Ethidium Bromide Induced Demyelination of The Central Nervous System in A Dog Model of Secondary Progressive Multiple Sclerosis

Ahmed Nour-Eldine Abdallah1*, Ashraf Aly Shamaa2, Omar Salah El- Tookhy2

1) Pathology department, Animal Health Research Institute, Dokki, Giza, Egypt.
2) Surgery, Anesthesiology and Radiology Department, Faculty of Veterinary Medicine, Cairo University, Egypt.

*Corresponding author: Light_system@hotmail.com Received: 5/3/2020 Accepted: 24/3/2020

ABSTRACT

Demyelinating lesions induced by intraspinal injection of gliotoxin have been studied for many years in order to gain insights into reasons for remyelination failure and in order to improve the understanding of the axonal conduction disorders in multiple sclerosis (MS). This work aimed to develop a model resembling clinical human-progressive MS where Ethidium bromide induced demyelination in dogs’ spinal cord is experimented. All animals received intraspinal injection of 20 µl of 0.1 % Ethidium bromide in the lateral columns using a microneedle syringe attached to a capillary tube. All animals were evaluated clinically with gait analysis, MRI imaging of the spinal cord and electron microscopic analysis. Results showed progressive clinical disability beginning from the third day post induction till 28 days; confirmed by the appearance of sclerotic plaques by MRI and hyperintense regions at the lateral columns of the spinal cord. The electron microscopic pictures showed progressive degenerative lesions characterized by death of oligodendrocytes and astrocytes leading to demyelination and vacuolation followed by axonal damage without signs of endogenous regeneration. Unique features were revealed compared to previous models; this dog model reached a more progressive form of MS where spontaneous remyelination was not observed till 28 days post induction. These findings support that dogs provide an alternative large model to study progressive MS.

Keywords: Demyelination - Dog Model - Ethidium Bromide – Spinal Cord - Multiple Sclerosis

INTRODUCTION

Multiple sclerosis (MS) is a multifactorial chronic demyelinating disease of central nervous system (CNS) with a great socioeconomic impact. It may lead to numerous physical and mental symptoms, and often progresses to physical and cognitive disability (Pachner, 2011). MS affects areas of the brain and the spinal cord known as the white matter. Specifically, the protective sheath (myelin) by destroying Oligodendrocytes (the cells that produce and maintain the myelin sheath) that covers nerve fibers and causes communication problems between your brain and the rest of your body. Eventually, the disease can cause permanent damage or deterioration of the nerves (Stadelmann, 2011) causing paralysis of the limbs, sensation, visual and sphincter problems. The disease is believed to occur by an autoimmune mechanism: the immune system produces antibodies and cells that attack the self-myelin antigens, causing demyelination (Lassmann, van Horssen, & Mahad, 2012). Damage to this myelin sheath protecting the nerve cells in the brain and spinal cord leads to retardation, distortion, or loss of messages to/from the brain and presents as a relapse of neurological disability, a flare-up of symptoms lasting from 24 hours to several
months (Compston & Coles, 2008). Damage or destruction of these important axons (nerve fibers) over time can also lead to irreversible neurodegeneration, causing progression of the disease and an increase in disability (Lassmann et al., 2012; Stadelmann, Wegner, & Broock, 2011; Stadelmann, 2011).

Researchers have only limited access to early and immunologically active MS tissue samples, and the modification of experimental circumstances is much more restricted in human studies compared to studies in animal models. The complex nature of MS and its different forms and stages makes it difficult to fully replicate the disease using in vivo models. This said, different models mimic certain stages of the disease course are needed to clarify the underlying immune-pathological mechanisms and test novel therapeutic and reparative approaches (Johnson & Rodriguez, 2011) but none of them studied a large animal models as dogs (Star, Vogel, Kipp, & Amor; Sandra, 2012).

The most commonly studied animal models of MS are the autoimmune experimental autoimmune/allergic encephalomyelitis (EAE) (Peireira, Cruz-Höfliing, Dertkigil, & Graça, 1996). The Virally induced models, mainly Theiler’s murine encephalomyelitis virus (TMEV) infection in mice and rats. (Deb et al., 2009; Gilden, 2005) and gliotoxin-induced models of demyelination, including the cuprizone, lysolecithin and Ethidium Bromide models that causes destruction of oligodendrocytes leading to demyelination (Graça & Blakemore, 1986).

Dogs, as animals, suffer from diverse neurological disturbances; not limited to the effects of injuries and trauma, nervous system disorders may include congenital defects, infections and inflammations, poisoning, metabolic disorders, nutritional deficiencies, degenerative diseases, or cancer (Amude, Alfieri, & Alfieri, 2010). Although Canine distemper virus (CDV) infection is an important etiological agent of demyelinating encephalomyelitis in dogs, holds a cross relation of MS etiology in humans and Distemper encephalitis was initially described as “acute MS of the dog” by human neuropathologists making them an ideal model for studying MS (Amude et al., 2010), but very few studies were conducted on dogs.

This work aimed to evaluate and study the demyelination process and the changes occurring in the spinal cord of the dog using a gliotoxin Ethidium Bromide to develop a model resembling clinical human-progressive MS.

**MATERIALS AND METHODS**

**Study Design:** Twelve dogs were used in this study, equally and randomly allocated in 4 groups according to the period of observation 3, 7, 14, 28 days. All dogs were subjected to a pre-study evaluation excluding any animals suffering from any nervous manifestations as paralysis, paraplegia, tremors, paresis, lameness, head tilts, etc...

Experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Cairo University. Approval ID#: CU/II/S/23/16.

**Induction:** Dogs were anesthetized using SC injection of atropine sulfate 0.05mg/kg; Then IV injection of a mixture of ketamine 10 mg/kg and Xylazine 1mg/kg. The anesthesia was maintained with 2.5% thiopental sodium administered IV. A dorsal midline incision was applied on the area to be exposed from T12 to L2 under aseptic precautions. Subcutaneous fat and fascia were incised until the dense lumbodorsal fascia was reached, the fascia and supraspinous ligament were incised around spinous process and, on the Midline, and the incision was deepened to the laminae to complete the midline muscle separation. Then the multifidus lumborum
muscle was sharply elevated from the spinous process and then bluntly elevated from the laminae laterally to the mammillary processes which was sufficient for exposure of the dorsal laminae. Then using a dental drill a bilateral hole was made through the dorsal lamina of L1 (Fig. 1) and a single injection of 20 µl of 0.1 % Ethidium Bromide was injected directly through each drilled hole into the spinal cord using a micro syringe enclosing a capillary tube (Blakemore, 1982). The lumbodorsal fascia was sutured at the dorsal midline. The subcutaneous fat, fascia, and skin were closed routinely. The animals were given a systemic course of antibiotics (Ceftriaxone® 1gm) for 5 days and daily dressing of the wound with antiseptic was applied.

Under general anesthesia MRI was performed using a 1.5 Tesla closed magnetic resonance unit. The spinal imaging protocol included sagittal and dorsal T2-weighted (TR/TE 2880/111 ms) and T1-weighted (TR/TE 623/1 ms), transverse T2-weighted (TR/TE 3290/99 ms) and T1-weighted (TR/TE 651/12 ms) and sagittal STIR (TR/TE/TI 3310/61/140 ms) sequences. The sagittal and dorsal spinal sequences were performed from T11 to L3 (vertebral body), and the transverse sequences used T11 to L3 (vertebral body). Animals were humanely euthanized at the end of each study period and the whole vertebrae (T11 to L3) were fixed in 10% Neutral Buffered Formalin for 24 hours then the spinal cord was extruded from the vertebra and divided into 2 samples and coronal sections of 1mm of the spinal cord were taken for histopathological evaluation using routine H&E stain on 10% Neutral Buffered Formalin as preservative and for electron microscopy on 5% gluteraldehyde (Graça & Blakemore, 1986; Kiernan, 2010). All samples were blindly examined to avoid any bias and photographed by SC30 Olympus® camera for H&E sections and by CCD digital camera Model XR- 41 for electron microscopic sections.

**Fig. (1):** The induction procedures of MS: a) drilling of the hole in the dorsal lamina of L1, b) the hole made after drilling exposing the spinal canal, c) the microneedle used to inject EB, d) injection of the EB in the lateral column of the spinal cord.

**Evaluation:** Changes in dogs gait were measured according to the corresponding score (Olby *et al.*, 2014) for the hind limb ataxia, tail movements and proprioception as shown in the following table.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>Paraplegic, no nociception.</td>
</tr>
<tr>
<td>1</td>
<td>Paraplegic plus nociception.</td>
</tr>
<tr>
<td>2</td>
<td>Paraplegic with voluntary tail wag.</td>
</tr>
<tr>
<td>3</td>
<td>Minimal non-weight bearing protraction of pelvic limb (movement of 1 joint)</td>
</tr>
<tr>
<td>4</td>
<td>Non-weight bearing protraction of pelvic limb with &gt; 1 joint involved &lt; 50% of time.</td>
</tr>
<tr>
<td>5</td>
<td>Non-weight bearing protraction of pelvic limb with &gt; 1 joint involved &gt; 50% of time.</td>
</tr>
<tr>
<td>6</td>
<td>Weight bearing protraction of pelvic limb &lt; 10% of time.</td>
</tr>
<tr>
<td>7</td>
<td>Weight bearing protraction of pelvic limb 10-50% of time.</td>
</tr>
<tr>
<td>8</td>
<td>Weight bearing protraction of pelvic limb &gt;50% of time.</td>
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Results

Clinical Evaluation:
Clinical manifestations appeared on day 2 after induction and were progressing over time (Fig. 2). After 3 days, animals showed full weight bearing all the time with ataxic gait which appeared mainly as lack of fine coordination as crossing of the pelvic limbs, standing on dorsum of foot, and skipping steps. The tail movement was normal, no change in sensation of the pelvic limbs and no changes in urination and defecation frequency was noticed. After 7 days, animals showed full weight bearing with clear ataxic gait mainly characterized by walking multiple steps on the dorsum of the foot, dragging the pelvic limbs and skipping steps. Tail movement began to weaken and delayed proprioception and nociception reflexes of the foot. Urination and defecation were normal. After 14 days, post induction animals showed weight bearing of the pelvic limb less than 50% of the time. Animals needed assistance to stand, were reluctant to move, with very weak reflexes and sensation of the foot. Urination and defecation frequency began to increase. After 28 days, animals showed non weight bearing of the pelvic limbs most of the time. The urination and defecation were completely uncontrolled and muscle atrophy began to appear in pelvic limbs but animals were able to move 1 joint at a time (Figure 3).

MRI Results:
MRI of the spinal cord lesions showed the appearance of hyperintense lesions on T2 weighted images on both sides of the spinal cord on axial scans and a diffuse hyperintense lesions on sagittal scans which began to appear from the 1st group (after 3 days’ post induction) and increased progressively at the 2nd group to greater areas of hypointensity. The 3rd and 4th groups showed large areas of hypointensity on T1 weighted images on axial scans referred to as black holes (Figure 3).

Histopathological Results:
3 days’ post injection of the Gliotoxin Ethidium bromide, spinal cord of the dogs showed a partial degree of demyelination appearing as remnants around the axons, Edema around blood vessels, and varying degrees of axonal damage appearing as axonal swelling and axonal degeneration and all sections showed low proliferation of glial cells. After 7 days’ post induction, areas of well circumscribed vacuolations appeared and white matter showed a higher degree of wallerian degeneration which appeared as complete demyelination around axons accompanied by axonal degeneration and higher proliferation of glial cells. After 7 days’ post induction, areas of well circumscribed vacuolations appeared and white matter showed a higher degree of wallerian degeneration which appeared as complete demyelination around axons accompanied by axonal degeneration and higher proliferation of glial cells. Few macrophages (Gitter cells) appeared by its foamy cytoplasm engulfing the degenerated myelin and axons leaving a clear vacuole. Neuronal damage was detected in the grey matter appearing as nuclear damage with chromatorhexis and chromatolysis and neuronophagia.

While after 14 days’ post induction, showed increase in vacuolated areas with large areas of wallerian degeneration, axonal swelling and axonal degeneration. Low proliferation of gitter cells and phagocytotic activity was observed with high degree of loss of architecture. And 28 days’ post induction, showed large areas mass demyelination and other areas of loss of architecture, vacuolation and axonal degeneration (Fig. 4).

Electron Microscopy Results:
Examination of the first group samples revealed that the glia cells either astrocytes or oligodendrocytes showed vacuolation with partial disintegration of its cell organelles in their processes. Few nerve fibers showed healthy myelin sheath as well as morphologically normal axonal appearance (1). Some nerve fibers showed splitting of the myelin lamella with variability in shape and size. The axons of these nerves showed shrinkage and partial separation from myelin lamellae. The second group samples showed that the processes of the glia cells had variable sized vacuolation. The myelin sheath of the nerve fibers showed many affections as complete destruction (D) or disintegration to splitting of the lamella forming spaces(s) in between. Vacuolation as well as shrinkage of the nerve axons were common (V). Also, some fibers especially those of destructed myelin were completely destructed (D).

The third group showed marked vacuolation of the processes (v) with partial necrosis or disintegration of the cell organelles (d) of glial cells. Nearly the myelin sheath of the all nerve fibers showed variable degree of affections from splitting of the myelin lamella forming spaces(s) variable in shape and size to complete destruction. Nearly all the nerve axons or neurofilaments of the nerve fibers showing pathological changes in form of marked shrinkage (2) and presence of multiple vacuoles(V). The fourth group showed that the processes of the astroglia and oligodendroglia with marked large sized vacuolations (v) as well as complete disintegration of the cell organelles(d). The myelin sheath of the nerve fiber was degenerated which appeared with low electron density. The axon of the nerve fiber showed vacuolation, fragmentation and degeneration.

Fig. (2): showing the progressive deficit in clinical manifestation according to the gait score analysis
Fig. (3): showing MRI findings a) axial section showing hyperintense lesions on both sides of the spinal cord represented by the arrows 3 days post induction of the disease; b) larger hyperintense lesions are present after 7 days post induction represented by the arrows. C) sagittal section showing large area of hyperintensity in the spinal cord (sclerotic plaque) 7 days post induction; d) axial section showing a hypointense lesion in the spinal cord represented by the arrow lateral to hyperintense lesion 14 days post induction; e) axial section showing larger hypointense lesion next to hyperintense lesion 28 days post induction. f) sagittal section showing hyperintense lesion in the spinal cord 28 days post induction.

Fig. (4): showing some of the histopathological pictures of the spinal cord of dogs where a) represents the lesions after 3 days’ post induction showing gliosis (arrow), partial demyelination and axon swelling. b) represents the lesions after 7 days post induction showing Wallerian degeneration, demyelination and appearance of multinucleated foamy gitter cells (arrow). c) represents the lesions after 14 days showing multiple vacuolated areas (arrows), axonal degeneration and and loss of architecture. d) represents the lesions after 28 days post induction showing severe loss of architecture and axonal degeneration (arrow). H&E Stain 200X, Bar 20 µm.
Fig. (5): showing the Transient Electron Microscopy analysis of the white matter where a) represents the first group samples showing normal axon and myelin sheath (1), other axons showed splitting of the myelin lamellae, axon separation from lamellar sheath (2), vacuolations (v), other areas showed disintegrated cell debris (d) and completely destructed axons (D) with remnants of myelin. Oligodendroglia (o) shows nuclear degeneration. b) represents the second group samples showing separation of the axons from the myelin sheath (s) and multiple vacuolations (v). c) represents the third group showing axonal shrinkage (2) with separation of the myelin sheath (s) and disintegration of the cell processes and vacuolations (v). d) represented the fourth group where all the axons appeared destructed and disintegrated with large vacuolations.

DISCUSSION

Demyelinating lesions induced by intraspinal injection of gliotoxins have been studied for many years in small animals as rats and mice (Graça and Blakemore, 1986; Woodruff and Franklin, 1999; Fernandes et al., 2002; Bondan et al., 2009; Kuypers et al., 2013) in order to gain insights into reasons for failure of remyelination and to improve understanding of the axonal conduction disorders in MS (Abdel-Salam, Khadrawy, & Mohammed, 2012; Kuypers et al., 2013) and developing a similar model system in large animals would be extremely useful. Demyelinating disorders in dogs were widely reported (Gough, 2004; Kortz et al., 1997; Levine, Budke, Levine, Kerwin, & F, 2008; Millán et al., 2010; Miller et al., 2009) as dogs are affected by congenital and hereditary hypomyelinating diseases, toxic demyelination, nutritional demyelination and axonopathy, and viral demyelination (Amude et al., 2010; Vandevelde & Zurbriggen, 2005) which holds a great correlation of the pathological picture and some reported etiology of human MS. Other diseases related to MS pathology is old dog encephalitis which affects many dog breeds ranging 3-4 years of age (Adams et al., 1975). EB was used to induce focal areas of demyelination by direct injection into the spinal cord. It was selected as the gliotoxic agent in this experiments because the demyelination it induces is delayed from the time of injection (Graca & Blakemore, 1986; Mothe & Tator, 2008; Yajima & Suzuki, 1979), thereby potentially more clearly separating the effects of demyelination from the effects of trauma inherent to the injection procedure. In order to define more clearly the effects of demyelination and remyelination in this system. EB intercalates with nucleic acids (Bondan et al., 2009) and although cells show signs of intoxication soon after exposure most retained their integrity for at least 7 days in the present lesions. Thus, intoxicated oligodendrocytes will support their myelin sheaths despite inhibition of nucleic acid and protein synthesis and although astrocytes will retain their structure, they would not be able to respond to external stimuli because of inhibition of nucleic acid and protein synthesis. The results clearly demonstrated that EB injection at the spinal cord of dogs led to decrease
in locomotor efficiency which began to appear 3 days after injection represented by imbalance and incoordination and walking on the dorsum of the foot and decrease in proprioception reflexes. Clinical signs continued to aggravate throughout time to reach a nearly paraplegic state after 28 days. These findings agrees with previous studies (Graça & Blakemore, 1986; Woodruff & Franklin, 1999; Yajima & Suzuki, 1979) stating that EB intraspinal injection leads to glial intoxication beginning 24 hours post-injection but large scale demyelination does not occur until 7-14 days post-injection. Other studies also stated that the initial clinical signs of locomotor deterioration were attributed to the direct trauma and acute intoxication followed by the injection of the EB whereas the clinical effects following 11-14 days were attributed to the demyelination and consequent axonal degeneration (Graça & Blakemore, 1986).

The MRI results showed bilateral hyperintensity at the sites of the injection of the spinal cord after 3 days and increased to reach all the lateral columns after 7 days which confirms that EB injection leads to intoxication and inflammation (Lycklama et al., 2003). While the other groups showed the appearance of hypointense lesions which was more attributed to axonal degeneration and demyelination. These findings comes in closer correlation with the normal course of MS progressive lesions where consequent axonal damage was observed following active demyelination and referred to in MRI as the Black holes of MS (Charil et al., 2006; Pirko, Nolan, Holland, & Johnson, 2008).

The histopathological picture showed high resemblance to the progressive MS pathology where primary gliosis followed by demyelination and axonal swelling and aggravated to axonal degeneration and vacuolation and loss of architecture. these findings are similar to those shown in cats and rats (Graça & Blakemore, 1986; Jeffery & Blakemore, 1997) but differs that the axonal degeneration and vacuolation was extensive with very low macrophage attacks and remyelination didn’t occur until 28 days. The electron microscopy pictures were confirmative and explanatory to the histopathology, the clinical and radiological findings, where EB injection showed signs of intoxication to oligodendroglia and astrocytes with separation of the myelin sheath that showed splitting of its lamellae which agrees with all the previous studies in rats and cats (Graça & Blakemore, 1986; Jeffery & Blakemore, 1997) stating that the myelin sheath was transformed into lattices of membranous profiles that persisted around axons leading to delayed macrophage attack and clearance of myelin debris. This observation indicates that lattices develop and persist when myelin sheath breakdown occurs in the absence of, or fails to induce, a normal macrophage response following the death of oligodendrocytes (Graça & Blakemore, 1986). These lesions became more extensive in later groups with large areas of vacuolation and disintegration of glial cells. On the contrary of most of the studies of toxic demyelination (Blakemore & Franklin, 2008; Crang & Blakemore, 1991; Fushimi & Shirabe, 2002) in rats and cats, not only macrophage activation and rapid processing of myelin was very low in all groups but also remyelination was not observed until the last group neither by Schwann cells nor oligodendrocytes which was a unique feature of demyelination in dogs but rather extensive vacuolation, axonal degeneration and destruction was more prominent. This might be due to the absence of macrophage activation that stimulates the migration of Schwann cells from peripheral nerve roots and blood vessels, the toxic effect on astrocytes that induce oligodendrocyte progenitor cells proliferation and the toxic effect on the mature oligodendrocytes and oligodendrocyte progenitor itself. The pathological picture of the last group confirmed the clinical signs of this group where dogs reached a state of paraplegia where most of the axons appeared destructed and degenerated.

CONCLUSION

Using ethidium bromide induced demyelination in dogs’ spinal cord the fore mentioned results demonstrate that dogs offer an alternative model of toxic demyelination with unique clinical, radiological and pathological pictures when compared to other animal models that reaches a more progressive form of Multiple Sclerosis where spontaneous remyelination was not observed till 28 days post induction.
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REFERENCES


