

## Endothelin-1 Detection in Bronchial Biopsy of Horses

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**Abstract:** Endothelin-1 (ET-1) is the most potent vasoconstrictor molecule up to now identified. Endothelial cells, smooth muscle cells and epithelial cells of airways are able to produce this potent proinflammatory, secretagogues and bronchoconstrictors mediator also implicated in the pathogenesis of inflammatory diseases of airways in human being. Some studies demonstrated the presence of endothelin-1 in respiratory apparatus of the horse and also that ET-1 cause bronchial contraction on bronchial rings of horses *in vitro*, especially in horse with respiratory disease.

**Key words:** Horse, respiratory disease, endothelin, biopsy, immunohistochemical analysis, proinflammatory

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### INTRODUCTION

Endothelin-1 (ET-1) is the most potent vasoconstrictor molecule up to now identified. Endothelial and smooth muscle cells, epithelial cells of airway, macrophages, fibroblasts, cardiac myocytes, neurons of the brain and pancreatic islets are able to produce ET-1 (Kawanabe and Nauli, 2011). Two main receptors have been individuate for it: Endothelin receptor A (ET<sub>A</sub>) and Endothelin receptor B (ET<sub>B</sub>). ET<sub>A</sub> induced stimulation leads to vasoconstriction while ET<sub>B</sub> receptors activation at pulmonary level leads to bronchoconstriction (Fagan *et al.*, 2001). ET-1 is also a potent proinflammatory, secretagogues and bronchoconstrictors mediator and is implicated in the pathogenesis of airway inflammatory diseases in human being (asthma) (Aoki *et al.*, 1994) and also in several inflammatory and non-inflammatory diseases (Fagan *et al.*, 2001).

Benamou *et al.* (2003, 1998) demonstrated that ET-1 levels in venous blood (but not in arterial blood) and in Bronchoalveolar Lavage Fluid (BALF) of horses affected by Recurrent Airway Obstruction (RAO) are significantly higher than those of control horses. These differences are statistically significant only in horses during exacerbation of the disease but not during remission periods when horses have an intermediate value between RAO horses in exacerbation and healthy horses. Similar results were found in a further recent study (Costa *et al.*, 2009) and analogous situation can be found in humans (Aoki *et al.*, 1994). Moreover, they observed that in contrast with normal animals, horse with RAO had a negative arterial-

venous difference of ET-1. There are also evidences that exercise can cause an increase of ET-1 in BALFs of horses affected by Chronic Obstructive Pulmonary Disease (COPD) but not in those of normal horses (Benamou *et al.*, 1999). However, McKeever *et al.* (2002) found an increased level of ET-1 in venous blood immediately after exercise on treadmill in healthy horses. In spite of all these important information, the exact site of production for ET-1 in respiratory apparatus is still unclear.

Although, ET-1 has a potent vasoconstrictive action (ET-A receptors mediated) on the pulmonary and systemic circulation of the horse, it seems to have a role neither in the hypoxic pulmonary vasoconstriction in response to acute hypoxia (Benamou *et al.*, 2001 a, b) nor in the pathogenesis of exercise-induce pulmonary haemorrhage (Padilla *et al.*, 2006). ET-1 cause concentration-dependent bronchial contraction on bronchial rings *in vitro*. This contraction is greater on bronchial rings of SPAOPD (Summer Pasture Associated Obstructive Pulmonary Diseases) affected horse than on rings of unaffected horses and seems to be due to the activation of pulmonary ET<sub>B</sub> 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<sub>B</sub> are overexpressed on post-euthanasia collected samples of peripheral lung of RAO affected horses (Polikepahad *et al.*, 2006, 2008). In the knowledge, this is the first study about endothelin-detection in biopsy samples collected from live horses.

## MATERIALS AND METHODS

Bronchial biopsies have been endoscopically performed on five horses with no clinical signs of respiratory diseases. Horses were brought at the Veterinary Teaching Hospital of Camerino University for problems not related to the respiratory system but requiring sedation for diagnostic or therapeutic purpose. Owners were asked to give the permission to make bronchial sample collection.

About 0.02 mg kg<sup>-1</sup> of acepromazine were administered intravenously to each horse approximately 15 min before positioning in stocks. An additional intravenous injection of butorphanol (0.02 mg kg<sup>-1</sup>), acepromazine (0.02 mg kg<sup>-1</sup>) and xylazine (0.2 mg kg<sup>-1</sup>) were then performed. During sample collection a twitch-nose was applied.

An 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. From three to five bronchial septum at different distance from carina were chosen as sites for sample collection.

The 10 mL of 1% lidocaine were topically instilled on the mucosa through the operative channel and samples collected using a forcep passed in the same channel (Fig. 1). 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 immunohistochemistry was carried out by the Streptavidin-Biotin-Peroxidase (ABC) complex method. After microwave antigen unmasking (8' at 650W for two times) and inhibition of endogenous peroxidase activity (60 with H<sub>2</sub>O<sub>2</sub> in 0.3% distilled water), the slides were incubated overnight with monoclonal antibody



Fig. 1: Endoscopic collection of the bronchial tissue

against ET-1 (Sigma, St. Louis, USA, 1:150). Positive control is naturally present in all examined tissues in endothelial cells of vessels; negative controls were made by replacing the primary antibodies with TBS.

## RESULTS AND DISCUSSION

All animals well tolerated the biopsy procedure and did not show any clinical consequences neither during nor after samples collection. Although, in some instance the amount of sampled tissue was not suitable to be examined, in all cases at least two samples (one from upper and one from lower airways) has been obtained from each horse. The histological examination of biopsy samples showed a normal architecture of bronchial tissue without cellular alterations (Fig. 2). Immunohistochemically, a specific reaction to ET-1 in normal epithelial cells of both upper and lower respiratory tract has been found in all horses. In both bronchial and bronchiolar epithelial cells the cytoplasm showed a brown, finely granular appearance (Fig. 3). The intensity of reaction appeared quite uniform among the several airways tissues collected. The endothelial cells resulted always positive in all samples. The present study

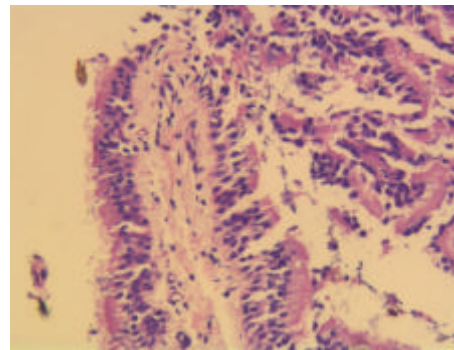


Fig. 2: Bronchial biopsy (E-E-20X)

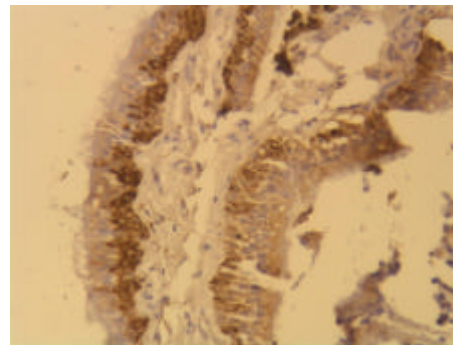


Fig. 3: Bronchial biopsy (ET-1 Immunostain-20X)

demonstrated for the first time, the possibility to use biopsy sampling as a technique to individuate the presence of endothelin-1 in airways of live animals. The topographic distribution of ET-1 in normal bronchus in live animals has been examined founding the presence of this molecule in both epithelial and endothelial cells. Since, now ET-1 was found in other biological samples (venous and arterial blood, bronchoalveolar lavage fluid) but never in bronchial biopsies collected from sedated patients.

Regarding the technique since, the small dimension of the biopsy forcep sometimes gave not enough tissue to be processed and examined, researchers suggest (when using a long forceps as researchers have done) to repeat the sampling on at least three sites before to retrieve the scope from the respiratory tract. Recurrent Airway Obstruction (RAO) and Inflammatory Airway Disease (IAD) are worldwide respiratory condition causing chronic cough, poor performances and labored expiratory effort in horse.

Aethiopathogenesis is still unclear and therapeutic management is usually long term, unsatisfactory and expensive. Evaluation of ET-1 concentration in BALF and/or in serum seems to be a not enough reliable method to assess the real status of airway in horse with RAO or IAD. Lungs act as a clearance system and it is possible that ET-1 can sometimes be found in the airway even if not acting on it.

Furthermore, in the current literature there not enough clear statistically significant differences between sick and healthy horses. That is why in the present study, the normal ET-1 status has been assessed with immunohistochemistry techniques in horse with normal respiratory system, trying to avoid the interference of metabolic condition external to the cells.

This mean that ET-1 can be produced by the airways in normal horse and that after an appropriate stimulation, respiratory cells could enhance their production, directly triggering some the common pathogenetic events of respiratory diseases (e.g., hypersecretion and bronchospasmus).

Comparing the presence and distribution of ET-1 (other than ET<sub>A</sub> and ET<sub>B</sub> receptors) between healthy and diseased animals (especially RAO and IAD) it will be the next step of the present research, trying to understand the possible potential use of endothelin for diagnostic and prognostic purposes and of ET<sub>A</sub> and ET<sub>B</sub> receptors to assess the possible therapeutic use of receptor antagonist as bosentan and sitaxentan that has been recently approved for treatment of selected human patients with Pulmonary Arterial Hypertension (PAH) (Kawanabe and Nauli, 2011).

## CONCLUSION

In the present study, detection of endothelin in sample collected from live healthy horses has been tried using endoscopic technique for collection and immunohistochemical analysis for detection. All horse resulted positives for ET-1 both in endothelial and epithelial bronchial cells. This is the first report of ET-1 detection in tissue samples of live horses and could represent the first step for diagnostic, prognostic and therapeutic advance in respiratory diseases of horses.

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