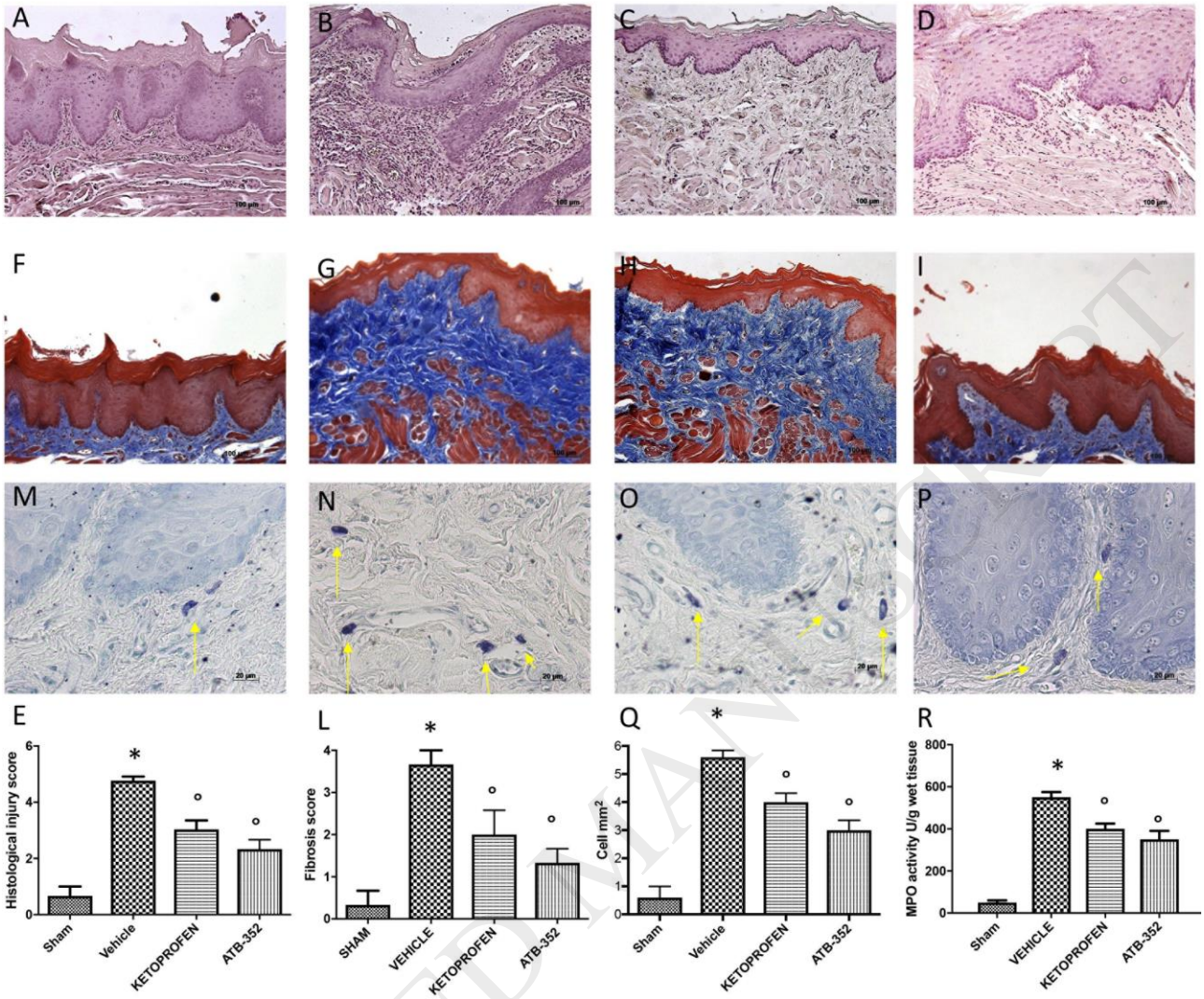
Western blot analyses performed on samples of gingivomucosal tissue from LPS-injected rats, compared to sham group, displayed an increase of NGF expression, that was found reduced in ATB-352-treated rats (A and A1). Densitometric analyses of blots were performed normalizing bands for b-actin. Blots are representative of 3 different gels. Data in are means ± SEM of 10 mice for each group. $^{*}P < 0.05$ *vs* sham group. $^{\circ}P < 0.05$ *vs* LPS group.

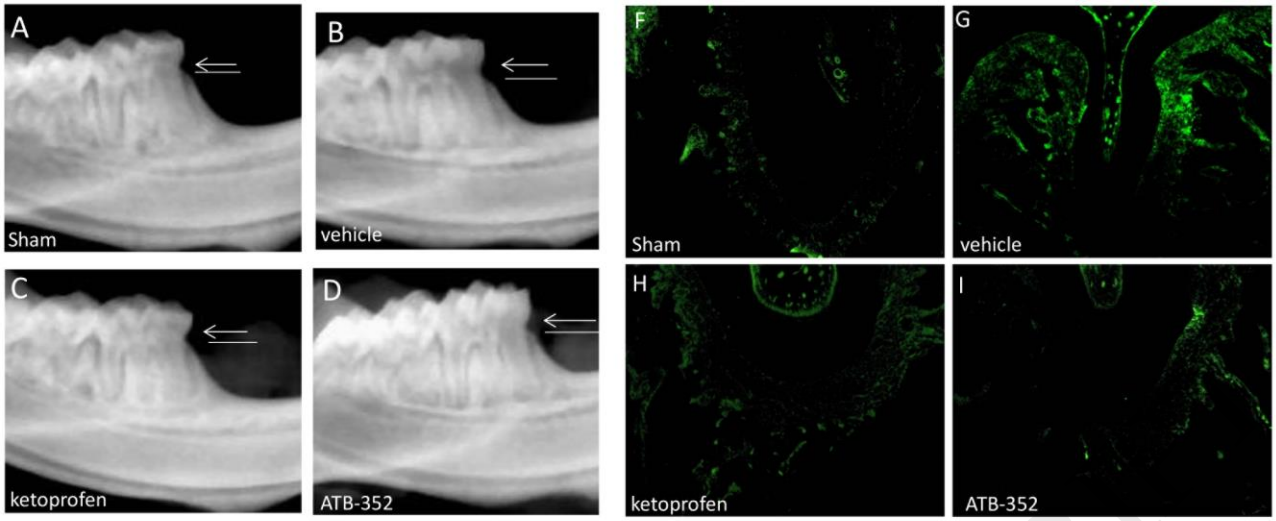
Figure 7 Effect of ATB-352 on apoptosis pathway

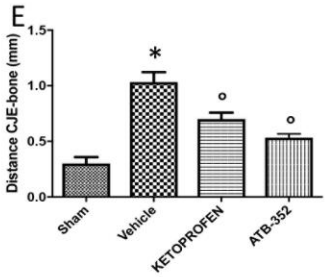
Basal level of Bax was present in the tissue from-sham rats, Bax band is more evident in the tissue from LPS-injected rats, The Bax expression is significantly reduced by ATB-352 treatment (A, A1). Moreover a basal level of Bcl-2 was present in the tissue from sham rats. Bcl-2 expression in significantly reduced in tissue from vehicle group. Treatment with ATB-352 restores the expression of Bcl-2 similar to the sham group (B, B1). Blots are representative of 3 different gels. Data in are means ± SEM of 10 mice for each group. $^{*}P < 0.05$ *vs* sham group. $^{\circ}P < 0.05$ *vs* LPS group.

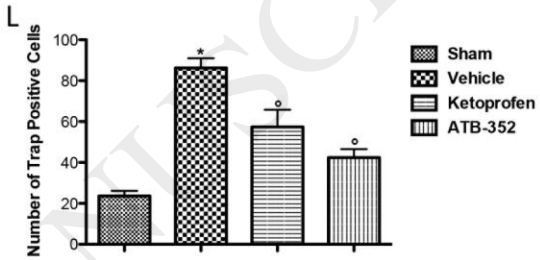
Figure 8 Effect of ATB-352 on gastric damage

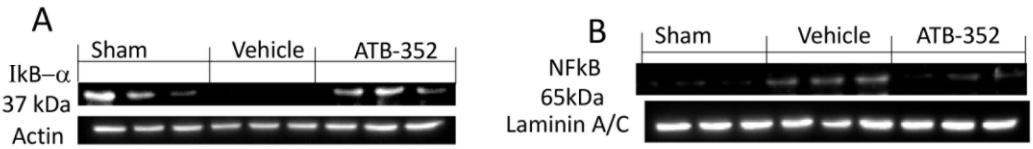
The histological analisys of sham group (A) showed no alteration of the surface epithelium. The vehicle group showed mild (B) alteration of the surface epithelium, while the group treated with ketoprofen (C) showed extensive damage to the gastric mucosa and also the presence of edema and infiltration of the submucosal layer. The group treated with ATB-352 (D) showed moderate disruption of the surface epithelium and a mild presence of edema and infiltration into submucosal layer. Values are expressed as mean ± SEM (N =10 rats in each group). $^{\circ}P < 0.05$ *vs* LPS group.

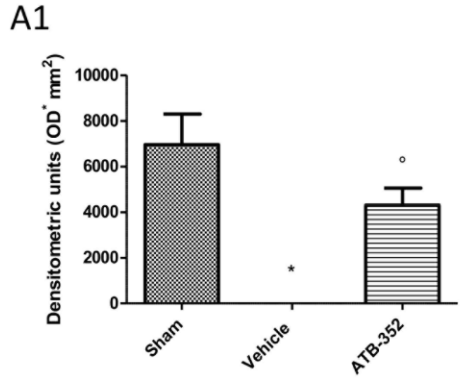


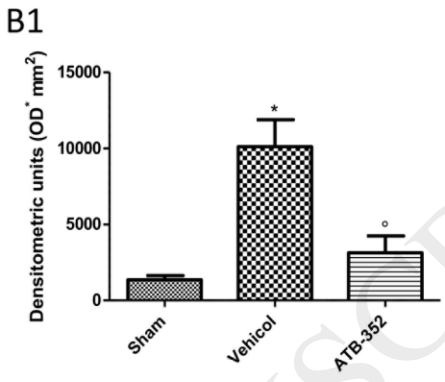


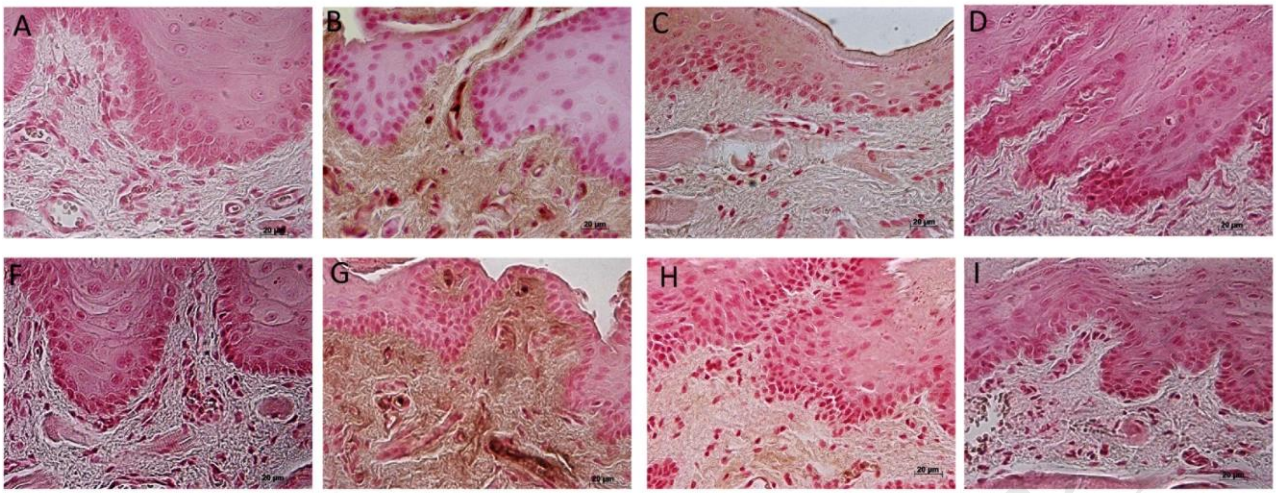


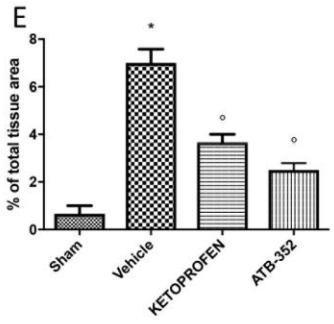


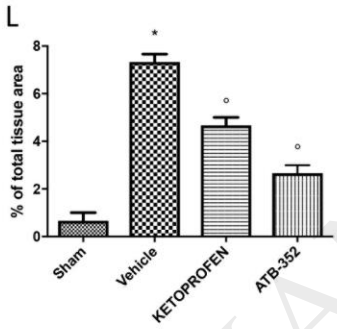


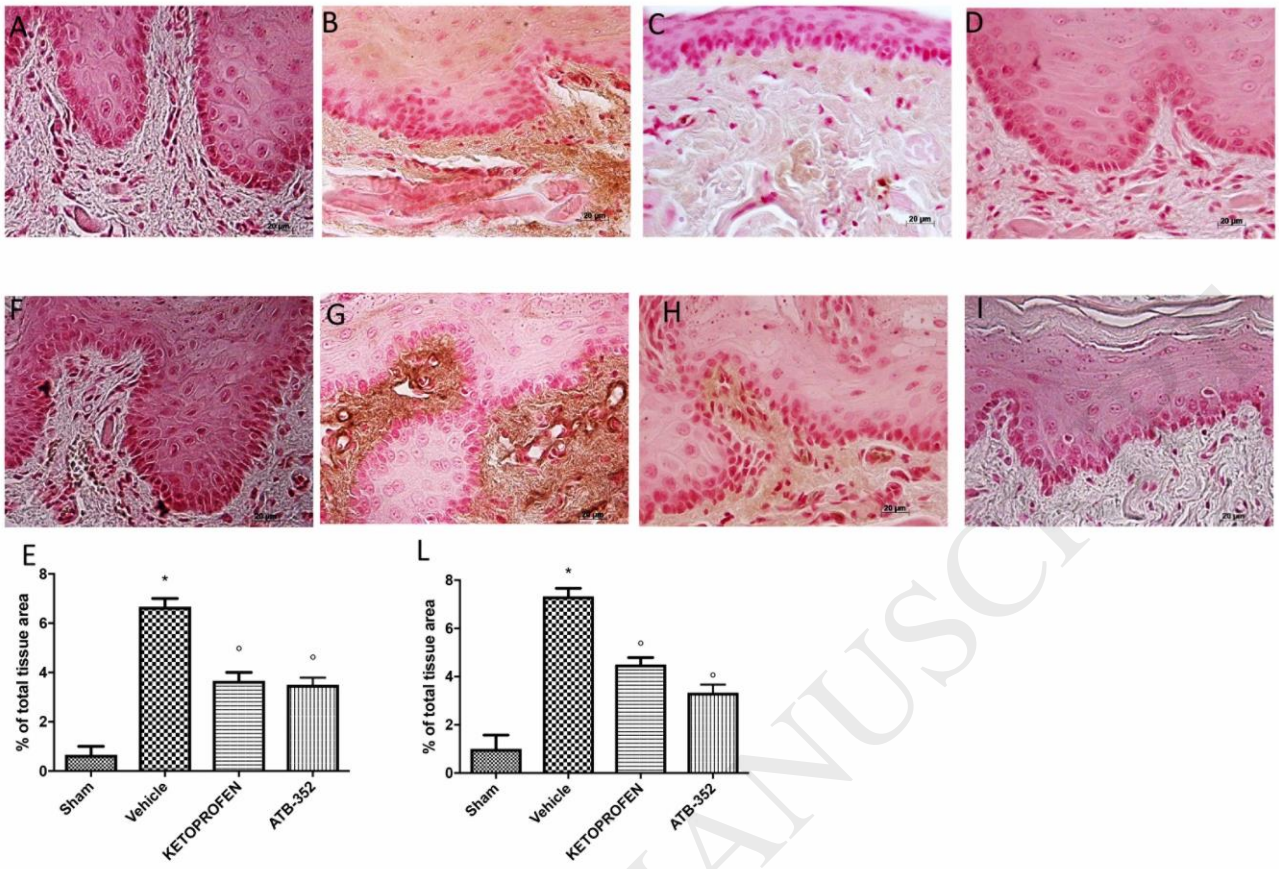












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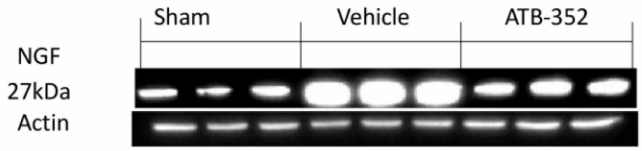

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