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Inflammation Research

Antiinflammatory effects of etoricoxib alone and combined with NSAIDs in LPS-induced reactive arthritis

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Abstract. *Objective:* Nonsteroidal anti-inflammatory drugs constitute the primary therapeutic approach in reactive arthritis. Here, we compared etoricoxib, a specific COX-2 inhibitor, with other cyclooxygenase inhibitors on articular incapacitation, edema, leukocyte migration, and gastric damage, in a model of LPS-induced reactive arthritis in rats.
Methods: E. coli Lipopolysaccharide was injected into a carrageenan-primed knee-joint of rats. The effects of etoricoxib, piroxicam, indomethacin, as well the combination of etoricoxib either with piroxicam or indomethacin, were evaluated on articular incapacitation and edema. Afterwards, the synovial leukocyte content and the stomach bleeding points were counted.
Results: Etoricoxib, piroxicam, and indomethacin dose-dependently inhibited incapacitation and edema. However, only etoricoxib inhibited both mononuclear and polymorphonuclear leukocyte migration. Piroxicam inhibited only mononuclear migration, while indomethacin even increased polymorphonuclear content in inflamed synovia. Associating etoricoxib with either subeffective doses of piroxicam or indomethacin did not enhance the hyponociceptive or the antiedematogenic effect, but prevented the anti-leukocyte migration effect and increased gastric damage.
Conclusion: The present results suggest that the selective COX-2 inhibitor etoricoxib could be a better option than non-selective COX inhibitors, since it presented a potent inhibitory effect on the clinical signals and also a potent inhibition on cell migration.

Key words: Etoricoxib – Piroxicam – Indomethacin – LPS-induced reactive arthritis – Leukocyte migration – Gastric damage

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Introduction

NSAIDs (non steroidal anti-inflammatory drugs) are the most commonly used medication for the therapy of pain induced by inflammatory and degenerative joint diseases including reactive arthritis [1–4]. Their primary mechanism of action is considered to be the blockade of the two isoforms of cyclooxygenases, i.e. COX-1 and COX-2, thus reducing prostaglandin synthesis. Actually, the therapeutic effects of NSAIDs are attributed to their ability to inhibit COX-2 isoform that is induced in inflamed tissues, whereas some of their most frequent adverse effects are related to the inhibition of the COX-1 isoform that generally plays a homeostatic role. One of the most frequent adverse effect induced by NSAIDs is damage to the gastrointestinal tract. In contrast, the newer selective COX-2 inhibitors (coxibs) are safer in patients with an increased risk for adverse gastrointestinal effects [5–6]. This heterogeneous class of drugs, however, can act by additional mechanisms not always related to COX inhibition, e.g. change the leukocyte activity and kinetics, that may contribute to the final analgesic and antiinflammatory effects, but can also aggravate the adverse effects or even the underlying pathology [7–8]. In spite of the fact that leukocyte migration constitutes a critical event for the development of reactive arthritis [9–14], COX inhibitors are not chosen or discarded for their ability to modify cell migration. Therefore, these drugs must continuously be reassessed by different approaches to properly select the compounds that are most appropriate for each inflammatory condition. In this study, we compared the effect of etoricoxib, a specific COX-2 inhibitor, to that of non-selective cyclooxygenase inhibitors on three different inflammatory parameters: articular incapacitation, edema, and leukocyte migration in the model of LPS-induced reactive arthritis in rats.

Material and Methods

Drugs and Reagents

Carrageenan multiple type kappa/lambda was from BDH Chemicals Ltd. (UK), Escherichia coli lipopolysaccharide serotype 055:B5 (LPS) from DIFCO (USA), etoricoxib - MW 358.84 and indomethacin - MW 357.79 from Merk, Sharp and Dohme Ltd. (Brazil) and piroxicam - MW 331.35 from Galena Química Farmacêutica Ltd. (Brazil).

Animals

Experiments were performed on male Wistar rats (250–300 g), which were housed in temperature-controlled rooms (21 ± 2 ºC) under a 12/12 h light/dark cycle with free access to water and food until the experimental sessions. All the experiments were conducted according to the ethical guidelines of the International Association for the Study of Pain [15] and the experimental procedures were previously approved by local committee for the ethical use of animals (CEUA – UFSC).

Monoarthritis model

This procedure was reported in the present form elsewhere [16]. In order to induce inflammatory incapacitation, 20 µl of a boiled carrageenan solution (15 mg/ml), suspended in physiological saline (0.9 %), was injected into the right knee-joints of naïve rats (300 µg/knee). A second challenge, with LPS in saline 0.9 %, was performed in the same joint (30 ng/knee; 50 µl) 3 days after the previous carrageenan sensitizing stimulus. Without the carrageenan priming, this amount of LPS does not induce incapacitation or edema. Intraarticular injections were quickly made through a 26-gauge needle. The animals were gently handled with the aid of a soft rubber cone, and kept calm in a supine position without sedation. The injection site was previously shaved and treated with a 10 % povidone-iodine (10) solution.

Algesimetric test

For the rat knee-joint incapacitation test [17], the rats are placed on a revolving cylinder (30 cm diameter; 3 r.p.m.) for 1-min period and a computer-assisted device measures the total time that the right hind paw was not in contact with the cylinder surface – the paw elevation time. Before LPS stimulus, the animals display a paw elevation time of approximately 10 to 15 s. LPS injection in carrageenan-primed knee-joints increases this value only in the affected limb. The paw elevation time was evaluated immediately before (time 0) and hourly after LPS injection during 6 hours.

Edema measurement

In order to quantify the inflammatory edema induced by LPS, the rats were restrained as for the intraarticular injection procedure. A micrometer was used to measure the articular diameter of the inflamed knee-joint through the medio-lateral axis. The articular diameter was taken just after each paw elevation time evaluation. The data are presented as the difference between the articular diameter values taken hourly after LPS injection and values taken just before LPS injection.

Synovial leukocyte count

Six hours after the LPS injection the rats were euthanized, and the knee-joint cavities exposed. Five microliters of the synovial fluid was collected for a smear slide preparation. After, the synovial cavity was washed out with 100 µl of a saline 0.9 %: EDTA 5 % solution. This lavage was diluted in Turk's solution (1:20) for 5 minutes and a sample of this lavage was pipetted and placed in a Neubauer counting chamber for total leukocyte count (TC; cells/mm3). The May-Grünwald-Giemsa stained smear was used for mononuclear (MON) and polymorphonuclear (PMN) leukocytes percent count by visual identification under 100x optical magnification.

Gastric damage evaluation

The stomachs were removed and opened along the greater curvature. They were rinsed with saline solution 0.9 % and macroscopically examined and scored for point-like bleeding erosion. Data were presented as the number of bleeding points per stomach.

Experimental procedures

Etoricoxib (oral), piroxicam (oral), indomethacin (i.p.), as well as the combination of etoricoxib either with piroxicam or indomethacin were applied as pretreatment 1 hour before LPS stimulus. The animals were submitted to a 12-h fasting period before drug pretreatments. Etoricoxib or piroxican were diluted in distilled water and indomethacin in a 1.29 % sodium bicarbonate solution (pH = 7.8). Control groups received distilled water (0.1 ml/kg, oral) or sodium bicarbonate solution (0.1 ml/kg, i.p.) wherever needed. Paw elevation time and articular diameter evaluation were made just before and hourly after LPS injection, through 6 hours. Just after the in vivo data recording, the rats were euthanized by cervical dislocation under chloral hydrate (15 %) deep anaesthesia for synovial leukocyte count and gastric damage evaluation.

Statistical analysis

All the statistical analyses were carried out using GraphPad Prism 3.0® software. Multiple comparisons were performed using one-way ANOVA for repeated measures followed by Tukey's Multiple Comparison Test. The minimum significance level was $P<0.05$. Data were presented as mean ± s.e. of mean. Experimental groups comprised six animals.

Results

Inflammation-induced paw elevation time (PET) and articular diameter increase (AD) were dose-dependently inhibited by etoricoxib 5 (14) and 10 (28) mg(µmol)/kg (PET and AD: $p<0.001$; Fig. 1A, B), piroxicam 2.5 (7.5) and 5 (15) mg(µmol)/kg (PET2.5: $p<0.001$ and AD2.5: $p<0.01$; PET5 and AD5: $p<0.001$; Fig. 2A, B), indomethacin 2.5 (7.1) mg(µmol)/kg (PET and AD: $p<0.001$; Fig. 3A, B). The inhibitory effect was observed at the first hour after LPS-injection, and lasted by the six hours of observation. On a molar basis, indomethacin produced a more potent inhibitory effect than the other drugs, which was 4-fold greater than that of etoricoxib and 2-fold than that of piroxicam. However, indomethacin was less toxic than piroxicam, producing 2 bleeding points in 3 stomachs, in contrast to piroxicam which produced 10 bleeding points in 3 stomachs. Piroxicam was proportionally less antiedematogenic than the other drugs, although producing a similar hyponociceptive effect at the higher dose. When the combined treatments were used, etoricoxib (5 mg/kg)/piroxicam (0.5 mg/kg) did not produce hyponociception and antiedematogenic effect higher than etoricoxib alone, although etoricoxib / indomethacin (0.5 mg/kg) combined treatment produced an antiedematogenic effect higher than etoricoxib alone. No sign of gastric damage was found in

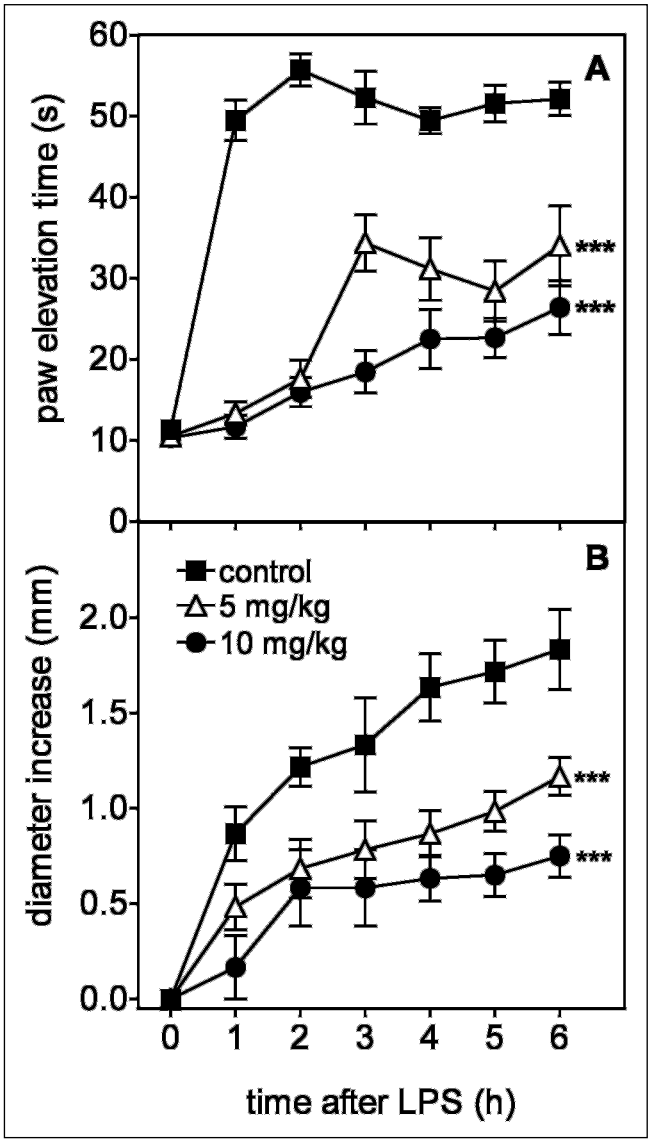

Fig. 1. Inhibitory effect of etoricoxib on incapacitation (A) and articular diameter (B) measured hourly through 6 h. Etoricoxib (oral) was administered 1 hour before LPS stimuli (30 ng; 50 µl; i.art.). Control groups received only distilled water (1 ml/kg; oral). Data are shown as mean ± s.e. of mean (n = 6). *** indicate statistical difference from the control group with $p < 0.001$ (One way ANOVA for repeated measures followed by Tukey's Multiple Comparison Test).

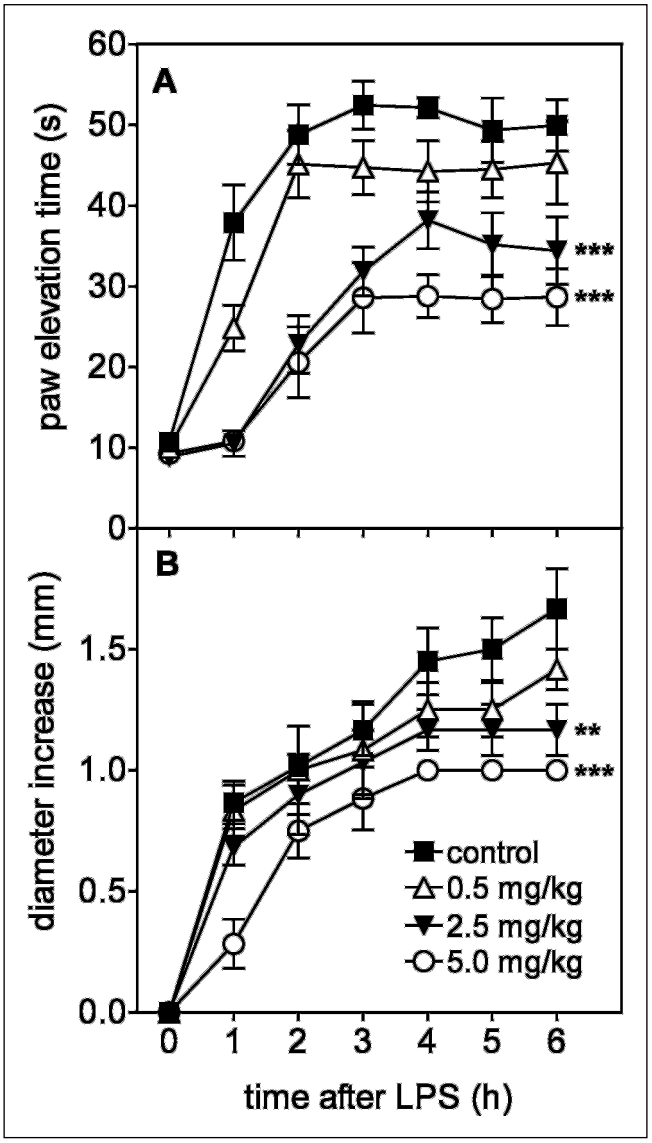

Fig. 2. Inhibitory effect of piroxicam on incapacitation (A) and articular diameter (B) measured hourly through 6 h. Piroxicam (oral) was administered 1 hour before LPS stimuli (30 ng; 50 µl; i.art.). Control groups received only distilled water (1 ml/kg; oral). Data are shown as mean ± s.e. of mean (n = 6). ** and *** indicate statistical difference from the control group with $p < 0.01$ and 0.001, respectively (One way ANOVA for repeated measures followed by Tukey's Multiple Comparison Test).

the rats treated with the higher dose of etoricoxib, but the combined treatment of the coxib (5 mg/kg) with a sub-therapeutic dose of indomethacin (0.5 mg/kg) produced more gastric damage than the NSAID given alone (29 bleeding points in 6 stomachs). Etoricoxib associated with piroxicam (0.5 mg/kg) did not apparently enhance gastric damage (14 bleeding points in 6 stomachs), when compared to a high dose of piroxicam alone.

Table 1 shows the ability of these drugs in modifying synovial leukocyte content. Etoricoxib 5 and 10 mg/kg inhibited total leukocyte (TC) content by -29.8 ± 9.6 and -52.1 ± 6.9 % ($p < 0.05$), respectively. The inhibition on total leukocyte content produced by the lower dose did not reach statistical significance, but it reduced significantly the proportion of mononuclear (MON) cells (-42.5 ± 6.1 %, $p < 0.05$) when compared to control group. The higher etoricoxib dose significantly inhibited both leukocyte types (MON: -49.3 ± 11.4 %, $p < 0.01$; PMN: -43.5 ± 13.2 %, $p < 0.05$). Piroxicam (5 mg/kg) inhibited TC (-46.6 ± 8.5 %, $p < 0.05$), particularly due to an inhibition on MON migration (-47.1 ± 8.7 %, $p < 0.05$). In contrast, indomethacin (0.25 and 2.5 mg/kg) produced a significant increase of PMN migration (0.25 mg/kg: 324.0 ± 60.1 %, $p < 0.05$ and 2.5 mg/kg: 390.4 ± 95.5 %, $p < 0.01$) without increas-

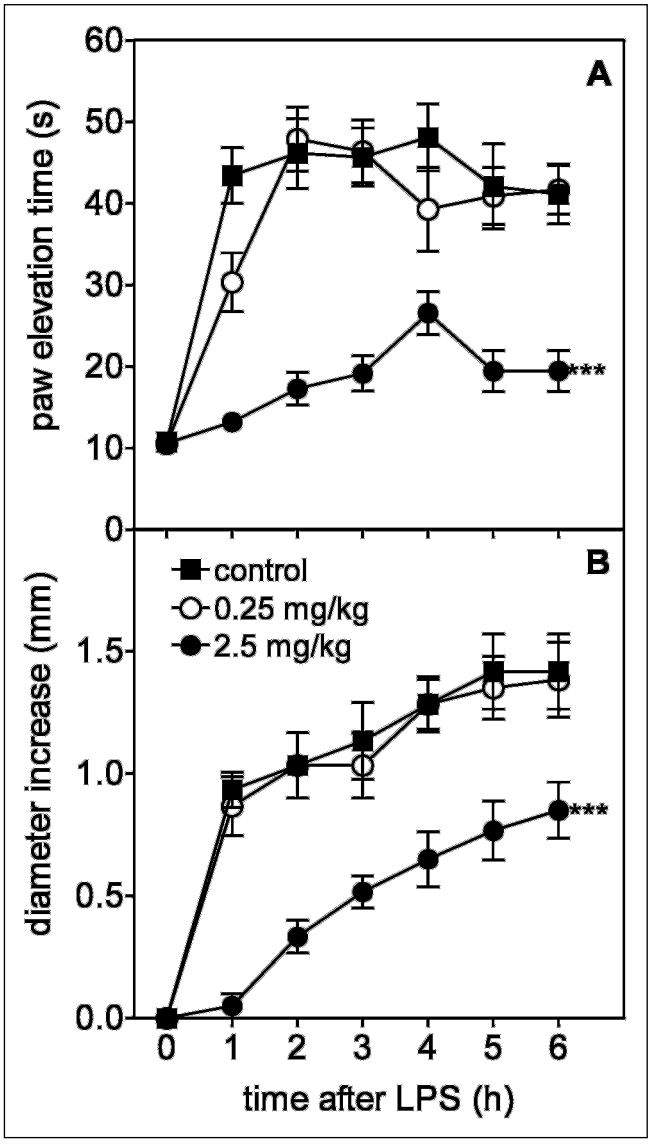

Fig. 3. Inhibitory effect of indomethacin on incapacitation (A) and articular diameter (B) measured hourly through 6 h. Indomethacin (i.p.) was administered 1 hour before LPS stimuli (30 ng; 50 µl; i.art.). Control groups received only bicarbonate solution 1.29 % (1 ml/kg; i.p.). Data are shown as mean ± s.e. of mean (n = 6). *** indicate statistical difference from the control group with $p < 0.001$ (One way ANOVA for repeated measures followed by Tukey's Multiple Comparison Test).

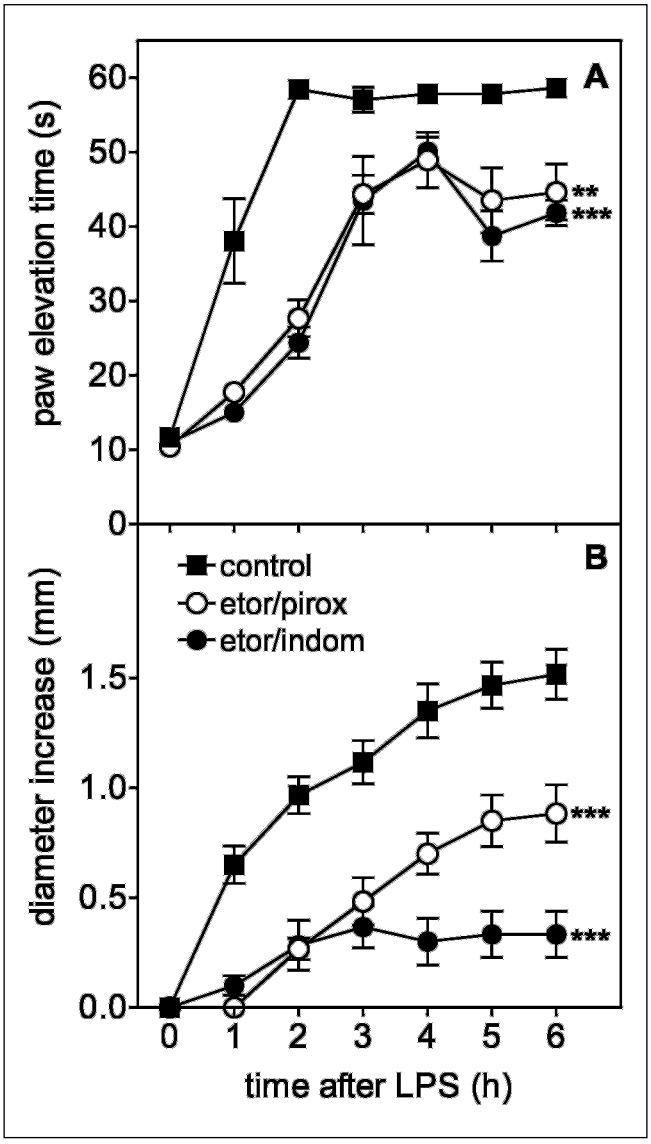

Fig. 4. Inhibitory effect of the association of etoricoxib either with piroxicam or indomethacin on incapacitation (A) and articular diameter (B) measured hourly through 6 h. Etoricoxib (5 mg/kg; oral), piroxicam (0.5 mg/kg; oral) and indomethacin (0.5 mg/kg; i.p.) were administered 1 hour before LPS stimuli (30 ng; 50 µl; i.art.). Control groups received only distilled water (1 ml/kg; oral). Data are shown as mean ± s.e. of mean (n = 6). ** and *** indicate statistical difference from the control group with $p < 0.01$ and 0.001, respectively (One way ANOVA for repeated measures followed by Tukey's Multiple Comparison Test).

ing TC (0.25 mg/kg: -17.6 ± 7.0 % and 2.5 mg/kg: -5.9 ± 12.9 %), since mononuclear cells decreased proportionally (0.25 mg/kg: -45.8 ± 6.1 % and 2.5 mg/kg: -38.6 ± 8.1, $p < 0.01$). These effects on cell migration were observed with the lower dose, which did not affect incapacitation and edema, and were not significantly different from that observed with the higher dose. On the other hand, piroxicam effectively produced hyponociception and edema inhibition at a dose below that produced leukocyte inhibition. Finally, the sub-therapeutic doses of indomethacin and piroxicam prevented the leukocyte inhibition produced by etoricoxib (5 mg/kg).

Discussion

In the present study, etoricoxib, indomethacin and piroxicam were evaluated in a model of LPS-induced reactive arthritis [16]. This model was developed based on the clinical condition known as reactive arthritis (ReA) that may occurs after an extra-articular primary infection with a Gram-negative bacteria. In significant cases the respective lipopolysaccharide endotoxin (LPS), but never the viable pathogen, is found in the synovial fluid of the patient's affected joints [18, 19]. LPS seems to be essential for arthritis induction and maintainance of the inflammatory host response in the joints of

Table 1. Leukocyte count from synovial fluid (MON, mononuclear; PMN, polymorphonuclear; TC, total cells). Etor, etoricoxib; pirox, piroxicam; indo, indomethacin. The doses (in parentheses) are in mg/kg. Data are presented as the mean percent difference from the control group.

Treatment	MON (%)	PMN (%)	TC (%)	TC (cell/mm^3)
control				5791 ± 959
etor (5)	–42.5 ± 6.1 (*)	–34.2 ± 19.1	–29.8 ± 9.6	4066 ± 558
etor (10)	–49.3 ± 11.4 (**)	–43.5 ± 13.2 (*)	–52.1 ± 6.9 (*)	2775 ± 401 (*)
control				3541 ± 451
pirox (0.5)	1.3 ± 10.4	–43.7 ± 12.1	–3.1 ± 9.2	3383 ± 323
pirox (2.5)	–30.7 ± 6.1	–29.7 ± 31.2	–13.2 ± 14.3	2458 ± 255
pirox (5)	–47.1 ± 8.7 (*)	–12.1 ± 23.2	–46.6 ± 8.5 (*)	1891 ± 302 (*)
control				4216 ± 298
indo (0.25)	–45.8 ± 6.1 (**)	324.0 ± 60.1 (*)	–17.6 ± 7.0	3475 ± 295
indo (2.5)	–38.6 ± 8.1 (**)	390.4 ± 95.5 (**)	–5.9 ± 12.9	3967 ± 548
control				7033 ± 423
etor (5) / pirox (0.5)	18.4 ± 6.2	8.2 ± 7.6	13.3 ± 4.9	7966 ± 348
etor (5) / indo (0.5)	10.3 ± 19.2	–27.8 ± 9.0	–10.4 ± 8.1	6300 ± 567

*: $p<0.05$
**: $p<0.01$

susceptible patients with reactive arthritis [20, 21]. Although ReA has one well-known genetic marker (HLA-B27) highly correlated to a significative number of cases [1], an articular trauma story may also be an important factor that predisposes people to this condition [23, 24]. Carrageenan knee-joint priming attempts to mimic this previous joint trauma in a controlled way, which predispose the animals to the long-lasting LPS response observed here. Therefore, this model may be a useful tool to identify some important mechanisms underlying the ongoing disease [16], supplying important clues for the pharmacological treatment of reactive arthritis.

All the NSAIDs studied presented similar hyponociceptive and antiedematogenic effects, inferred from their capacity to inhibit the inflammation-induced incapacitation and periarticular diameter increase. These effects are likely to reflect the blockade of prostaglandin production. Etoricoxib is considered a highly selective COX-2 inhibitor in several assays [25], while indomethacin and piroxicam are non-selective, although preferential COX-1 inhibitors [25–28]. Thus, these data suggest that etoricoxib could be as effective as non-selective NSAIDs for the control of the clinical signs in potentially similar situations, such as reactive arthritis.

However, in spite of the similar symptomatic relief, these drugs showed differential ability to modify leukocyte migration implying that in long-course therapies some NSAIDs could worsen to the underlying inflammatory condition. This is probably the case with indomethacin, which increased migration of PMN leukocytes in the present study. We observed no significant change in total leukocyte migration, but a proportional increase in PMN counts with a reduction in monocyte percentage suggesting the potential of indomethacin to change the leukocyte profile in synovial fluid without modifying the number of migrated cells in this model. A previous study showed that indomethacin causes an increase in PMN adhesion to endothelial cells, associated with the upregulation of CD11b/CD18 (MAC-1) molecules [29]. In addition, PMN exposure to indomethacin promoted myeloperoxidase release and superoxide generation, important events for tissue damage of arthritic joints [30, 31]. Indeed, cartilage damage has been observed after indomethacin treatment and was also attributed to an increase in leukocyte migration into inflammatory exudates [32, 33] or to the inflamed synovial lining [34]. In addition, indomethacin can reduce the normal articular perfusion and blood supply, which may also contribute to cartilage damage [35, 36]. Our data, however, conflicts with reports that indomethacin inhibits, or even has no effect at all on, pleural leukocyte infiltration [37–42]. This differential ability of indomethacin to modify cell migration depending on the inflammatory model was also seen with other NSAIDs. For example, piroxicam was inhibitory to total cell migration in the present model and this effect was consistent with previous findings in carrageenan-induced rat pleurisy [43] or paw inflammation [44], while it contradicts results found in LPS-induced rat peritonitis [42].

Etoricoxib-produced hyponociception and antiedematogenic effects were accompanied by the inhibition of synovial leukocyte migration. The specific inhibitory action of etoricoxib on mononuclear cell migration may also be of interest, since T-lymphocyte synovial infiltration is thought to be pivotally involved in ReA pathogenesis [9–14]. Furthermore, the inhibitory effect on total cell migration also explains why systemic therapy with etoricoxib retards alveolar bone loss [45], reinforcing the idea of additional benefits of etoricoxib. The mechanism underlying the effect on cell migration is not understood, and COX-independent mechanisms as NF-kappaB and CREB transcription factors inhibition might contribute to the reduction of several inflammatory mediators involved in cell migration [8]. However, two other potent coxibs, celecoxib and rofecoxib, have also been found to interfere negatively with leukocyte mobility [33, 42, 46, 47] indicating that COX-2 inhibition might also be the mechanism of etoricoxib for this effect. The same explanation may also be true for piroxicam effect, since only the higher dose inhibited synovial leukocyte count, which is consistent with its minor selectivity to COX-2 isoform than the coxibs. On the other hand, indomethacin-produced in-

crease in leukocyte count was at a dose lower than needed to produce hyponociception and antiedematogenic effects, which may implicate COX-1 inhibition.

Several studies highlight the favorable cost/benefit ratio for the use of selective COX-2 inhibitors, instead of traditional NSAIDs, in patients with high risk of gastrointestinal complications. However, the cost/benefit ratio is clearly unfavorable when the risk of a cardiovascular event in such patients is high. Based on the idea of the altered homeostatic balance between endothelial (COX-2) PGI2/ platelet (COX-1) TXA2 production, the cardio-protective effect of low dose aspirin by reducing platelet TXA2 production, could also reduce the coxib risk of cardiovascular events. Unfortunately, the combined treatment with coxibs and low dose aspirin greatly enhances the occurrence of upper gastrointestinal bleeding, in a way not explained by simple additive inhibition on COX-1 enzyme [48, 49]. Whereas the coxib inhibition of lipoxin synthesis due to the blockade of the acetylated-COX-2 may be a cause for the enhanced gastric damage in subjects undergoing a combined treatment with aspirin and coxib [48], our present findings with the co-treatments used here bring us to suppose that a more general mechanism is needed to explain the toxicity of the association NSAID/coxib. This mechanism may be the coxib blockade of prostacyclin produced by COX-2. Prostacyclin exerts vasodilation and antiagregant effects in stomach mucosa, which is thought to protect from the damage induced by COX-1 blockade [50]. As an additional drawback, these associations also prevented the inhibitory effect of the coxib on leukocyte migration to the synovia. Such sub-therapeutic doses of indomethacin and piroxicam are thought to be acting mainly on COX-1 enzymes, since these drugs have lower IC50 values for COX-1 than COX-2. Therefore, the inhibitory effect of etoricoxib on leukocyte migration may be dependent on the inhibition of COX-2, but also on the fully functional COX-1 isoform.

NSAIDs constitute the primary therapeutic approach in ReA. Indeed, non-selective COX inhibitors should be used early, and regularly over several weeks or months to allow the patient to become more active and functional [1]. For this reason, the use of gastric-sparing COX-2 inhibitors could be of interest. Our present results suggest that the selective COX-2 inhibitor etoricoxib could be a better option than non-selective COX inhibitors, since it presented a potent inhibitory effect on the clinical signals and also a potent inhibition on cell migration. However, special attention must be paid to patients receiving concomitant cardioprotective doses of NSAIDs, since this association may prevent the anti-leukocyte effect of etoricoxib.

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