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Endothelin in Bronchial Biopsy Specimens from Horses with Recurrent Airway Obstruction

[1]Laus Fulvio, [1]Paggi Emanuele, [1]Mariotti Francesca,
[1]Cerquetella Matteo, [2]Sgariglia Stefano and [1]Tesei Beniamino
[1]School of Veterinary Medical Sciences, University of Camerino,
Via Circonvallazione 93/95, 62024-Matelica (MC), Italy
[2]Practitioner, Fermo (FM), Italy

Abstract: Recurrent Airway Obstruction (RAO) is a chronic, worldwide recognized diseases of horses. Despite the well known clinical presentation and the ease of diagnosis, the exact etiology and pathogenesis are still unclear which makes it difficult prevention and treatment. Recurrent obstruction of airway is caused by mucus production, bronchospasm and bronchial edema and the most important clinical signs are chronic cough and expiratory effort. The ET-1 is the most potent vasoconstrictor molecule up to now identified, produced by several cells as endothelial cells, smooth muscle cells and epithelial cells of airways. The ET-1 is a potent proinflammatory, secretagogues and bronchoconstrictors mediator. ET-1 can cause bronchial contraction on bronchial rings of horses *in vitro*, especially in horse with respiratory disease.

Key words: Horse, recurrent airway obstruction, endothelin, biopsy, immunohistochemical analysis

INTRODUCTION

Recurrent Airway Obstruction (RAO) is a respiratory diseases of horses characterized by periods of airway obstruction caused by mucus production, bronchospasm, bronchial edema and neutrophil accumulation. The most important clinical signs are chronic cough and expiratory effort. Although, RAO is a well known disease, much debate still occurs concerning its causes and pathogenesis (Reed *et al*., 2004).

Endothelin-1 (ET-1) can be produced by endothelial and smooth muscle cells, epithelial cells of airway, macrophages, fibroblasts, cardiac myocytes, neurons of the brain and pancreatic islets (Kawanabe and Nauli, 2011). ET-1 is the most potent vasoconstrictor molecule up to now identified and its action are regulated by two main receptors: Endothelin receptor A (ET_A) and Endothelin receptor B (ET_B). The ET_A stimulation leads to vasoconstriction while ET_B receptors activation at pulmonary level leads to bronchoconstriction (Fagan *et al*., 2001).

ET-1 is considered to be involved in several human diseases like primary and secondary pulmonary hypertension, systemic hypertension, hearth failure and ovarian cancer and airway diseases (Kawanabe and Nauli, 2011; Fagan *et al*., 2001). Immunostaining for ET-1 in biopsy specimens collected from asthmatics human patients have shown an increase in ET-1 in the bronchial epithelium that correlates with asthma symptoms (Springall *et al*., 1991).

Involvement of ET-1 has been demonstrated in mammary and ovarian tumours of bitch (Borzacchiello *et al*., 2010) other than in cardiac conditions (Prosek *et al*., 2007). Furthermore, ET-1 can be deteted in airway of dogs with idiopathic pulmonary fibrosis but not in healthy dogs (Krafft *et al*., 2011).

The ET-1 seems to be not involved in the pathogenesis of hypoxic pulmonary vasoconstriction in response to acute hypoxia in horse (Benamou *et al*., 2001a, b). Likewise, ET-1 does not seem to play a major role in the pathogenesis of exercise-induce pulmonary haemorrhage (Padilla *et al*., 2006). On the other hands, ET-1 levels in venous blood (but not in arterial blood) and in Bronchoalveolar Lavage Fluid (BALF) of horses affected by RAO have been demonstrated to be higher than those of healthy horses (Benamou *et al*., 1998). Such results were statistically significant in horses during exacerbation but not during remission periods when horses have an intermediate value. Costa *et al*. (2009) found similar results and analogous situation occurs in humans (Aoki *et al*., 1994). In contrast with normal animals, horse with RAO had a negative arterial-venous difference of ET-1 (Costa *et al*., 2009). An increased level of ET-1 in venous blood immediately after exercise on

Corresponding Author: Laus Fulvio, School of Veterinary Medical Sciences, University of Camerino, Via Circonvallazione 93/95, 62024-Matelica (MC), Italy

treadmill can be found in healthy horses (McKeever *et al*., 2002). Nevertheless, there are also evidences that exercise can cause an increase of ET-1 in BALFs of horses affected by Chronic Obstructive Pulmonary Disease (COPD, old name of RAO in horse) but not in those of normal horses (Benamou *et al*., 1999).

In vitro studies, demonstrated that ET-1 cause concentration-dependent bronchial contraction on bronchial rings. This contraction is greater on bronchial rings of SPAOPD (Summer Pasture Associated Obstructive Pulmonary Diseases, a condition similar to RAO) affected horse than on rings of unaffected horses and seems to be due to the activation of pulmonary ET_B receptors (Venugopal *et al*., 2006) although, some researchers ascribe the effect to both receptors (Benamou *et al*., 2003). There are evidence that ET receptors, especially ET_B are overexpressed on post-euthanasia collected samples of peripheral lung of RAO affected horses (Polikepahad *et al*., 2006, 2008).

In the last few years, many studies have focused their attention on molecules involved in the pathogenesis of RAO, in order to understand the exact sequence of events occurring in this disease. In the knowledge of the researchers, the present study is the first study on detection of endothelin in bronchial biopsies of horses with RAO.

MATERIALS AND METHODS

Nine horses have been selected on the basis of clinical signs. Criteria for inclusion in the study were the presence of expiratory effort lasting at least from 2 years, chronic cough, dilated nostrils, wheezing at lung auscultation and absence of fever or other systemic symptoms.

Recurrent Airway Obstruction (RAO) were diagnosed in all horses at the Veterinary Teaching Hospital of Camerino University by bronchoalveolar lavage at least 2 years before the biopsy collection. At the moment of biopsy all horse had clear signs of RAO exacerbation with all above mentioned respiratory symptoms. Owners were asked to give the permission to make bronchial sample collection during the routine 6 months clinical and endoscopical examination of respiratory system before to start any drugs administration.

After the clinical examination which confirmed the presence of the above mentioned clinical signs, horses were sedated (acepromazine, 0.02 mg kg^{-1} IV followed after 15 min by butorphanol, 0.02 mg kg^{-1} IV, acepromazine, 0.02 mg kg^{-1} IV and xylazine 0.2 mg kg^{-1} IV) a twitch-nose was applied and the endoscopic examination were then performed.

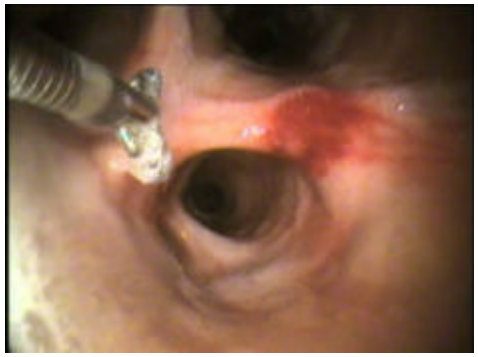

Fig. 1: Endoscopical collection of the bronchial tissue in RAO horse. Note the abnormal exudation, the thickening of bronchial wall and the evidence of bronchial rings (indicating bronchospasmus)

The 9.8 mm diameter, 320 cm length endoscope (Mercury Endoscopia Italiana) was passed through the ventral meatus of the nostril, to reach the carina passing through the larynx and trachea. The endoscope was then inserted in the left main bronchus. Two bronchial septum at different distance from carina were chosen as sites for sample collection for a total of eighteen specimens. Samples where collected using a forcep passed in the operative channel (Fig. 1) after topical application of 10 mL of 1% lidocaine.

Each sample was fixed in 10% buffered neutral formalin and embedded in paraffin wax then cut in 4 μm thick sections; one slide was stained with haematoxylin-eosin, the others were used for the immunohistochemical analysis. The immunohistochemestry was carried out by the Streptavidin-Biotin-Peroxidase (ABC) Complex Method. After microwave antigen unmasking (8' at 650 W for two times) and inhibition of endogenous peroxidase activity (60' with H_2O_2 in 0.3% distilled water) the slides were incubated overnight with monoclonal antibody against ET-1 (Sigma, St. Louis, USA, 1:150).

Positive control is naturally present in all examinated tissues in endothelial cells of vessels. As positive controls from healthy horses, the samples collected in a earlier research performed in the hospital (were the same materials and procedures were applied) have been used (Fulvio *et al*., 2012).

RESULTS AND DISCUSSION

All clinical diagnosis of RAO were confirmed by the endoscopical appearance of the airways were signs of altered mucus secretion, edema of bronchial wall and bronchospasmus were evident. The biopsy procedure did not cause any clinical complication neither during nor after samples collection.

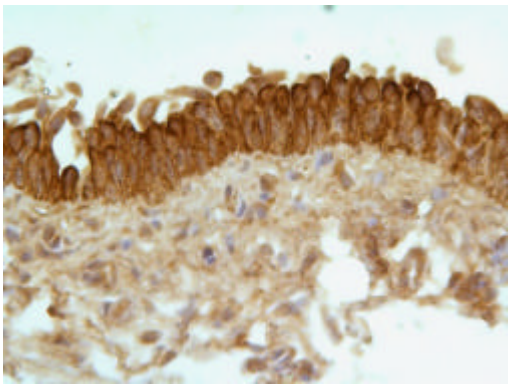

Fig. 2: Endothelin-1 in horses with RAO. All epithelial cells are positives. ABC; 20x

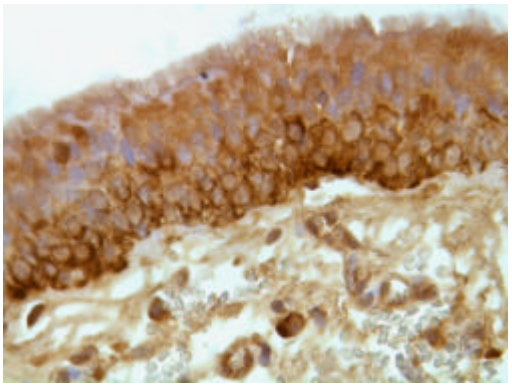

Fig. 3: Endothelin-1 in horses with RAO. High magnification showing the specific positivity of lower epithelial cells. ABC, 40x

At histological examination the bronchial epithelial cells of both upper and lower respiratory tract were intensely positive to ET-1. The Fig. 2 shows a tract of bronchiolar epithelium in which in all the cells were present a fine granular brown stain associated with a diffuse edema of the mucosal lamina propria. The bronchial epithelium were iperplastic and the intensity of immunoreaction were more strong in lower epithelial cells (Fig. 3).

When compared with normal bronchial tissue sampled from healthy horses and immunostained for ET-1 in a earlier research (used as positive control), some important differences can be noted: the immunoreaction to ET-1 is more pale and evident in few epithelial cells while the endotelial cells and macrophages appeared intensely stained (Fig. 4).

An earlier research of the researchers, Fulvio *et al.* (2012) demonstrated the presence of ET-1 in bronchial

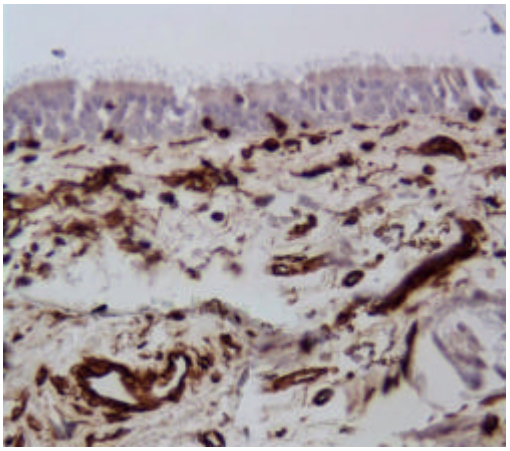

Fig. 4: Endothelin-1 in normal bronchus. Only few epithelial cells are positives. ABC; 20x

biopsy of healthy horses. In that study, a specific reaction to ET-1 in normal epithelial cells of both upper and lower respiratory tract was found in all healthy horses. The cytoplasm of some bronchial and bronchiolar epitelial cells showed a brown, finely granular appearance and the intensity of reaction appeared quite uniform among the several airways tissues collected.

Histological samples from horse with RAO showed a strongly intense reaction for ET-1. It is interesting to notice that there is an association between the intensity of reaction and those of respiratory condition. The observation that the epithelial cells of the lower layers resulted more positive than the upper cells confirms that the hyperplastic process is continuus and active and it could be hypothesized a role of ET-1 on it and therefore, a dual pathogenic role of endothelin. Futhermore, epithelial cells are probably the main site of production for ETs in horses.

This represent the first time demonstration of the rising in ET-1 content of epithelial cells in naturally occurring disease in live horses. The same result have been found in human patient affected by asthma (Redington *et al.*, 1997) a respiratory condition sharing some clinical and pathogenetic similarity with RAO and the researchers suggest an involvement of ET in the pathogenesis of human asthma. Furthermore, asthmatic patients experience a dose-dependent bronchoconstriction to inhaled ET-1 (Chalmers *et al.*, 1999).

In equine pathology, other investigations will be necessary to explain the up-regolation of ET-1 in airways and to know its role in the pathogenesis of RAO. For this purpose could be important to examine the distribution of

ET-receptor (ET-A) in the peripheral pulmonary tissues of RAO affected and unaffected horses. Since, ET_B receptors are overexpressed on post-euthanasia collected samples of peripheral lung of RAO affected horses (Polikepahad *et al*., 2006, 2008), researchers suggest the need for a clinical trial based on the receptor blockade with specific active principles.

CONCLUSION

An earlier research demonstrated the presence of Endothelin-1 (ET-1) in bronchial biopsy of healthy horses while in the present one, the results of immunohistochemical analysis of bronchial biopsy sampled from horses affected by reurrent airway obstruction are presented and compared with healthy horses. Histological samples from horse with RAO have a much more intense reaction for ET-1, especially epithelial cells. These results confirmed the involvement of ET-1 in pathogenesis of respiratory condition of horse and the possible use of its receptors as pharmacological target.

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