Immunohistochemical Detection of P-glycoprotein in Tissues from Horses Afflicted with Equine Motor Neuron Disease

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P-glycoproteins (P-gp) are transmembrane glycoproteins associated with the multidrug resistance (MDR) phenotype. The P-gp functions as an ATP-dependent transporter of a diverse xenobiotic and pharmacologic compounds to effectively lower their intracellular concentration. As such, P-gp is believed to be a significant component of the blood-brain-barrier (BBB) in mammals. We carried out a study to investigate whether P-gp could be detected in normal and in horses afflicted with equine motor neuron disease (EMND). Fresh samples were harvested at necropsy from seven horses diagnosed with EMND and three control horses and immediately frozen in liquid nitrogen until processing. The samples included tissues from the adrenal gland, kidney, liver, small intestine, brain stem, and spinal cord. These tissues were evaluated for P-gp immunoreactivity using monoclonal antibody (C494). Normal and EMND afflicted horses expressed the P-gp at similar levels in the adrenal cortex (100%), the renal proximal tubules (57%), and small intestinal enterocytes (60%). There was no significant difference in the P-gp expression rates between the EMND affected horses and controls. None of the liver and central nervous tissue samples exhibited any P-gp staining. The overall results of our investigation indicate that abundant P-gp expression can be detected in some of the equine tissues using the immunohistochemical methods. The ability to detect P-gp in equine tissues might help in determining its specific role in the etiology of EMND.

Key words: EMND, equine, immunohistochemistry, MAb C494, P-glycoproteins

Equine Motor Neuron Disease (EMND) is an acquired sporadic neuromuscular disease of horses that affects animals of all breeds and sex [2]. Since it was first reported in 1990, more than 100 cases have been diagnosed in North America, Europe, Japan and Brazil, indicating that EMND is more prevalent and geographically widespread than was initially perceived [5, 11, 17, 24]. The clinical and pathological changes in EMND are similar to amyotrophic lateral sclerosis (ALS), or more precisely, to progressive muscular atrophy (PMA) in humans [2, 26]. However, a definitive etiology for EMND has not yet been determined.

Previous studies on EMND have revealed that affected horses show increased lipopigment (ceroid) accumulation in the endothelium of spinal cord vessels, similar to that associated with vitamin E deficiency in monkeys [3, 18]. Plasma levels of vitamin E have been reported to be significantly lower in affected horses, with no significant changes in glutathione peroxidase activity in red blood cells or selenium in the serum [5]. Vitamin E has an important role in biologic membrane integrity, as an antioxidant and stabilizing agent. Membrane derangement could increase blood-brain barrier (BBB) permeability, rendering the central nervous system vulnerable to potential toxicants. Some affected horses have also shown an elevation of protein and IgG levels in the cerebrospinal fluid (CSF) without concomitant leukocytosis [2, 5]. These findings have suggested a possible damage to the BBB.

In vertebrates, membrane P-glycoproteins (P-gp)
mediate the influx and efflux of a large number of hydrophobic amphipathic molecules from the cell. The P-gp protects cells from the effect of endogenous and exogenous cytotoxic compounds and they are found in high concentrations in the blood-tissue barriers. The increased expression of P-gp in tumor cells has been associated with the multidrug resistance phenotype. Multidrug resistance is a general term that refers to a phenotype of neoplastic cells exhibiting simultaneous resistance to chemically dissimilar cytotoxic compounds. The multidrug resistance (MDR) phenotype results from one of several specific mechanisms operating alone or simultaneously to reduce the action or concentration of a cytotoxic agent at the target [19, 23]. Though many mechanisms of drug resistance exist, one of the most important involves the overexpression of the MDR1 gene. The MDR1 gene produces a 170-kDa integral membrane protein called P-glycoprotein (P-gp) which acts as an ATP-dependent efflux pump and is expressed normally in a variety of tissues [20, 25]. P-gp expressed in normal tissues functions as a transmembrane metabolic or detoxification system in the hepatic biliary epithelium and renal proximal convoluted tubule and as a barrier to xenobiotic exposure in the gastrointestinal tract and endothelium of the BBB [15]. Even low levels of transcription may confer substantial drug resistance [4, 6]. Therefore, accurate methods of detecting MDR1 transcript expression would be a key factor in determining the possible effects of neurotoxins via the BBB.

As a step towards developing a comprehensive appreciation for the BBB in the equine we designed this study to investigate whether horses afflicted with EMND express P-gp in various tissues. Inherent objective in this initial investigation is to optimize the immunohistochemical protocol to detect P-gp in equine tissue, to determine whether P-gp expression can be detected with this method in equine tissue, and to ascertain the relative expression of P-gp in various tissues.

**Materials and Methods**

The methods used in this study were adapted from a procedure described previously in canines [14]. Tissues were harvested from EMND afflicted and control horses and examined for the expression of P-gp using a monoclonal antibody using an immunohistochemistry approach.

**Animals and Tissues**

Samples were collected from horses enrolled in a field trial to assess the role of antioxidants on the risk of EMND and from controls. The EMND horses had been previously diagnosed with the disease showing a varying degree of pathological involvement. Table one shows the demographic characteristics of the horses enrolled in the study. There were 7 EMND-afflicted horses and three controls. Tissue samples were harvested from these horses at necropsy in liquid nitrogen. The harvested tissue samples included: adrenal gland, kidney, liver, small intestine, brain stem, and spinal cord. Similar tissues were harvested from three control horses. The control horses had no clinical signs and were enrolled in a trial aimed towards assessing the dietary effect on osteochondrosis (Table 1).

The tissue samples were anchored in optimum cutting temperature (OCT) compound in a freezing boat and immersed in chilled isopentane (in liquid N2) for 30–60 sec. The boats were subsequently sealed with foil and frozen at −70°C. At the time of processing, frozen blocks were warmed to −30°C in cryostat and sectioned at 8 µm, air dried and fixed on cleaned slides in cold acetone for 15 min. Slides were then air dried for 30 min and stored in the freezer at −70°C until use.

**Immunoperoxidase Staining: Frozen Tissues**

Slides were brought to room temperature and were blocked with 10% goat serum in 0.01 M phosphate buffered saline (PBS) (Zymed Labs, San Francisco, CA, USA) for 30 min at room temperature in a humid chamber on a rotator. Slides were then blotted and

<table>
<thead>
<tr>
<th>Horse ID</th>
<th>Age years</th>
<th>Sex</th>
<th>Breed of horse</th>
<th>Clinical Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>MC*</td>
<td>Standardbred</td>
<td>EMND</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>Fb</td>
<td>Thoroughbred</td>
<td>EMND</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>F</td>
<td>Thoroughbred</td>
<td>EMND</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>F</td>
<td>Standardbred</td>
<td>EMND</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>MC</td>
<td>Quarterhorse</td>
<td>EMND</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>MC</td>
<td>Mixed breed</td>
<td>EMND</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>F</td>
<td>Thoroughbred</td>
<td>EMND</td>
</tr>
<tr>
<td>8d</td>
<td>13</td>
<td>F</td>
<td>Thoroughbred</td>
<td>Control</td>
</tr>
<tr>
<td>9d</td>
<td>12</td>
<td>F</td>
<td>Quarterhorse</td>
<td>Control</td>
</tr>
<tr>
<td>10d</td>
<td>8</td>
<td>MF</td>
<td>Thoroughbred</td>
<td>Control</td>
</tr>
</tbody>
</table>

*: Male castrated; b: Female; c: Male intact; d: Controls

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Table 1. Demographic data on horses afflicted with EMND and controls used in the P-glycoprotein study
primary antibody specific for the MDR1 epitope, murine-origin IgG subclass monoclonal C494 (Signet Laboratories, Dedham, MA, USA), was added. Optimal primary antibody dilution was determined by initial trials of serial dilutions (1:10, 1:20, 1:40, 1:80, 1:160, 1:320) on sections of each type of different tissue. Subsequently, primary antibody was always added at 1:40 dilution in 0.01 M PBS for 2 hr at 37°C in humid chamber. Two negative controls were used for each tissue sample: a primary negative mouse reagent (V-
Series, Dako Laboratories, Carpinteria, CA, USA) and normal mouse ascites, both diluted to 1:40. The negative reagent controls were added in the place of the primary antibody to duplicate sections cut from the same sample block. Slides were washed with 0.01 M PBS for 2 min, 3 times each. Pre-diluted biotinylated secondary antibody, goat anti-mouse (Zymed Labs, San Francisco, CA, USA), was added for 15 min at room temperature in a humid chamber on a rotator. Slides were washed with 0.01 M PBS for two min three times each. Slides were incubated with pre-diluted streptavidin peroxidase (Zymed Labs, San Francisco, CA, USA) for 15 min at room temperature in a humid chamber on a rotator. Slides were washed with 0.01 M PBS for 2 min, 3 times each. Slides were then incubated with aminoethyl carbazole (AEC) (Zymed Labs, San Francisco, CA, USA), prepared as instructed on the kit, for 5 min in a humid chamber on rotator. Slides were washed with tap water for 2 min, 3 times each. Slides were then counterstained with Gill’s Hematoxylin for 1 min, mounted in Fluoromount and sealed with nail polish.

Scoring of immunoreactivity

The expression of P-gp was independently assessed by 2 observers who were blinded about the clinical status of the horse. The distribution of P-gp expression was semi-quantitatively assessed by estimating the proportion of positively stained cells in the respective tissue. Samples were considered negative for expression of protein based on the staining of the negative control as a reference. Samples with more than 10% of the cells stained positive for C494 were regarded as positive. Samples were scored on a scale from zero to 3, for zero being negative and 3 where 100% of the cells are stained. Slides were viewed independently and scored by the two senior authors on a scale of 0 to 3.

Statistical analysis

The Wilcoxon rank sum test was used to compare the level of expression of P-gp in different tissues between the EMND cases and control horses. A two-tailed P-value of <0.05 was considered to be significant. The statistical analysis was performed using STATIIX 8 software (Analytical Software, Tallahassee, FL, USA).

Results

Tissues from ten horses were evaluated in this study for the expression of the P-gp (Table 1). Horses in the study appeared to be similar with respect to breed and sex. There was no significant difference in the age between the EMND horses and controls in this study (P=0.78, in the t-student test).

The optimization of the test involved several dilutions of the monoclonal antibodies, C494 at two-fold dilutions starting at 1:10 up to 1:160. Immunohistochemical detection of P-gp in frozen tissues fixed with acetone was best realized for all tissues processed with a primary antibody dilution of 1:40; this produced sections with minimal background and distinctive labeling (Fig. 1). In addition, counterstaining with Gill’s Hematoxylin for more than one minute resulted in a too intense counterstain, washing out the initial P-gp staining.

All 10 adrenal gland samples processed exhibited distinctive P-gp staining with a median score of three for both EMND-affected and control horses (Table 2). The cells of the adrenal cortex labeled diffusely with the C494 monoclonal antibody. The intensity of the labeling varied among cells with some having intensely labeled borders and/or prominent perinuclear stained foci (Fig. 1b). It appeared that the cells of the adrenal medulla did not pick up the stain well. There was no significant difference in the immunohistochemistry score between the EMND-affected and control horses (P>0.05).

P-gp expression was demonstrated in kidney samples collected from four out of the seven EMND afflicted horses (57%). The median immunohistochemistry score among these samples was 2 (Table 2). The proximal tubular epithelium stained more often and

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Median score (range)</th>
<th>EMND-horses</th>
<th>Control horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland</td>
<td>3 (2–3)</td>
<td>3 (2–3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>2 (0–3)</td>
<td>2 (0–3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>57%</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Small Intestine</td>
<td>2 (0–3)</td>
<td>1 (0–3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60%</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>Nervous tissues</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
</tbody>
</table>

Percent of samples with respective (score)
intensely than the distal tubular epithelium relative to the control (Fig. 1 c,d). The cytoplasm also labeled diffusely eliciting a more intensely stained luminal border within the proximal tubular epithelium. This pattern of staining was consistent among the EMND-cases and control horses and there was no significant difference in the immunohistochemistry score between the two groups (P>0.05) (Table 2).

Although three out of the five EMND afflicted horses showed evidence of expression of P-gp in samples collected from the small intestine the rate of expression was relatively low, only 60% of the samples tested positive in the immunohistochemistry (Table 2) (Fig. 1 e, f). There was no significant difference between the EMND cases and control horses in the immunohistochemistry score (P>0.05). No detectable P-gp could be found in the liver or the nervous tissues in samples collected from either EMND-cases or control horses.

Discussion

The primary purpose of this study was to determine whether P-gp expression in equines could be detected using conventional methods, immunohistochemistry. To our knowledge, no studies have been undertaken to investigate the expression of the P-gp in the horse before. The interest in the P-gp, in relation to the risk of EMND, stems from the role of the MDR in protecting the brain against neurotoxic substances. Other studies have established the relation between the deficiency of the P-gp expression and the risk of neurotoxicity in other animals [13, 22]. The BBB is formed by the endothelial cells lining the brain microvessels with tight junctions linking adjacent cells which make brain capillaries around 100 times tighter than peripheral capillaries to small hydrophilic molecules. As a result, only drugs and chemicals that are lipophilic are able to cross the brain endothelium via the lipid membranes. However, the efflux carriers of the BBB, particularly P-gp, control such lipophilic substances. In normal brain tissue P-gp is expressed almost exclusively by endothelial cells [22]. The barrier phenotype of the brain endothelium is induced and maintained by chemical factors released by brain cells, particularly perivascular astrocytic end feet. In several neuropathological conditions, the BBB is disturbed, either as a result of pathology of the endothelium, or of the cells responsible for barrier induction and maintenance. There is evidence that under such pathological conditions such as in EMND, ‘second line defense’ mechanisms in perivascular glia may be up-regulated, including expression of P-gp and other drug efflux transporter. Therefore, we have undertaken this study to investigate if the expression of P-gp in EMND afflicted horses and controls. Lending credence to pathological changes in the endothelial cells capillaries is the finding of the lipofuscin inclusions in these cells among EMND afflicted horses [3].

This study indicates that P-gp can be detected in frozen normal equine tissues via immunohistochemical methods using a commercially available antibody. The C494 appears to be a viable antibody for use in immunohistochemical P-gp detection in equine tissues, which coincides with the results of studies that have developed immunohistochemical methods of P-gp detection in other animals [7, 14]. Hemmer et al., [10] compared tissue specificity of three different commercial monoclonal antibodies:C494, C219, and JSB-1 and a polyclonal antiserum MDR(Ab-1). They concluded that there was variation in tissue specificity among these antibodies and part of this variation could be attributed to the method of histological fixation and decalcification procedure. In our study we used only one monoclonal antibody, C494, which it could explain the observed variation among the tissues that examined in lieu of the finding in Hemmer’s et al., [10] study.

Other studies had evaluated the efficacy of commercially available P-gp antibodies for detection the expression of these membrane proteins and reported variability which depends on the tissue preparations (fresh vs. formalin fixed), tissue fixation, and the type of tissue (hepatic, adrenal, nervous, or gastric) [7, 10, 12]. We examined the expression of the P-gp on freshly frozen tissues using the C-494 antibodies an approach that was consistently recommended in those comparison studies [7, 10, 12].

Although we were able to detect the expression of the P-gp proteins in tissues from the adrenal gland, kidney, and small intestine, no P-gp was identified on hepatic tissues. This was likely due to technical variation rather than true lack of MDR proteins in this tissue. This finding is supported by the observation made by Bittl et al., [1] who indicated that the C-219 monoclonal antibody for P-gp was more sensitive for P-gp detection in hepatic tissues than the C-494 antibody. Our rationale for using fresh frozen samples in the immunohistochemical approach was to minimize the

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potential impairment or masking of some epitopes that might take place during formalin fixation.

Also none of the tissues harvested from the brain stem or the spinal cord demonstrated the expression of the P-gp. Apparently this is not an unusual finding where other studies eluded to the fact that it is difficult to detect P-gp expression in nervous tissues [7, 28]. The P-gp in the brain tissues are mainly expressed in the endothelial cells of the BBB, however, this expression could increase in medical conditions such as seizure [4]. In addition these studies also confirmed the fact that immunohistochemical detection may vary by the fixation conditions of the nervous tissue and the type of monoclonal antibody used [1, 7, 28]. Given these factors and the fact that the expression rate of the P-gp in nervous tissues is generally low, we are not surprised by the observed results in our study. Because any of the above mentioned factors could have contributed to the observed absence of expression of the P-gp in the nervous tissues harvested from EMND afflicted horses and the controls.

In this preliminary study, equine kidney, adrenal cortex and small intestine were immunoreactive for P-gp expression suggesting similar distribution of P-gp expression as in human tissues [8, 9, 27]. The consistent lack of P-gp labeling in the equine liver and nervous tissues suggests a relatively lesser or undetectable expression of P-gp in these tissues. It is important to note though, that these characterizations were not the primary goal of this study, and thus, more extensive investigation into differentiating the specifics of P-gp expression in each respective equine tissue would be necessary before being able to draw any absolute conclusion.

The ability to detect P-gp in equine tissue is an important first step in determining specific role for the P-gp in the maintenance of the blood-brain barrier, and in a larger context, in the etiology of EMND. The recent finding that P-gp deficiency in a subpopulation of CF-1 mice enhances the neurotoxicity of ivermectin [13, 21] emphasizes the role of the P-gp in the protection of the central nervous system. In the colllies hypersensitivity to neurotoxic drugs such as ivermectin has been attributed to the dysfunctional molecule due to mutation of the mdr-1 gene responsible for the P-gp expression [16, 21, 22]. In humans, genetic polymorphisms of the MDRI gene influence the function of P-gp and thus may alter distribution of substrates into sensitive normal tissues such as the brain. It is likely such polymorphisms exist in other species including the horse.

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