Endothelin in Bronchial Biopsy Specimens from Horses with Recurrent Airway Obstruction

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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 (Borzacchello et al., 2010) other than in cardiac conditions (Prosek et al., 2007). Furthermore, ET-1 can be detected in airway of dogs with idiopathic pulmonary fibrosis but not in healthy dogs (Kraft 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 hand, 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
treadmill can be found in healthy horses (McKeever et al., 2002). Nevertheless, there are also studies that show an increase in ET-1 in BALF 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 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, are overexpressed on post-ethanization collected samples of peripheral lung of RAO affected horses (Polikarpahad 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 inspiratory 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 endoscopic examination of respiratory system before to start any drug 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.

![Endoscopic 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)](image)

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 forceps 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 immunohistochemistry 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 H₂O₂ 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 examined 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 endoscopic 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.
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 hyperplastic 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 endothelial 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 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 epithelial 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 continuous and active and it could be hypothesized a role of ET-1 on it and therefore, a dual pathogenic role of endothelin. Furthermore, 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-regulation 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-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 recurrent 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.

**REFERENCES**


