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The Role of Dopaminergic Neurodegeneration in Equine Pituitary Pars Intermedia Dysfunction (Equine Cushing's Disease) (21-Nov-2003)

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Abstract
Equine pituitary pars intermedia dysfunction (PPID) is a well-recognized disease in aged horses and ponies, yet the etiology and pathophysiology of this condition remains poorly understood. Immunohistochemistry of formalin-fixed pituitary tissue was used to demonstrate dopaminergic neurodegeneration and oxidative stress in the pars intermedia in horses with PPID. The role of oxidative stress in the pathophysiology of this disease is a new finding. Further research is warranted to elucidate the mechanism resulting in oxidative stress in horses with PPID.

1. Introduction

Equine pituitary pars intermedia dysfunction (PPID, equine Cushing's disease) is one of the most common diseases in horses 15 yr and older. It is a naturally occurring, progressive condition characterized by hypertrophy and hyperplasia of the pituitary pars intermedia resulting in an increased expression of proopiomelanocortin (POMC) peptides [1]. Affected horses have an increased circulating concentration of POMC-derived peptides, ultimately leading to the development of clinical signs through an as yet poorly defined sequence of events. Horses with PPID show a number of clinical signs including hirsutism, lethargy, infertility, polydipsia, and polyuria. Approximately 25 - 80% of affected horses develop laminitis, often necessitating euthanasia [2,3]. Altered energy metabolism may result in muscle wasting, weight loss, or abnormal fat distribution. Affected horses have an increased susceptibility to both parasitic and bacterial infections with sinusitis, dermatitis, and pneumonia being common. Despite clinical recognition of this disease for over 70 yr, the pathophysiology remains poorly understood.

The mechanism of pars intermedia hypertrophy, hyperplasia, and adenoma formation in PPID is not clear and may be the result of a primary lesion of the pituitary, or alternatively, a loss of hypothalamic inhibition. The pars intermedia is comprised of a single cell type, the melanotrope, that is under inhibitory control by dopamine, released in the pars intermedia by local nerve terminals [4,5]. In rodents and cats, dopaminergic neurons have been demonstrated to originate in the periventricular nucleus of the hypothalamus, adjacent to the third ventricle [6,7]. The neurons project through the infundibulum, terminating in the pars intermedia. Dopamine released from these neurons inhibits the secretion of POMC-derived peptides through interaction at D2 receptors on the melanotropes [8]. Surgical disruption of the periventricular hypophyseal dopaminergic tracts in rats results in increased expression of pars intermedia melanotropes [6]. In addition, D2 receptor knockout mice develop pars intermedia lesions similar to PPID [8]. Based on these studies and others in horses, it is probable that equine PPID results from a loss of inhibition of the pars intermedia because of degeneration of the periventricular hypophyseal dopaminergic neurons. Millington et al., [9] demonstrated an 80% decrease in dopamine and dopamine metabolite concentrations in the pars intermedia of affected horses compared with age-matched controls. Orth et al., [10] showed that systemic supplementation of dopamine or a dopamine agonist to horses with PPID resulted in a decrease in plasma concentration of POMC peptides. A number of investigators have reported anecdotal clinical response of affected horses to treatment with dopamine agonist, pergolide [11,12]. In addition, horses treated with pergolide have been reported to have improvement of the biochemical abnormalities associated with the disease [13,14].

If in fact PPID is a dopaminergic neurodegenerative disease, it raises the following questions. 1) What causes the neurons to degenerate? 2) Why are only some horses affected? Based on the literature, we speculate PPID may result from dopaminergic

neurodegeneration secondary to oxidative stress. Oxidative stress results in modification of cellular components including proteins, DNA, and cell membrane lipids caused by excessive exposure to exogenous or endogenous sources of oxidants. This cellular damage ultimately leads to cell death, or in the case of neurons, neurodegeneration. Chronic exposure to oxidants in excess of an animal's antioxidant capacity results in accumulation of functionally impaired cellular components. Measurement of these altered cellular components is useful as a marker of historical exposure to oxidative stress [15]. Dopaminergic neurons are particularly vulnerable to oxidative damage. Chronic oxidative stress is considered to be an inciting factor in the development of other diseases associated with dopaminergic neuronal degeneration, such as Parkinson's disease. In people with Parkinson's disease, antioxidant capability is *decreased*, whereas the concentration of oxidatively damaged cellular components is *increased* compared with age-matched healthy individuals [16]. Markers of oxidative stress, including 3-nitrotyrosine, have been demonstrated to be increased in patients afflicted with Parkinson's disease [16]. In animal models of Parkinson's disease, mutation of the genes for antioxidant enzymes results in animals more susceptible to developing dopaminergic neuronal degeneration after oxidative stress [17,18].
We hypothesize that PPID results from loss of dopamine inhibition of the pars intermedia secondary to degeneration of the periventricular hypophyseal dopaminergic neurons ("dopaminergic neurodegeneration"). Furthermore, based on studies in other species, we hypothesize that chronic oxidative stress is the cause of the dopaminergic neurodegeneration. The purpose of this paper is to present results from research done in our laboratory demonstrating the role of oxidative stress and dopaminergic neurodegeneration in the development of PPID.

2. Materials and Methods

Immunohistochemical evaluation of archived formalin-fixed pituitary tissue was used to assess dopaminergic neuronal density and oxidative stress in the pars intermedia. Three study groups of horses were examined: 1) young (<15 yr old) unaffected horses, 2) old (> 15 yr old) unaffected horses, and 3) horses with PPID. Twelve horses were used in each group. Horses with PPID were selected based on clinical signs and confirmed to have the disease at post-mortem examination because of the presence of pars intermedia hyperplasia and micro- or macroadenomas. Mares late in pregnancy or lactating and horses under 1 yr of age were excluded. Comparison of young and old unaffected horses was necessary to determine the impact aging on dopaminergic neurodegeneration and oxidative stress in horses in the absence of concurrent PPID.
Dopaminergic neuronal density was assessed by immunohistochemistry (IHC) using a polyclonal antibody recognizing tyrosine hydroxylase, a marker of dopamine neurons. After IHC, six fields within the pars intermedia were selected in a masked fashion, and positive tyrosine hydroxylase signal was counted using a computer imaging program [a]. The average count for the six fields was calculated for each horse, and results were expressed as tyrosine hydroxylase positive signal per unit area (field). Results from the three study groups were compared using one-way analysis of variance.
To demonstrate oxidative stress, paraffin-embedded pituitary tissue was examined by IHC using a monoclonal antibody for the oxidative marker, 3-nitrotyrosine. After IHC, for each slide, all fields of view within the pars intermedia were examined and graded as either positive or negative. A positive field had a minimum of two cells positive for 3-nitrotyrosine. The percent positive fields were calculated for each horse, and results were compared by one-way analysis of variance.

3. Results

Tyrosine hydroxylase immunohistochemistry - The mean age of the PPID horses was well matched to the aged control group (19.17 and 20.75 yr, respectively). There was a significant decrease in tyrosine hydroxylase staining in horses with PPID compared with both the aged and young controls ($P < 0.001$). There was no observed difference between the young and aged controls (Table 1; Fig. 1).

Table 1. Tyrosine Hydroxylase Immunohistochemistry

Mean (SE)	PPID	Young (< 15 yrs)	Old (> 15yrs)
Age	19.17 (1.86)	6. 0 (1.5)	20.75 (1.24)
TH signal/field	28.68 (6.51)	139.8 (12.4)	132.5 (10.2)

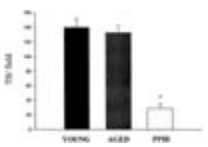

Figure 1. Tyrosine hydroxylase immunohistochemistry of equine pituitary pars intermedia. - To view this image in full size go to the IVIS website at www.ivis.org . -

3-Nitrotyrosine immunohistochemistry - The mean age of the PPID and aged control group were well matched (19.33 and 21.62 yr, respectively). There was a significant increase in the total number of fields of view in the pars intermedia of affected

horses compared with controls (Table 2). This is expected as the number of fields of view parallels the total area, and PPID is characterized by hyperplasia of the pars intermedia. There was a statistically significant increase in the absolute and percent of fields positive for the presence of 3-nitrotyrosine in the PPID group ($P < 0.001$). In addition, there was an increase in the presence of 3-nitrotyrosine in the aged controls compared with the young controls ($P < 0.05$) (Fig. 2).

Table 2. 3-Nitrotyrosine Immunohistochemistry

Mean (SE)	PPID	Young (< 15 yrs)	Old (> 15 yrs)
Age	19.33 (1.87)	6.17 (1.49)	21.08 (1.26)
Number of fields counted	57.08	18.83	21.62
Number of positive fields	28.15 (3.63)	0.250 (0.179)	4.62 (2.15)
Percentage of positive fields	0.4804 (0.0579)	0.0208 (0.0144)	0.1398 (0.0488)

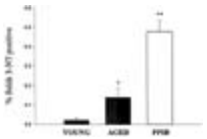

Figure 2. 3-Nitrotyrosine immunohistochemistry of equine pituitary pars intermedia. - To view this image in full size go to the IVIS website at www.ivis.org . -

4. Discussion

The results of these experiments support the hypothesis that PPID is a dopaminergic neurodegenerative disease. Horses with PPID had five times less tyrosine hydroxylase staining, indicating a five-fold reduction in the number of dopaminergic terminals in the pars intermedia. This is consistent with the marked reduction in dopamine and dopamine metabolite concentration in the pars intermedia of affected horses reported by Millington et al., [9]. We are currently pursuing further confirmation of neurodegeneration by quantifying the dopaminergic cell bodies in the hypothalamic periventricular nucleus of horses with PPID and unaffected controls.

The role of oxidative stress in the pathophysiology of PPID is a new finding. Oxidative stress may result from excessive exposure to environmental oxidants, increased metabolism, inflammation, or deficiency in antioxidant capacity. The sporadic nature of PPID makes exposure to environment toxicants a less likely cause; however, it may be a contributing factor in development of disease individuals with poor antioxidant capacity. Chronic inflammation is also an unlikely cause of PPID, because there is no histological evidence of this. In addition, in our study, many of the aged control horses had chronic inflammatory diseases, such as osteoarthritis or chronic obstructive pulmonary disease. Despite this, they were free of the oxidative stress marker 3-nitrotyrosine. Therefore, we suspect horses with PPID may be predisposed to oxidative stress because of either a deficiency in antioxidant capacity or altered glucose metabolism. Chronic hyperglycemia and type II diabetes are both associated with oxidative stress in other species [19]. We are currently investigating the relationship between abnormal glucose regulation and oxidative stress in horses as well as documenting the antioxidant capacity in horses with PPID.

The increase of oxidative stress in the aged control group was not unexpected, because age-related accumulation of markers of chronic oxidative stress has been reported in other species [20]. It was, however, surprising that there was not an age related decrease in tyrosine hydroxylase staining, because progressive dopaminergic neurodegeneration is known to occur with age in other species [21]. One possible explanation for this failure to appreciate an age-related neuronal loss is because, in our study, the aged control group may in fact have been too young. Evaluation of a larger number of older horses may provide insight as to whether this age-related phenomenon occurs in horses.

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Footnotes

[a] Bioquant TCW98, V3.50.6 MST 1993 - 1998, R & M Biometrics Inc., Nashville, TN, 37209.

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