

Effects of nonselective and selective cyclooxygenase inhibitors on the contractions of isolated bronchial smooth muscle in the horse

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Abstract

We evaluated the effects of nonselective cyclooxygenase (COX)-1/COX-2 inhibitors (acetylsalicylic acid, indomethacin, ibuprofen, flunixin meglumine, phenylbutazone), preferential COX-2 inhibitors (diclofenac, meloxicam, carprofen), selective COX-1 inhibitor (SC-560), and selective COX-2 inhibitors (celecoxib, firocoxib, parecoxib) on the contractions of isolated bronchi induced by electrical field stimulation (EFS). Bronchial rings, obtained from lungs of slaughtered horses, were put in isolated organ baths, and the mechanical activity was measured by means of isotonic transducers. Electrical Field Stimulation was applied to the preparations, and the effects of drugs on the amplitude of evoked contractions were measured. Nonselective COX inhibitors did not modify EFS-induced contractions to a relevant degree, except indomethacin which caused a concentration-dependent decrease of the contraction amplitude. Conversely, preferential COX-2 inhibitors enhanced the contractions in a concentration-related fashion, whilst the selective COX-1 inhibitor reduced them. Among selective COX-2 inhibitors, parecoxib increased EFS-evoked contractions whereas celecoxib and firocoxib were ineffective. These results suggest that the inhibition of prostanoid synthesis does not modify the electrical field-stimulated contractions of isolated horse bronchi. Since EFS-induced contractions of horse bronchi were previously shown to be of full cholinergic nature, the increase caused by diclofenac, meloxicam, carprofen, and parecoxib could be due to an inhibition of acetylcholinesterase; in accordance, these drugs potentiated exogenous acetylcholine-induced but not carbachol-induced bronchial contraction. Indomethacin and SC-560 might instead decrease bronchial contractions by inhibiting calcium currents. Clinical use of meloxicam and carprofen in horses with bronchial hyper-responsiveness requires caution for a potential risk of causing adverse effects due to bronchoconstriction.

COX-1, COX-2, NSAIDs, equine, bronchi

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in clinical practice for their anti-inflammatory, analgesic and antipyretic properties both in humans and animals. Nonsteroidal anti-inflammatory drugs are enzymatic inhibitors which target cyclooxygenase (COX), and thus prevent the synthesis of prostaglandins and thromboxanes. However, the depletion of prostanoids reduces not only their pro-inflammatory effects but also their protective ones, and this is recognised as the main cause of the well-known adverse effects of these drugs.

The breakthrough discovery of two distinct COX isoforms (COX-1 and COX-2) (Xie et al. 1991) propelled the development of novel drugs which, by selectively binding to the inducible enzyme, COX-2, would have been able to block the noxious effects of prostaglandins, while sparing the beneficial ones, unlike traditional NSAIDs, which inhibit both COX isoforms. Much of the initial enthusiasm for these “safer” NSAIDs has been extinguished by further studies, though, which revealed that, like COX-1, COX-2 is also important for the maintenance of homeostasis in several tissues (Kargman et al. 1996;

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Zimmermann et al. 1998). As a matter of fact, only very few COX-2 selective NSAIDs are presently approved for use in humans, and two of them, rofecoxib and valdecoxib, were quickly withdrawn from the market due to the concern for an increased risk of causing serious cardiovascular adverse effects (Bombardier et al. 2000; Atukorala et al. 2013).

Nonselective COX inhibitors such as phenylbutazone, flunixin meglumine, ketoprofen, or acetylsalicylic acid (ASA) are administered to horses for the treatment of musculoskeletal lesions, osteoarthritis or after surgery, and are known to cause adverse effects which include gastrointestinal ulcers, colitis, and bowel motility disorders (MacKay et al. 1983; MacAllister et al. 1993; Dabareiner et al. 1995). Preferential COX-2 inhibitors meloxicam and carprofen, and selective COX-2 inhibitor firocoxib, are also registered for horses as analgesic and anti-inflammatory agents. Although it is known that prostanoids modify the bronchial tone in several species (Horton et al. 1965; Gardiner 1975; Hamberg et al. 1975), COX inhibitors seem mostly devoid of major effects on the contraction of bronchial muscle *in vitro* (Douglas et al. 1987). However, NSAIDs were found to potentiate the contraction of isolated trachea induced by agonists in the guinea pig and dog (Orehek et al. 1973; McGrogan et al. 1996); and there is abundant evidence (Steinhoff et al. 2014) that ASA and other NSAIDs may be responsible for bronchospasm in human patients, or for exacerbating the clinical signs of asthma (Bianco et al. 1985; Looney et al. 2005; Parker et al. 2016). It has been suggested that NSAIDs could increase bronchial contractions by either reducing the production of myorelaxant prostaglandin E₂ (PGE₂), which is synthesized in response to bronchoconstriction caused by disease or drugs (Smith et al. 1976), or by shifting the conversion of arachidonic acid towards the 5-lipoxygenase-dependent production of leukotrienes, which are potent smooth muscle spasmogenic agents (Christie et al. 1991). Since bronchial PGE₂ seems to be mainly produced by COX-1 (Harrington et al. 2008), COX-2 selective inhibitors are indeed thought to be safer than traditional NSAIDs in asthmatic patients. It is possible that PGE₂ could play a protective role also in horse bronchi, since for instance a decrease in the synthesis of this prostanoid in horses with recurrent airway obstruction (RAO) was detected (Gray et al. 1992).

The aim of the present study was to assess the effects of nonselective COX-1/COX2 inhibitors (ASA, indomethacin, ibuprofen, flunixin meglumine, phenylbutazone), preferential COX-2 inhibitors (diclofenac, meloxicam, carprofen) and selective COX-1 (SC-560) or COX-2 (celecoxib, firocoxib, parecoxib) inhibitors (Futaki et al. 1994; Brideau et al. 2001), on the contraction of isolated bronchial smooth muscle of the horse, as so far there has been a lack of knowledge regarding the ability of NSAIDs to modify the motility of equine respiratory tract.

Materials and Methods

Samples of lung were collected from 46 male horses (2–8 years of age), slaughtered at a private abattoir; the experiments were performed between September 2016 and August 2017. The samples were excised from the apical lobes of lungs and were stored in ice-cooled modified Krebs-Henseleit solution of the following composition (mM): NaCl 113.0, KCl 4.7, MgSO₄·7H₂O 1.2, CaCl₂·2H₂O 1.8, KH₂PO₄ 1.2, NaHCO₃ 25.0 and dextrose 11.2, for the 10-min transport from the slaughterhouse to the laboratory. Bronchial segments were then isolated from the surrounding parenchyma, and bronchial rings of 0.7–1 cm diameter, without epithelium, (12 from each horse) were obtained to be used in motility experiments. Each ring was put into a 10 ml organ bath at 37 °C, containing the solution above described, continuously bubbled with 95% O₂ and 5% CO₂ (pH 7.4). The bronchial rings were left to stabilize for 60 min and then the mechanical activity was measured by means of an isotonic transducer (Basile, Milan, Italy) connected to the preparation, developing a passive stretch of 1 g throughout the entire experiment. Pilot experiments were performed to establish the optimum load to get the best contractile activity. The viability of bronchial rings was assessed by the ability of acetylcholine (ACh) (10⁻⁷ M) to evoke a contractile response (> 0.1 cm shortening). Electrical Field Stimulation (EFS) was applied by means of two coaxial platinum electrodes positioned 10 mm from the longitudinal axis of the preparation and used to deliver trains of square wave pulses (1 ms duration, 50 V amplitude) every 90 s to the tissue, at the

frequency of 15 Hz. For each experiment, the intensity was adjusted to a level giving 70–80% of the maximum tissue response (usually 250–300 mA), in order to be able to measure either an increase or a decrease of the contraction amplitude. Regular phasic contractions were obtained, and the effects of drugs were measured as the percentage of variation of pre-drug amplitude of contractions, assumed as 100%. In a separate set of experiments, concentration-response curves of ACh or nonselective muscarinic agonist, carbachol, were constructed in absence and after 30 min of incubation with a single aliquot of 10^{-5} M diclofenac, meloxicam, carprofen, or parecoxib. The potency of the agonists was expressed by the concentration giving 50% of maximum effect (EC_{50}) from individual concentration-response curves, and expressed with $-\text{Log } EC_{50}$.

The effects of NSAIDs on the basal tone of horse bronchi was also assessed, by adding the drugs in the organ baths at increasing concentrations, without applying EFS. Moreover, in order to detect a possible relaxant activity by NSAIDs, their effects on the sustained contraction induced by carbachol, a nonselective muscarinic agonist, were studied. For this purpose, a single concentration (10^{-5} M) of carbachol was added to the organ bath solution, and the effect was observed until a constant plateau of contraction was achieved; increasing concentrations of the COX inhibitors were then added into the bath solution, and the effect on the amplitude of contraction plateau was measured. For the generation of all concentration-response curves, drugs were added cumulatively into the bath solution in 1-log unit increments of concentration. Each concentration-response curve was fitted to nonlinear regression with variable slope using a commercial software (GraphPad Prism ver. 6.05, GraphPad Inc., San Diego, CA, USA).

Drugs

Acetylsalicylic acid, indomethacin, ibuprofen, flunixin meglumine, phenylbutazone, diclofenac, meloxicam, carprofen, SC-560, celecoxib, firocoxib, and parecoxib were purchased by Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA). Flunixin meglumine was dissolved and diluted to the final concentration in distilled water; all other drugs were dissolved and diluted to the final concentration in 1% dimethyl sulphoxide (DMSO), and then diluted to the final concentration with distilled water. Such concentration of DMSO was previously tested and did not modify the contractility of preparations or the effect of drugs. All solutions were freshly prepared before each experiment and proper aliquots (10 to 100 μ l) were added to the organ baths to achieve the desired molarity.

Statistical analysis

All data were expressed as means \pm SEM from eight experiments. Differences among data were evaluated by means of unpaired parametric Student's *t*-test. A *P* value < 0.05 was considered significant. All calculations were performed using GraphPad Prism software.

Results

Nonselective COX-1/COX-2 inhibitors, ASA, phenylbutazone, and ibuprofen (10^{-8} – 10^{-4} M), did not modify the amplitude of EFS-evoked contractions, whereas flunixin meglumine induced only a mild increase of the contractions at 10^{-7} M– 10^{-5} M (Fig. 1A). Conversely, indomethacin reduced the amplitude of EFS-induced contractions in a concentration-dependent manner, reaching a maximal effect of $-30.4 \pm 12.2\%$ at 10^{-4} M (Fig. 1A).

Preferential COX-2 inhibitor, diclofenac, enhanced EFS-induced contractions in a concentration-related fashion, with a maximal effect of $+19.8 \pm 3.2\%$ at 10^{-6} M, whereas meloxicam and carprofen increased the amplitude of contractions at the higher concentrations only (Fig. 1B). The maximum effects at the concentration of 10^{-4} M were $+28.9 \pm 8.15\%$ and $+29.2 \pm 9.8\%$ for meloxicam and carprofen, respectively.

Selective COX-1 inhibitor, SC-560, induced a concentration-dependent decrease of the contraction amplitude, reaching $-26.9 \pm 5.6\%$ at 10^{-5} M (Fig. 1C). Selective COX-2 inhibitors celecoxib and firocoxib did not induce relevant modifications of EFS-evoked contractions, whereas parecoxib caused a marked and concentration-dependent increase of the contractions, with a maximal effect of $+69.1 \pm 22.6\%$ at 10^{-4} M (Fig. 1D).

None of the studied NSAIDs, in the range of concentrations 10^{-9} – 10^{-4} M, was able to affect the basal tone of the bronchial preparations (not shown).

Diclofenac, meloxicam, and parecoxib at the concentration of 10^{-5} M potentiated the concentration-response curves of ACh, while carprofen caused a minor leftward shift of the ACh curve (Fig. 2A–D). The pD_2 values for ACh curves were significantly increased after

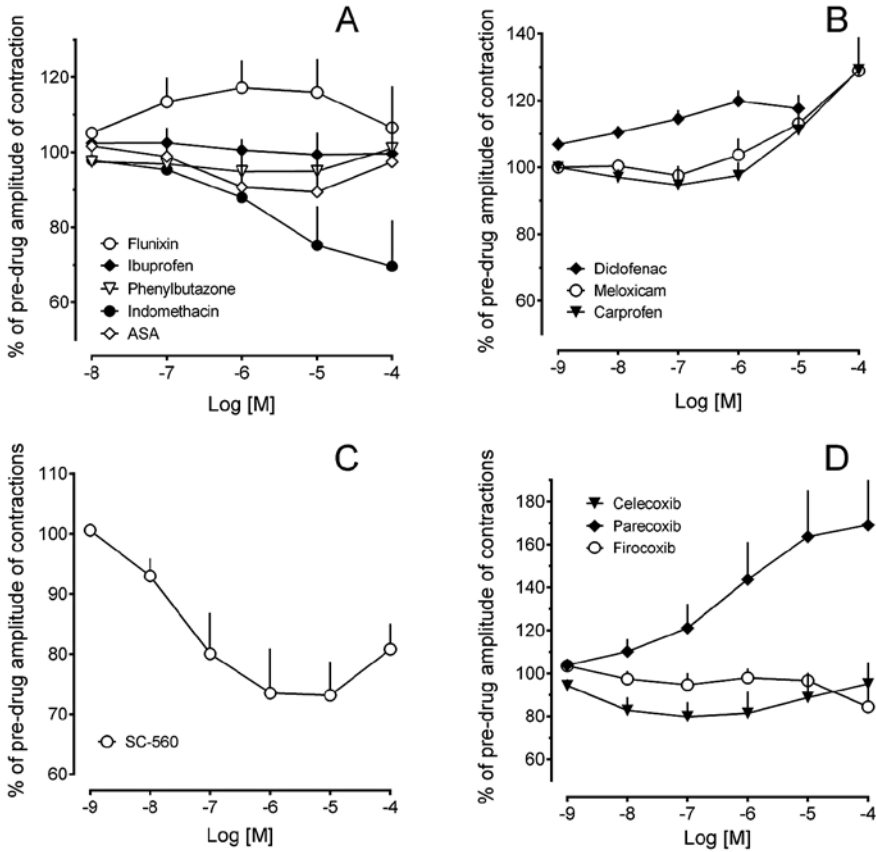


Fig. 1. Effects of nonselective cyclooxygenase (COX)-1/COX-2 inhibitors (A), preferential COX-2 inhibitors (B), selective COX-1 inhibitor (C), and selective COX-2 inhibitors (D), on electrical field-stimulated contractions of the horse's bronchial smooth muscle

incubation with a single 10^{-5} M aliquot of diclofenac, meloxicam, and parecoxib (Table 1). On the contrary, diclofenac, meloxicam, carprofen and parecoxib were unable to potentiate the concentration-response curve of carbachol (not shown).

Both indomethacin and compound SC-560, up to 10^{-5} M, were devoid of measurable effects on the contraction plateau of bronchial smooth muscle induced by 10^{-5} M carbachol (data not shown).

Discussion

At present, available data about the effects of NSAIDs on the motility of the respiratory tract are controversial, and their interpretation may be complex. Prostanoids exert variable effects on airway smooth muscle of different species. Prostaglandin E_2 and prostaglandin I_2 (PGI_2) relax the tracheal muscle of guinea pigs, whereas prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2) and thromboxane A_2 (TXA_2), induce a contraction (Hamberg et al. 1975); human bronchial muscle contracts under the effect of $PGF_{2\alpha}$, and is relaxed by prostaglandin E_1 (PGE_1), whereas PGE_2 induces either contraction or relaxation (Gardiner

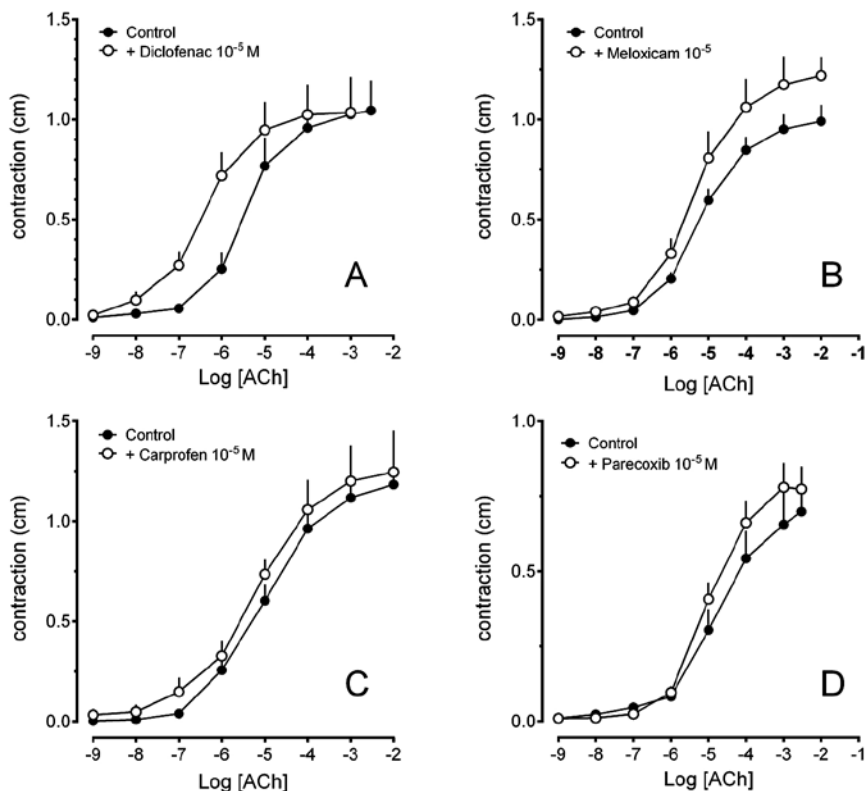


Fig. 2. Acetylcholine-induced contraction of the horse's bronchial smooth muscle in absence (black dots) and after the incubation with 10^{-5} M diclofenac (A), meloxicam (B), carprofen (C), and parecoxib (D)

1975). Furthermore, PGE_1 , PGE_2 and $PGF_{2\alpha}$ relax the smooth muscle of the cat trachea (Horton and Main 1965).

Orehek et al. showed that indomethacin and ASA, two NSAIDs with different chemical structures, reduced the basal tone of trachealis strips and enhanced the contractions caused

Table 1. Potency values ($-\text{Log EC}_{50}$) calculated for concentration-response curves of acetylcholine in absence (Control) and after incubation with 10^{-5} M diclofenac, meloxicam, carprofen, and parecoxib in the horse's bronchial smooth muscle.

	Control	+ NSAID	
Diclofenac	5.46 ± 0.11	$6.37 \pm 0.09^*$	$P = 0.0002$
Meloxicam	5.14 ± 0.06	$5.53 \pm 0.08^*$	$P = 0.021$
Carprofen	5.06 ± 0.12	5.22 ± 0.10	$P = 0.38$
Parecoxib	4.56 ± 0.05	$5.02 \pm 0.08^*$	

* $P < 0.05$ vs control

Data represent means \pm standard error of the mean from eight experiments

by different agonists in guinea pigs (Orehek et al. 1973; Orehek et al. 1975). Similarly, carbachol-induced contraction of the tracheal muscle was increased by indomethacin in the dog (McGrogan and Daniel 1996). On the contrary, despite effectively preventing the prostanoid synthesis in the bronchial muscle of men, NSAIDs did not modify the tone or the agonist-mediated contraction of such smooth muscle (Brink et al. 1980; Hays-Legrand et al. 1986). Previous experiments conducted

in vivo in humans yielded rather conflicting results, as although large doses of NSAIDs did not modify the bronchial tone in most asthmatic patients (Smith 1975; Bianco et al. 1985), in 4% of them a worsening of clinical signs occurred, and in 1% an amelioration was observed (Hanley 1986). The improvement is thought to be due to a depletion of prostanoids causing bronchoconstriction (Smith and Cuthbert 1976; Hanley 1986), whereas the worsening is likely caused by an increased conversion of arachidonic acid to spasmogenic leukotrienes by 5-lipoxygenase pathway (Christie et al. 1991; Parker et al. 2016). Why NSAIDs could induce contrasting effects in patients with the same disease remains to be clarified, even though an individual variability in the synthesis of prostanoids exerting opposite actions on the smooth muscle tone might be suspected. Collectively, previous evidence suggests that although COX enzymes and their products may be involved in the modulation of the airway motility, their role differ considerably based on species, airway portion, and experimental conditions.

The effects induced by NSAIDs observed in the present study suggest that the existence of a basal production of prostanoids modulating EFS-evoked contractions of isolated bronchi in the horse should be excluded. In fact, even though some of the studied NSAIDs caused a modification of the contraction amplitude, such effects do not seem related to the inhibition of prostanoid synthesis, since other drugs with similar COX-1/COX-2 selectivity were ineffective. Moreover, NSAIDs produced in some cases opposite effects on EFS-induced contractions: diclofenac, carprofen, meloxicam, and parecoxib caused an enhancement of the contractions, whereas indomethacin and SC-560 induced their decrease. No correlation between the chemical structure and effect was observed either, given that carprofen and parecoxib caused an increase of the contractions, whereas structurally similar NSAIDs ibuprofen, celecoxib, and firocoxib were ineffective. Indeed, although COX-2 selective inhibitors celecoxib and rofecoxib were proved to be safe in human patients with NSAID-sensitive asthma (Martin-Garcia et al. 2002; Martin-Garcia et al. 2003), two cases of severe bronchospasm following parecoxib administration were reported (Looney et al. 2005), suggesting that such adverse reactions may be caused by peculiar properties of the drug, rather than by the main mechanism of action.

The effects induced by NSAIDs on EFS-evoked contractions in our study are likely due to a modulation of ACh actions on the bronchial muscle of horses, since these contractions have been previously shown to be of fully cholinergic nature (Menozzi et al. 2014), and could involve either the release or the degradation of the neurotransmitter. An interaction by NSAIDs with post-synaptic receptors seems to be unlikely, since none of the studied drugs was able to alter the basal tone of the preparations. Even though it was proposed that prostanoids could affect the motility of the respiratory system by modulating neurotransmitter release (Shore et al. 1985; Zhao et al. 1994), and this might partially explain the difference between data obtained *in vivo* and *in vitro*, the results obtained in the present study suggest that this does not occur in isolated bronchi of horses. In accordance, indomethacin, meclofenamate or PGE₂ were previously found unable to modify the ACh release in equine bronchi (Zhao et al. 1994).

A hypothesis for the increase of the phasic contractions induced by diclofenac, meloxicam, carprofen, and parecoxib could be that these drugs might inhibit acetylcholinesterase, thus enhancing the effects of endogenous ACh released by EFS. The effects of these NSAIDs on exogenous ACh seem to confirm this, since an increase of agonist potency was observed, whereas the concentration-response curves of carbachol, which is resistant to degradation by acetylcholinesterase, were not significantly shifted. Indeed, NSAIDs with different chemical structures were previously shown to inhibit the acetylcholinesterase activity (Goverdhan et al. 2012; Parmar et al. 2017). In those studies, however, celecoxib, ibuprofen, and ASA, which were ineffective in our experiments, also displayed an acetylcholinesterase inhibitor activity. Further studies will be needed to clarify the reason for these discrepancies.

Indomethacin and SC-560 caused a reduction of neurogenic contractions of horse bronchi, and also this effect cannot be attributed to COX inhibition but could possibly involve an interference with ACh release. The lack of effect of both drugs on the basal tone or carbachol-induced contraction suggests that an interaction with post-synaptic receptors located on the smooth muscle is instead unlikely to be involved. Previous studies showed that the indomethacin-induced inhibition of the contraction of the guinea pig ileum induced by ACh or other agonists is unrelated to prostaglandin depletion (Aboulaflia et al. 1976). It could be speculated that indomethacin or SC-560 might inhibit calcium voltage-gated channels. In accordance with this hypothesis, Northover et al. (1971) found that indomethacin reduced the EFS-stimulated contractions of stomach in the guinea pig by decreasing the calcium uptake into muscle cells. This NSAID also seems to inhibit the motility of human uterus by blocking calcium ion currents (Sawdy et al. 1998).

The results obtained in this study may also have some interesting clinical implications. Among the NSAIDs commonly employed in horses, phenylbutazone and firocoxib could be considered safe with regard to the risk of causing adverse reactions due to bronchoconstriction. On the contrary, the use of meloxicam and carprofen might require some caution, especially when treating horses with bronchial hyper-reactivity or obstructive respiratory diseases like RAO, since these NSAIDs tend to enhance cholinergic contractions of isolated bronchi. Moreover, the perioperative use of these NSAIDs could also increase the risk of inducing respiratory side effects, given that horses seem to be particularly susceptible to respiratory stress connected to general anaesthesia (Grosenbaugh et al. 1998).

Indomethacin was shown to inhibit EFS-induced contractions of the horse bronchi, and thus might be potentially useful in different clinical conditions by increasing pulmonary ventilation. However, its safety profile in horses seems to be lower (Roberts 1982) with respect to other NSAIDs, such as phenylbutazone or firocoxib, and thus a future clinical use of this drug in equine practice is rather unlikely.

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