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Hydrogen sulfide releasing naproxen offers better anti-inflammatory and chondroprotective effect relative to naproxen in a rat model of zymosan induced arthritis

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Abstract. – OBJECTIVE: Hydrogen sulfide (H_2S) is rapidly gaining ground as a physiological mediator of inflammation, but there is no clear consensus as to its precise role in inflammation. Therefore, this study was undertaken to evaluate the effects of ATB-346 as a novel H_2S-releasing naproxen compared to naproxen, as a traditional non-steroidal anti-inflammatory drug on zymosan induced mono-arthritis in rats.

MATERIALS AND METHODS: Male Wistar rats (n=48) were randomly assigned to four main groups: normal control, untreated arthritis, Naproxen and ATB-346 treated groups. Mono-arthritis was induced by intra-articular injection of zymosan into the knee joints. Mechanical hypernociception and joint swelling were evaluated at 6 hours and 5 days. Inflammatory cellular recruitment and adherence, tumor necrosis factor alpha, nuclear factor kappa β, total sulfide levels, and histological changes were evaluated in knee lavages, blood or joint tissues at selected time points.

RESULTS: Zymosan injection evoked knee inflammation and pain as characterized by mechanical hypernociception, impaired gait, joint swelling with inflammatory exudation and histological changes. Treatment with ATB-346 attenuated nociceptive responses, inflammatory cellular and biochemical changes in comparison to naproxen. Only ATB-346 was able to suppress neutrophil adherence and to preserve normal articular structure.

CONCLUSIONS: H_2S releasing naproxen represents an advancement over the parent drug, naproxen. Apart from the superior anti-inflammatory and anti-nociceptive activity, ATB-346 offered a distinguished chondroprotective effect and is almost devoid from naproxen deleterious effects on articular cartilage.

Key Words:
Arthritis, Hydrogen sulfide, Inflammatory markers, ATB-346, Naproxen, Zymosan.

Introduction

The inflammatory response involves the activation of several biochemical mediators and cytokines[1,2]. Commonly used chemical models of inflammatory mono-arthritis include the zymosan-induced arthritis. The zymosan arthritis is characterized by an acute phase (within hours) of increased vascular permeability, edema formation, neutrophil infiltration and exudation, whereas the chronic phase (days to several weeks) resembles chronic rheumatoid synovitis[3].

Physiological gaseous mediators have been proposed to induce, inhibit and regulate the inflammatory process. Recently, an endogenous gas, hydrogen sulfide (H_2S), has been recognized as an inflammatory mediator. H_2S is synthesized in the human body from the amino acids cystathionine, homocysteine, and cysteine by the action of two main enzymes: cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS)[4]. Exogenous or altering endogenous H_2S may have unanticipated effects since H_2S plays a role in regulation of chemokines, cytokines and adhesion molecule expression[5]. However, the role of H_2S in inflammation is still a matter of debate[6]. In some studies, H_2S has been shown to be pro-inflammatory, while others have reported an anti-inflammatory effect of H_2S[7,8]. The mechanisms of such effects have not yet been fully investigated. Activation of nuclear transcription factor-κβ (NF-κβ) with subsequent intracellular adhesion molecule (ICAM)-1 upregulation has been reported to explain the H_2S pro-inflammatory activity[9]. Other researches on the other hand had demonstrated numerous anti-inflammatory ef-

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fects of H_2S by reducing adhesion and rolling of circulating leukocytes in inflamed microvasculature[10].

Non-steroidal anti-inflammatory drugs (NSAIDs) remain the most commonly used drugs for treating arthritis. To generate more effective anti-inflammatory drugs, there is a need to target more elements of inflammation than just prostaglandin synthesis. Hydrogen sulfide releasing derivative might provide an answer because it has recently been shown to exert several anti-inflammatory effects and to increase the resistance of the stomach to injury[11].

Therefore the aim of this work was to study the effect of exogenous H_2S in modifying knee joint inflammation; evaluating the role of tumor necrosis factor-α and NF-κβ in mediating the proposed pro- or anti-inflammatory effect of exogenous H_2S in arthritis. It was also designed to examine whether H_2S releasing NSAIDs would be superior to traditional NSAIDs in reducing the inflammatory reaction in a rat model of zymosan arthritis.

Materials and Methods

The experiments were approved and performed according to the guidelines of the Ethics Committee of Alexandria Faculty of Medicine-Ethical Animal Code. Permit number: 000075555.

Forty eight adult male Wistar rats (180-200 g) were used in the study; the animals were housed in standard plastic cages with food and water available *ad libitum*.

Induction of Arthritis

Arthritis was induced by injecting 1 mg zymosan (Sigma, St. Louis, Mo, USA), in 50 µl sterile saline into the right knee joint through the lateral margin of the patella tendon in each animal in a deep knee-flexed position under ether anesthesia using a 29-gauge needle[12]. An equivalent amount of sterile saline was injected into the left knee as a negative control for assessment of the knee circumference. Control rats received intra-articular (i.a.) saline injection in both knees to serve as a control for all other parameters.

Experimental Design

The animals were divided into 4 experimental groups (12 rats/group): Group I: Normal control; Group II; Arthritis group; rats in both groups received gum acacia orally as a vehicle; Group III; Naproxen treated group receiving naproxen (Naprofen®; 10 mg/kg) orally; Group IV; ATB-346 treated group receiving the H_2S releasing naproxen derivative in equimolar dose (ATB-346-Antibe Therapeutics Inc, 15.9 mg/kg) orally[13].

All groups were then subdivided into 2 subgroups A and B (6 rats each). The A subgroups received only single drug or vehicle treatment one hour prior to induction of arthritis and were used for studying the acute phase of arthritis. After 6 hours of induction, pain behavior and gait score were assessed; then, all animals were anesthetized and joint swelling was evaluated by measuring the knee circumference at marked points using a flexible tape in accordance with the procedure reported by Yu et al[14] and ratios of arthritic to normal joints circumferences were calculated. Animals were then euthanized and the synovial cavities of the knee joints were washed with 0.4 mL saline containing 10 mM EDTA. The synovial exudate was aspirated for determination of cell count and assay of neutrophil adherence. B subgroups, on the other hand, were studied for 5 days after induction of arthritis during which drug dose or vehicle was given twice daily. At the end of the study period, pain behavior, gait score and joint swelling were also evaluated as mentioned above. Blood was then collected by cardiac puncture; aliquoted with subsequent plasma, serum or peripheral blood neutrophils isolation for estimation of total sulfide, TNF-α and NF-κB respectively. After euthanization, knee joints were surgically isolated for histopathological examination.

Assessment of Pain Behavior by Pin Prick Test

Rats were tested for the presence of pain-like behaviors or secondary hyperalgesia using pin prick test as indirect measure in knee monoarthritis. Rats were placed in specialized cages with a metal mesh floor allowing access to the plantar surface of the hindpaw. The pin prick test was done by pressing the plantar surface of the hindpaw with the point of safety pin, at intensity sufficient to produce a reflex withdrawal response in normal animal, but without skin penetration. Mechanical stimuli were given ten times to the arthritic limb through the wire mesh floor. The occurrence of paw withdrawal in each of these ten trials was expressed as percent withdrawal frequency [(number of paw withdrawals/number of trials) × 100]. Avoidance responses such as lifting, shaking or licking the paw, and running away were regarded as positive responses[15].

Gait Score

Knee joint pain was evaluated by the functional measurement of animal behavior using a modified gait score based on walking pattern. In a quiet, dimmed room, each rat was placed on an open bench that enabled the animal to walk freely. The severity of disturbances of walking was graded as score 0 (normal; rat runs and walks normally), score 1 (mild disability; rat runs and walks with difficulty), score 2 (rat walks with difficulty due to intermittent loading of inflamed joint) or score 3 (rat stands on only three paws i.e. total joint immbolity)[16].

Cell Isolation and Preparation and Neutrophil Adherence Assay

Joint lavage fluids were centrifuged at 200 × g for 10 min at room temperature, and the pelleted cells were suspended in PBS. The viability of recovered cells was found to be more than 97% as detected by trypan blue dye exclusion test. The total cell yield was estimated using a Neubauer haemocytometer. Microscopic examination of a leishman stained film confirmed neutrophil predominance. Cells were then resuspended in normal rat serum and neutrophil adherence was assayed by a modification of the method of MacGregor et al[17] and Rodriguez et al[18]. Briefly, 1 ml of cell suspension was allowed to flow through the adherence columns prepared by packing 70 mg of nylon fiber into 23 cm pasteur pipettes at 37°C. After 10 minutes, the effluent fluid was collected and the percentage of neutrophil adherence is calculated by the following equation: % of Neutrophil adherence = 100-Neutrophil/ml in the effluent sample/Neutrophil/ml in the original sample × 100.

Preparation of Histological Sections of the Knee Joint

Knee joints were dissected and fixed in 10% paraformaldehyde. After dehydration with a gradient ethanol series and decalcification with 20% EDTA (pH 7.4), they were embedded in paraffin, and 5 μm-thick sagittal sections were prepared with a cutting plane. Whole knee joints were stained with hematoxylin and eosin (H&E) and examined by light microscopy to evaluate joint inflammation[19].

Biochemical Assay

Aliquots of plasma (100 μl) were immediately assayed for total sulfide as described previously using the zinc trap colorimetric assay[20]. Serum TNF-α (pg/ml) was measured by using ELISA (Quantikine Rat TNF- alpha Immunoassay e-Labscience, Carugate, MI, Italy) according to the manufacturer's instructions[21]. NF-κβ was detected in peripheral blood neutrophils by western blotting[22]. Briefly, neutrophils (95-98% purity) were isolated using Ficoll-Paque centrifugation and were subsequently purified by dextran sedimentation and hypotonic lysis of residual erythrocytes. Cayman's nuclear extraction kit was used to isolate nuclear protein according to the manufacturer's instructions. A modification of Lowry et al[23] method was then used for determination of protein. Protein (50 μg) was separated by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted with primary rabbit polyclonal anti-NF-κB/p65 antibody (1:100 Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). Equal loading was confirmed by reprobing the membranes with secondary antibody (Horse Radish Peroxidase). DAB (3, 3´-diaminobenzidine tetrahydrochloride) chromogenic substrate solution then hydrogen peroxide 30% were added. Detection was performed using the Corel paint shop pro X2 software, the color intensity of each band was converted to a number with red green blue (RGB) unit and divided by the protein concentration in each sample to be represented finally with RGB unit/mg protein[24].

Statistical Analysis

Using one sample Lilliefors test for normality, the distribution of each set of data was tested for normality before analysis. Data were expressed as mean ± SEM. Tests of significance were two tailed and $p < 0.05$ was considered statistically significant. Multiple variables were evaluated by one-way ANOVA test, followed by multiple comparison test using least significance difference procedure. Results for gait score were expressed as median values and analyzed by Kruskal-Wallis test followed by Mann Whitney U test for comparison. Analysis was conducted using Statistical Toolbox for the MATLAB (Matrices Laboratory software version R2008b).

Results

Gait Score and Pain Behavior

Zymosan injection resulted in significant impairment of the normal walking pattern (as shown by the increased gait score) and increased

paw withdrawal frequency % (PWF%) in animals 6 hours and 5 days in comparison to the saline injected subgroup. ATB-346 was able to significantly improve gait score following both treatment periods in comparison to arthritis subgroups. Treatment with naproxen or ATB-346 was associated with a significant decrease in PWF% compared to the arthritis subgroups in both study periods. ATB-346 treatment in either the 6 hour or the 5 days subgroups was associated with significant decrease in PWF% compared to naproxen treatment (Figure 1a, b, c).

Knee Joint Swelling

Examination of knee joints six hours after zymosan injection, revealed a significant increase in the ratio of joints circumferences as compared to control rats. With prolonged period of inflammation up to 5 days, this increase was still significant as compared to the control indicating a positive inflammatory reaction at different time point. Treatment of animals with ATB-346 in either the 6 hours or the 5 days subgroups resulted in significant reduction in the joint circumference ratio compared to the arthritis group. However, the naproxen treated rats showed a significant reduction of knee joint swelling only in the acute arthritis subgroup (Figure 2).

Leukocyte Adherence and Recruitments in Synovial Fluid

As shown in Table I, intra-articular injection of zymosan resulted in early leukocytic recruitment into the synovial cavity in all zymosan injected subgroups, while nearly no cells could be harvested from synovial lavage of saline injected control subgroup. Single treatment of the animals with naproxen or ATB-346 prior to arthritis induction resulted in a significant reduction in leukocytic count in the synovial fluid. Moreover, H_2S-releasing naproxen was found to significantly decrease neutrophil adherence from 74.9% in arthritic group to 50.3%, the latter effect was not observed with naproxen treatment.

Biochemical Estimates

Treatment of the arthritic rats with ATB-346 for 5 days resulted in a significant rise of plasma total sulfide concentration (38.2 ± 0.73 μM) compared to all other subgroups (Figure 3a). Conversely, naproxen treatment was associated with a significant total sulfide reduction.

Intra-articular injection of zymosan was associated with a significant increase in both TNF-α and NF-κβ after 5 days following induction of arthritis as compared to control rats.

The zymosan induced elevation of TNF-α was found to be significantly reduced after 5 days of treatment with either naproxen or ATB-346;

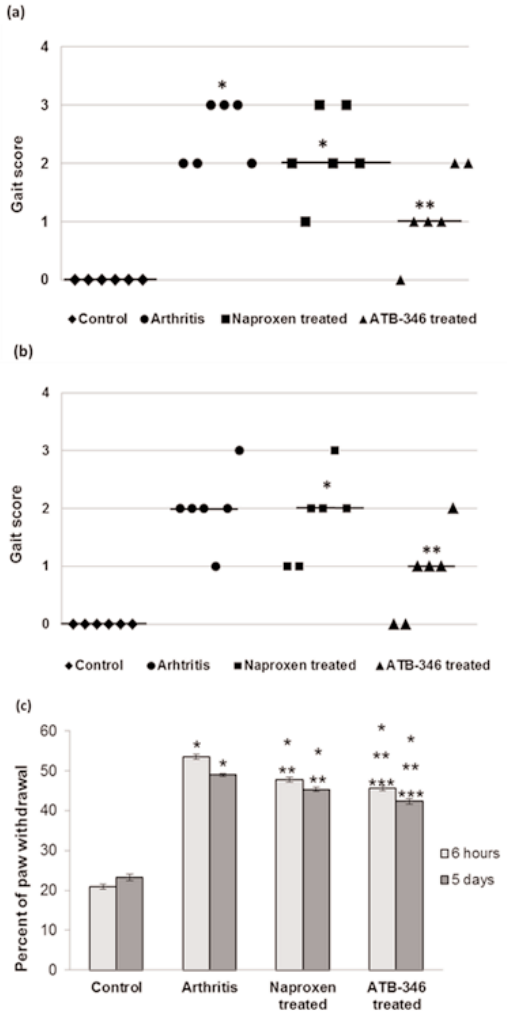

Figure 1. Effect of pretreatment with Naproxen and ATB-346 on Zymosan induced impairment on walking pattern and nociceptive responses. ***(A)*** Walking behavior or gait was scored on a scale from 0 (normal) to 3 (total joint immobility), 6 hours and ***(B)*** 5 days following intra-articular injection of zymosan. In ***(A)*** and ***(B)*** data are presented as a scatter plot of n=6 with the median values for each experimental group. Median value after 6 hours for arthritis subgroup is 2.5. *p = 0.001 versus normal control subgroup; **p = 0.001 versus arthritis subgroup after 6 hours and **p = 0.02 after 5 days. ***(C)*** Pain like behavior evaluated by pain prick test and expressed as paw withdrawal frequency percent in arthritic limb in all subgroups, 6 hours and 5 days after induction of arthritis. Data represent the mean ± SEM.*p = 0.001 significant versus normal control group; **p = 0.001 compared to arthritis group. *** p = 0.02 after 6 hours and p = 0.001 after 5 days compared to naproxen treated subgroup.

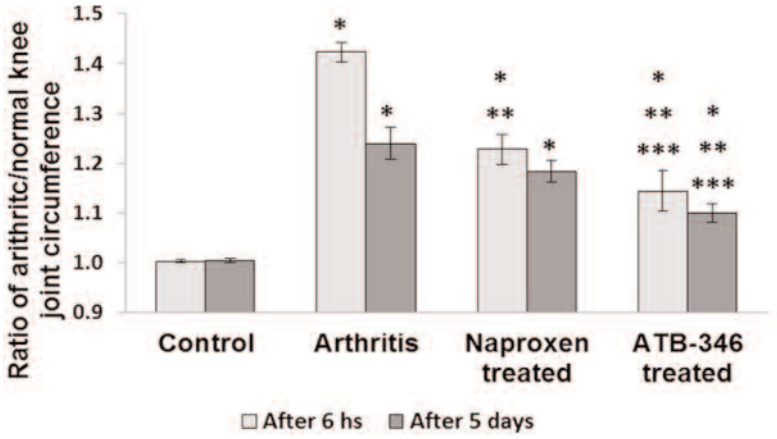

Figure 2. Effect of pretreatment with Naproxen and ATB-346 on zymosan induced knee joint swelling. Right knee joints received i.a. injection of zymosan (1mg/50μl saline), while left Knee joints were injected with saline. Control rats received i.a. of saline in both knee joints. Joint circumferences were measured 6 hours and 5 days after induction of arthritis and ratios of right to left joints circumferences were calculated. Data are presented as means ± SEM *p < 0.05 compared to control subgroup; **p < 0.001 compared to arthritis subgroup. ***p < 0.05 compared to naproxen treatment.

however, ATB-346 treatment achieved more reduction in TNF-α level compared to naproxen treatment. It is worth mentioning that H_2S-releasing naproxen treatment was able to almost normalize the serum level of TNF-α (p = 1.0).

Western blotting of NF-κB revealed a significant reduction after 5 days of drug treatment by 68% and 41% for ATB-346 and Naproxen, respectively (p = 0.001 for both). ATB-346 was significantly superior to naproxen in reducing NF-κβ (p = 0.001) (Figure 3b,c).

Table I. Effect of pretreatment with Naproxen and ATB-346 on cell count and adherence of leukocytes recovered from synovial lavage 6 hours after intra-articular injection of zymosan.

Subgroups	Arthritis (vehicle treated)	Naproxen treated	ATB-346 treated
Leukocytes count (x 106/cavity)	6.24 ± 0.13	4.65 ± 0.15*	1.56 ± 0.15*,**
Neutrophil adherence %	74.9% ± 0.3	76.2% ± 0.6	50.3% ± 0.7*,**

Zymosan injected animals were pretreated with either naproxen (10 mg/kg) or ATB-346 (15.9 mg/kg), or with a vehicle. Total cell counts in synovial exudate were determined 6 hours post injection and neutrophil adherence was assayed and expressed as percentage of adherent cells. Almost no cells could be harvested from joints of normal control rats. Data is presented as mean ± SEM of 6 rats per subgroup. One way ANOVA was conducted and the results of post hoc least significance difference comparison were shown. *p = 0.001 versus arthritis subgroup, **p = 0.001 versus naproxen treated subgroup.

Histological Finding

Examination of H&E-stained histological sections of normal non-arthritic knee joints (Figure 4a-c) revealed normal histological appearance with a clear joint space, intact synovial membrane and normal articular cartilage. Zymosan injection resulted in a pronounced inflammatory response as shown by prominent mononuclear cellular infiltration and proliferation with areas of increased vascularization. In addition, thickening of the synovial membrane and joint capsule, thinning of articular cartilage with areas of ragged articular surfaces were observed. The articular surface also exhibited densely-stained basophilic matrix with proliferating chondrocytes forming cell nests (Figure 4d-i). Rats treated with naproxen for 5 days had a tendency to exhibit less intense cellular infiltration in some specimens but still with thin articular cartilage with mild surface irregularity and chondrocytes showing dark pyknotic nuclei. Other specimens showed prominent cellular infiltration of the joint capsule that even extened into the joint space and covered the surface of the articular cartilage (Figure 4j-o). In contrast, treatment with ATB-346 for 5 days resulted in amelioration of most these structural derangements as evidenced by intact joint space, normal joint capsule with minimal cellular infiltration. The articular cartilage appeared with normal thickness and relatively smooth surface. Some proliferating chondrocytes were observed within their lacunae forming cell nests; however, the articular hyaline cartilage restored the pale basophilic staining of its matrix (Figure 4p-r).

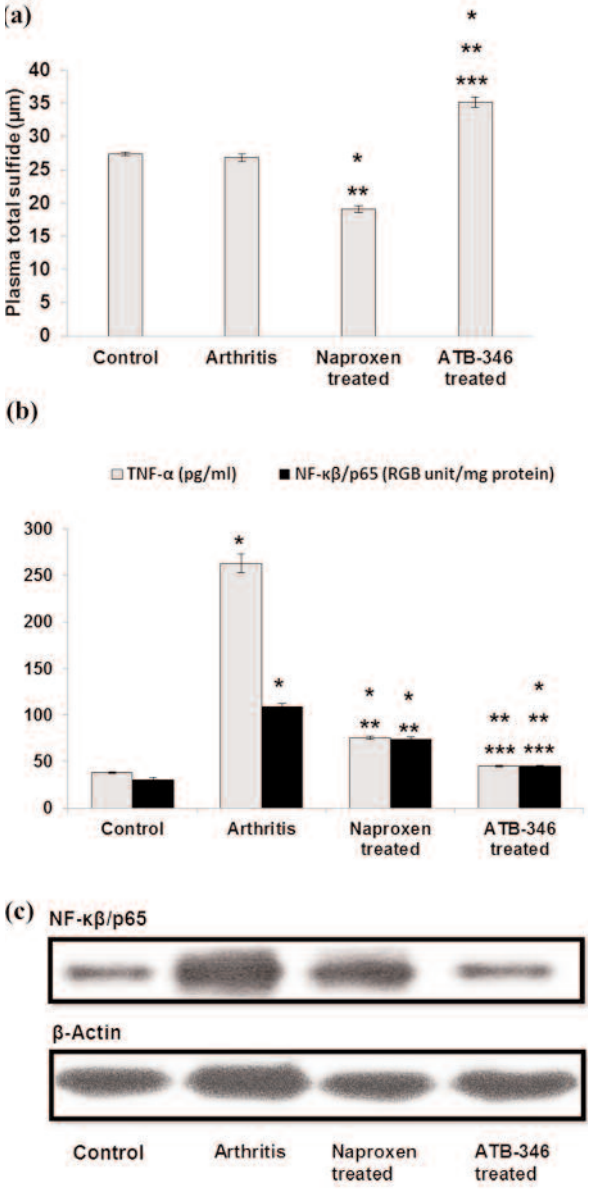

Figure 3. ***A,*** Plasma total sulfide. ***B,*** Serum TNF-α and peripheral blood neutrophil NF-κβ levels in the studied groups 5 days after induction of arthritis. Data are presented as means ± SEM *$p < 0.001$ compared to control group; **$p < 0.001$ compared to arthritis group. ***$p < 0.001$ compared to naproxen treatment. ***C,*** Western blot analysis of NF-κB in the studied groups using rabbit anti-NF-κB/p65 antibody. The color intensity of each band was converted to a number with red green blue (RGB) unit and divided by the protein concentration in each sample to be represented finally with RGB unit/mg protein as in ***(B)***.

Discussion

The administration of the H_2S releaser ATB-346 for five days was found to result in significant increase in the plasma total sulfide concentration in comparison to all other groups. Thus, the currently observed effects of ATB-346 over naproxen can be primarily attributed to the sulfide moiety and not to the parent drug naproxen. Since blood sampling was almost done around 3 hours after the last dose of administered drugs, we speculate that similar to the other S-N bond containing H_2S donors[25], ATB-346 slowly released H_2S from its carbamoylic moiety, an assumption that yet needs to be confirmed. Naproxen administration was associated with a significant decrease in plasma total sulfide. However, this finding may be explained by previous report that H_2S synthesis was significantly reduced following administration of NSAIDs, apparently through suppression of the expression and activity of cystathionine γ-lyase (CSE), one of the key enzymes for conversion of L-cysteine into H_2S[26].

In the present study we have demonstrated that both naproxen and ATB-346 decreased pain in the inflamed joint throughout the early and late phases of zymosan arthritis. These effects may be related in part to the inhibition of prostaglandins synthesis by both drugs. However, a possible role for H_2S release can be assumed since a better gait score was achieved by ATB-346 treatment in comparison to naproxen. A possible protective effect of H_2S against nociception is controversial, since anti-nociceptive, pro-nociceptive, and even no effect, were previously reported[16,27,28]. The cause of this controversy is not known; however, different animal models, different hydrogen sulfide donors, timing and method of pain assessment may be considered. The anti-nociceptive effect of H_2S may be related, at least in part, to the currently demonstrated inhibitory effect on acute joint inflammation, local tissue swelling and edema formation. Since trafficking of inflammatory cells is the key component of any tissue based response to injurious agents, the reduction of cellular infiltration seems to be the primary mechanism by which the ATB-346 modulates joint arthritis. In the earliest stages of inflammation, neutrophils are particularly prevalent, but later, monocytes and lymphocytes also migrate towards the site of injury[29]. The inhibitory effect of H_2S donor on leukocyte infiltration was previously explained by a local vasoconstrictor effect of H_2S[28]. Herein, we attribute it to the inhibitory effect of ATB-346 on neutrophil adherence as it is the initial step for inflammatory cellular recruitment to the site of inflammation. Since leukocyte adherence is known to increase after NSAIDs administration[26], the currently shown inhibitory effect on cellular adherence and

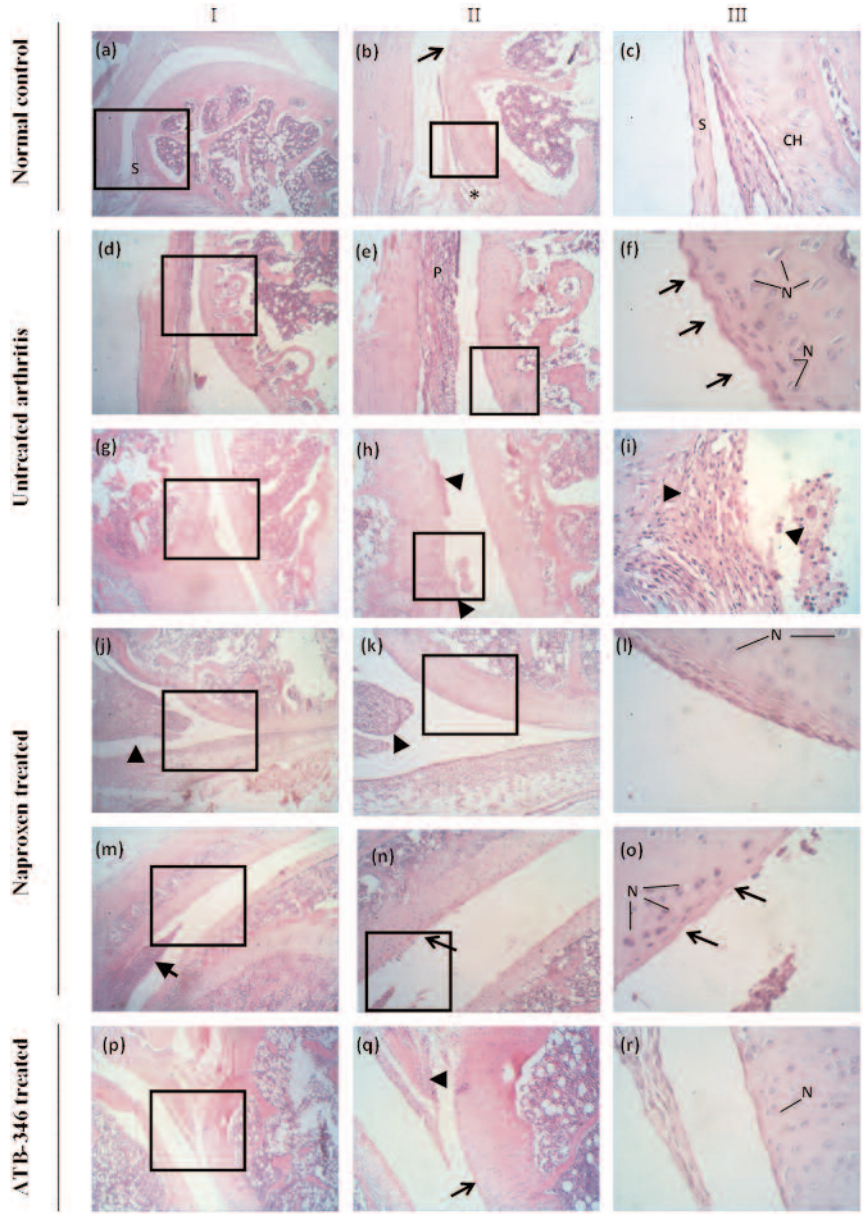

Figure 4. Representative photomicrographs of H&E stained sections of rat knee joints 5 days after receiving i.a. injections.; light microscopy with low (x40, column I); medium (x100, column II) and high magnification (x400, column III). ***A-C***, Micrographs of knee joint of control rats showing clear joint space with intact joint capsule. The inner synovial layer (S) is formed of low cuboidal cells with small amount of adipose tissue (*) seen between the 2 layers of the joint capsule. The articular cartilage *(arrow)* appears with smooth surface and pale basophilic matrix. The chondrocytes (CH) are seen in their oval lacunae with flattened nuclei. ***D-I,*** Micrographs of knee joints of untreated arthritic group, showing thickened joint capsule with prominent cellular proliferation (P). Mononuclear cellular infiltration and increased vascularization *(arrowheads)* were also observed within the synovial membrane, extending into the joint space and towards the peripheral boundary of the articular cartilage. Thinning of the articular cartilage with evident irregularity of its surface is seen (arrows). Notice the densely-stained irregular cartilage matrix and proliferating chondrocytes in cell nests (N). ***J-O,*** Micrographs of knee joints of Naproxen treated rats showing prominent thick joint capsule with cellular infiltration extending into the joint space and the articular surface *(arrowheads)*. The articular cartilage is thin with some ragging *(arrows)* and darkly-stained chondrocytes with pyknotic nuclei (N). ***P-R,*** Micrographs of knee joint of ATB-346 -treated rats, showing almost normal joint capsule and articular cartilage *(arrow)*. Minimal cellular infiltration is observed in the cellular layer of the capsule *(arrow head)*. Some proliferating chondrocytes are seen insides their cell nest (N). Notice the normal pale staining of the articular cartilage matrix.

recruitment represents an improvement of ATB-346 over the parent compound naproxen.

Several days after intra-articular injection of zymosan, the induced arthritis is known to resemble chronic rheumatoid synovitis[30]. Administration of either naproxen or ATB-346 was associated with decreased joint inflammation. However, ATB-346 further decreased synovial membrane thickenings with almost normal articular cartilage and clear joint space. Thus, the early modulation of acute inflammatory process by ATB-346 could significantly affect resolution of inflammation and ameliorate joint damage. A novel finding in this study is the modulatory effect of ATB-346 on cartilage matrix and chondrocyte survival in contrast to the observed negative effects of naproxen treatment. It seems that with naproxen treatment, the early adaptive response of increased chondrocyte proliferation and matrix formation in response to injury, turned maladaptive as evidenced by presence of pyknotic nuclei. Such response was enhanced towards repair with the use of ATB-346. Given the unique report that NSAIDs use in osteoarthritis accelerates articular cartilage breakdown[31], the possible role of H_2S-releasing naproxen in preventing chondrocyte apoptosis and matrix degradation needs further evaluation in different models of joint injury. The superior anti-inflammatory and antinociceptive effect of ATB-346 compared to naproxen may be also a consequence to its more inhibitory effect on TNF-α production. The ability of ATB-346 to totally mitigate the zymosan induced TNF-α rise is strongly supported by the finding of Li et al[32]. According to their work, H_2S inhibited the TNF-α-converting enzyme (TACE), which converts membrane-bound pro-TNF-α to mature and soluble TNF-α. Since TACE is a zinc-containing metalloproteinase, it was suggested to be a target for H_2S which has a high affinity for zinc, a property widely exploited for the measurement of H_2S levels. TNF-α is considered a pivotal cytokine in inflammatory arthritis whose antagonism significantly reduced both joint hypernociception and cell influx[33]. Taken together the fact that TNF-α is a principle cytokines initiating catabolic responses in chondrocytes[31], we assume that the ability of ATB-346 to totally abrogate TNF-α rise accounts for its chondroprotective activity.

It is also likely that inhibition of gene transcription through the NF-κB pathway is central in mediating the anti-inflammatory effect of exogenous H_2S. NF-κB is a key player in intracellular signaling pathways in response to inflammatory stimuli and is required for adhesion molecules and many pro-inflammatory cytokines production[34]. This is in accordance with previous reports that other potential H_2S donors, such as the garlic constituent, diallysulfide, also inhibited NF-κB activation in primary cultures of human chondrocytes[35]. Nevertheless, Stuhlmeier et al[36], failed to demonstrate any effect of H_2S on NF-κB neither *in-vitro* on human fibroblast-like synoviocytes nor *in-vivo* in mice. In contrast, several studies reported a pro-inflammatory effect of H_2S where inhibition of its production was associated with reduced mRNA level of many inflammatory cytokines including TNF-α coupled with reduced translocation and activation of NF-κB in lung, liver and pancreas in different rat models of sepsis or acute pancreatitis[9,37,38]. Beside the different inflammatory models used in these studies, their data were generated by either the use of H_2S synthesis inhibitors such as DL propargylglycine (PAG) or the H_2S donor NaHS. These agents have been previously criticized as tools for H_2S evaluation[39,40]. The ideal H_2S-donors for therapeutic purposes should generate H_2S with slow releasing rates such as H_2S-releasing organic polysulphide or H_2S releasing hybrid NSAIDs[40]. In our work the increase in total sulfide level still falls within the physiologic levels of H_2S in rat blood which ranges from 10-100 μM[41]. Accordingly we expect that like the other H_2S releasing hybrid compounds, ATB-346 is also slowly releasing H_2S. This assumption may be emphasized by reports on the biphasic effect of H_2S on inflammatory signaling in lipopolysaccharide (LPS)-treated murine macrophages, where low concentrations of H_2S inhibited LPS induced synthesis of PGE2, NO, IL-1β, IL-6 and NF-κB activity, while higher concentrations of NaSH promoted the synthesis of pro-inflammatory mediators[42]. In addition, a comparative study showing differences between fast and slow-release H_2S donors showed an increase in inflammatory markers with high concentrations of NaHS compared to inhibitory effects of GYY4137, a slow H_2S donor[43]. Thus, the rate and concentration of exogenously donated H_2S should be appreciated while analyzing and interpreting the results of the different studies.

Conclusions

This work provides preclinical evidence on the superior anti-inflammatory and anti-nociceptive effect of H_2S releasing naproxen relative to

naproxen in inflammatory arthritis. Apart from reduction of joint pain, swelling and inflammation, an important finding is the ability of ATB-346 to preserve cartilage chondrocyte and matrix homeostasis. The study also showed that H_2S release within physiologic ranges may be the key of promoting such effects. Thus, H_2S releasing hybrid molecules could represent an attractive therapeutic strategy to reduce the severity and progression of acute and chronic inflammatory joint diseases. Interestingly, ATB-346 nearly abolished TNF-α release and regulated the important cellular transcriptional machinery NF-κB, and so, it may have more therapeutic implications beyond its anti-inflammatory activity. This paves the way for further anti-inflammatory drug development, adding more therapeutic options in the clinical practice.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Fujiwara N, Kobayashi K. Macrophages in inflammation. Curr Drug Targets Inflamm Allergy 2005; 4: 281-286.
2) Whiteman M, Winyard PG. Hydrogen sulfide and inflammation: the good, the bad, the ugly and the promising. Expert Rev Clin Pharmacol 2011; 4: 13-32.
3) Penido C, Conte FP, Chagas MS, Rodrigues CA, Pereira JF, Henriques MG. Antiinflammatory effects of natural tetranortriterpenoids isolated from Carapa guianensis Aublet on zymosan induced arthritis in mice. Inflamm Res 2006; 55: 457-464.
4) Li L, Hsu A, Moore PK. Actions and interactions of nitric oxide, carbon monoxide and hydrogen sulphide in the cardiovascular system and in inflammation--a tale of three gases. Pharmacol Ther 2009; 123: 386-400.
5) Sidhapuriwala JN, Ng SW, Bhatia M. Effects of hydrogen sulfide on inflammation in caerulein-induced acute pancreatitis. J Inflamm (Lond) 2009; 6: 35.
6) Eng A, Ramasam T, Bhatia M. Hydrogen sulfide. Pancreapedia (DOI: 10.3998/panc.2011.18]
7) Zhang J, Sio SW, Moochhala S, Bhatia M. Role of hydrogen sulfide in severe burn injury-induced inflammation in mice. Mol Med 2010; 16: 417-424.
8) Wallace JL, Vong L, McKnight W, Dicay M, Martin GR. Endogenous and exogenous hydrogen sulfide promotes resolution of colitis in rats. Gastroenterol 2009; 137: 569-578.
9) Tamizhselvi R, Koh YH, Sun J, Zhang H, Bhatia M. Hydrogen sulfide induces ICAM-1 expression and neutrophil adhesion to caerulein-treated pancreatic acinar cells through NF-kappaB and Src-family kinases pathway. Exp Cell Res 2010; 316: 1625-1636.
10) Zanardo RC, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL. Hydrogen sulfide is an endogenous modulator of leukocyte mediated inflammation. FASEB J 2006; 20: 2118-2120.
11) Li L, Rossoni G, Sparatore A, Lee LC, Del Soldato P, Moore PK. Anti inflammatory and gastrointestinal effects of a novel diclofenac derivative. Free Radic Biol Med 2007; 42: 706-719.
12) Takahashi T, Muneta T, Tsuji K, Sekiya I. BMP-7 inhibits cartilage degeneration through suppression of inflammation in rat zymosan-induced arthritis. Cell Tissue Res 2011; 344: 321-332.
13) Cicala C1, Ianaro A, Fiorucci S, Calignano A, Bucci M, Gerli R, Santucci L, Wallace JL, Cirino G. NO-naproxen modulates inflammation, nociception and downregulates T cell response in rat Freund's adjuvant arthritis. Br J Pharmacol 2000; 130: 1399-1405.
14) Yu YC, Koo ST, Kim CH, Lyu Y, Grady JJ, Chung JM. Two variables that can be used as pain indices in experimental animal models of arthritis. J Neurosci Methods 2002; 115: 107-113.
15) Park HJ, Lee Y, Lee J, Park C, Moon DE. The effects of botulinum toxin A on mechanical and cold allodynia in a rat model of neuropathic pain. Can J Anesth 2006; 53: 470-477.
16) Ekundi-Valentim E, Santos KT, Camargo EA, Denadai-Souza A, Teixeira SA, Zanoni CI, Grant AD, Wallace JL, Muscará MN, Costal SK. Differing effects of exogenous and endogenous hydrogen sulphide in carrageenan-induced knee joint synovitis in the rat. Br J Pharmacol 2010; 159: 1463-1474.
17) McGregor RR, Spagnuolo PJ, Lentenck AL. inhibition of granulocyte adherence by ethanol, prednisone and aspirin, measured with an assay system. N Engl J Med 1974; 26: 642-646.
18) Rodriguez AB, Pariente AJ, Prieto P, Barriga C. Effect of cefmetazole, cefositin and imipenem on polymorphonuclear leukocytes. Gen Pharmacol 1987; 18: 613-615.
19) Chaves HV, Ribeiro Rde A, de Souza AM, Rodrigues e Silva AA, Gomes AS, Vale ML, Bezerra MM, Brito GA. Experimental model of zymosan-induced arthritis in the rat temporomandibular joint: role of nitric oxide and neutrophils. J Biomed Biotechnol 2011; 2011: 707985.
20) Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ, Anuar FB, Whiteman M, Salto-Tellez M, Moore PK. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J 2005; 19: 1196-1198.
21) Stolina M, Schett G, Dwyer D, Vonderfecht S, Middleton S, Duryea D, Pacheco E, Van G, Bolon B, Feige U, Zack D, Kostenuik P. RANKL inhibition by osteoprotegerin prevents bone loss without affecting local or systemic inflammation parameters in two rat arthritis models: comparison with anti-

TNF alpha or anti-IL-1 therapies. Arthritis Res Ther 2009; 11: R187-R202.

22) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.

23) Vancurova I, Miskolci V, Davidson D. NF-kB Activation in Tumor Necrosis Factor a-stimulated neutrophils is mediated by protein kinase C δ. J Biol Chem 2001; 267: 19746-19752.

24) Bers G, Garfin D. Protein and nucleic acid blotting and immunobiochemical detection. BioTechniques 1985; 3: 276-288.

25) Kashfi K, Olson KR. Biology and therapeutic potential of hydrogen sulfide and hydrogen sulfide releasing chimeras. Biochem Pharmacol 2013; 85: 689-703.

26) Fiorucci S1, Antonelli E, Distrutti E, Di Sante M, Morelli A, Cirino G, Wallace JL. Inhibition of hydrogen sulfide generation contributes to the gastric injury caused by anti-inflammatory non steroidal drugs. Gastroenterology 2005: 129: 1210-1224.

27) Maeda Y, Aoki Y, Sekiguchi F, Matsunami M, Takahashi T, Nishikawa H, Kawabata A. Hyperalgesia induced by spinal and peripheral hydrogen sulfide: evidence for involvement of Cav3.2 T-type calcium channels. Pain 2009; 142: 127-132.

28) Andruski B, McCafferty DM, Ignacy T, Millen B, McDougall JJ. Leukocyte trafficking and pain behavioral responses to a hydrogen sulfide donor in acute monoarthritis. Am J Physiol Regul Integr Comp Physiol 2008; 295: R814-20.

29) Roitt I. Introduction to the immune system. In: Roitt I, Brostoff J, (eds) Immunology, 6th ed. St. Louis: Mosby, 2001; pp. 1-26.

30) Neugebauer V, Han JS, Adwanikar H, Fu Y, Ji G. Techniques for assessing knee joint pain in arthritis. Mol Pain 2007; 3: 8.

31) Hauser RA. The acceleration of articular cartilage degeneration in osteoarthritis by nonsteroidal anti-inflammatory drugs. J Prolotherapy 2010; 1: 305-322.

32) Li L, Fox B, Keeble J, Salto-Tellez M, Winyard PG, Wood ME, Moore PK, Whiteman M. The complex effects of the slow releasing hydrogen sulfide donor GYY4137 in a model of acute joint inflammation and in human cartilage cells. J Cell Mol Med 2013; 17: 365-376.

33) Rocha FA, Silva FS Jr, Leite AC, Leite AK, Girão VC, Castro RR, Cunha FQ. Tadalafil analgesia in experimental arthritis involves suppression of intra-articular TNF release. Br J Pharmacol 2011; 164: 828-835.

34) Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. FASEB J 1995; 9: 899-909.

35) Lee HS. Lee CH. Tsai HC. Salter DM. Inhibition of cyclooxygenase 2 expression by diallyl sulfide on joint inflammation induced by urate crystal and IL-1beta. Osteoarthritis Cartilage 2009; 17: 91-99.

36) Stuhlmeier KM, Bröll J, Iliev B. NF-kappaB independent activation of a series of proinflammatory genes by hydrogen sulphide. Exp Biol Med (Maywood) 2009; 234: 1327-1338.

37) Zhang H, Zhi L, Moore PK, Bhatia M. Role of hydrogen sulfide in cecal ligation and puncture-induced sepsis in the mouse. Am J Physiol Lung Cell Mol Physiol 2006; 290: L1193-1201.

38) Zhang H, Zhi L, Moochhala S, Moore PK, Bhatia M. Hydrogen sulfide acts as an inflammatory mediator in cecal ligation and puncture-induced sepsis in mice by upregulating the production of cytokines and chemokines via NF-kappaB. Am J Physiol Lung Cell Mol Physiol 2007; 292: L960-971.

39) Bhatia M. Role of hydrogen sulfide in the pathology of inflammation. Scientifica (Cairo) 2012; 159680.

40) Martelli A, Testai L, Breschi MC, Blandizzi C, Virdis A, Taddei S, Calderone V. Hydrogen sulphide: novel opportunity for drug discovery. Med Res Rev 2012; 32: 1093-1130.

41) Wallace JL. Hydrogen sulfide-releasing anti-inflammatory drugs. Trends Pharmacol Sci 2007; 28: 501-505.

42) Whiteman M, Li L, Rose P, Tan CH, Parkinson DB, Moore PK. The effect of hydrogen sulfide donors on lipopolysaccharide-induced formation of inflammatory mediators in macrophages. Antioxid Redox Signal 2010; 12: 1147-1154.

43) Dufton N, Natividad J, Verdu EF, Wallace JL. Hydrogen sulfide and resolution of acute inflammation: A comparative study utilizing a novel fluorescent probe. Sci Rep 2012; 2: 499.