Ethidium Bromide Induced Spinal Cord Demyelination in a Dog a model of Multiple Sclerosis

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Multiple sclerosis (MS) is a multifocal demyelinating disease that leads to irreversible neurodegeneration. Over the past several decades, many animal models were developed in order to understand variety of aspects of MS. Although Canine Distemper encephalitis was initially described by human neuropathologists as "acute MS of the dog" making them an ideal model for studying MS, yet still very few studies were conducted on dogs. This work aimed to develop a model resembling clinical human-progressive MS where Ethidium bromide induced demyelination in dogs' spinal cord is experimented for the first time.

All animals received intraspinal injection of 20 $\mu$l of 0.1 % Ethidium bromide in the lateral columns using a microneedle syringe attached to a capillary tube. All animals were observed for clinical evaluation and gait analysis, MRI of the spinal cord and electron microscopy. 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 disease characterized by demyelination of the central nervous system (CNS) (Pachner, 2011). It may cause 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, MS destroys oligodendrocytes, which are the cells responsible for creating and maintaining the myelin sheath, which helps the neurons carry electrical signals. MS results in a thinning or complete loss of myelin and, less frequently, transection of axons (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, and 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 and 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 active MS tissue samples, and the modification of experimental circumstances is much more restricted in human studies compared to studies in animal models. For these reasons, animal models are needed to clarify the underlying pathological mechanisms and test novel therapeutic and reparative approaches (Johnson & Rodriguez, 2011). Over the past several decades, a number of animal models have been developed in order to understand multiple aspects of MS (Star, Vogel, Kipp, & Amor; Sandra, 2012). The most commonly studied animal models of MS are the purely autoimmune experimental autoimmune/allergic encephalomyelitis (EAE) (Peireira, Cruz-Höfling, Derlkigil, & Graça, 1996). Viral induced models, mainly Thelier’s murine encephalomyelitis virus (TMEV) infection and consequent chronic demyelination (Deb et al., 2009; Gilden, 2005) and toxin-induced models of demyelination, including the cuprizone model, focal demyelination induced by lyso-phosphatidylcholine (lysolecithin) and Ethidium Bromide model that causes destruction of Glia cells’ DNA leading to demyelination (Graça & Blakemore, 1986).

Dogs, as animals, suffer from a lot of nervous system injuries; in addition to the effects of injuries and trauma, nervous system disorders may include birth 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 close 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 the primary findings of 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 to be further studied for therapeutics evaluation.

MATERIALS AND METHODS

Study Design

Ethical Approval: We confirm that our methods were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. With the least number possible of animals and that the approval sheet from the Institutional Animal Care and Use Committee (IACUC) ID: CU/II/S/23/16, can be offered upon request.

Twenty-four dogs were used in this study (2-4 years of age and of both sexes), equally and randomly allocated in 2 groups:

Group 1: “Sham operated Control” where animals underwent the surgical procedures and injected normal saline

Group 2: Induction of demyelination lesions group using Ethidium bromide

Each group was then subdivided into 4 equal subgroups according to the period of observation 3, 7, 14, 28 days’ post induction. 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...

Induction of Demyelinating lesions

Dogs were anesthetized using an I/V mixture of injectable ketamine/xylazine/thiopental

A dorsal midline incision was applied on T12 to L2. 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. The incision was deepened to the laminae to complete the midline muscle separation. Then the multifidus lumborum muscle was elevated from the spinous process and bluntly moved laterally from the laminae to the mammillary processes which was sufficient for exposing the dorsal laminae. Using a dental drill, two bilateral holes were made through the dorsal lamina of L1 (Fig. 1). A single injection of 20 µl of 0.1 % Ethidium Bromide was injected into the spinal cord using a micro syringe enclosing a capillary tube (Blakemore, 1982) a. The lumbodorsal fascia was sutured at the dorsal midline. The subcutaneous fat, fascia, and skin were closed routinely. Animals were given a systemic course of antibiotics (Ceftriaxone® 1gm) for 5 days, no anti-inflammatories or analgesics were given to avoid interference with the clinical
evaluation and daily dressing of the wound with antiseptic was applied.

**Clinical Evaluation**

Changes in dogs gait were measured according to (Olby et al., 2014) standard score for the hind limb ataxia, tail movements and proprioception of the dogs.

**Magnetic Resonance**

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).

**Histopathology and electron microscopy**

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%glutaraldehyde (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.

**RESULTS**

**Clinical evaluation**

All animals tolerated the surgeries well and showed full weight bearing the day after surgery. Control group animals showed little or no clinical manifestations noticed from day 2 after surgery appearing as mild ataxia of the hind limb which was restored on day 3 to normal gait and no other observations were noticed till the end of the study period with normal gait, normal reflexes and normal defecation and urination. On the other hand, Clinical manifestations of the EB induction group progressively appeared from day 2 onward (Fig. 1).

![Gait Score Analysis](image)

**Figure 1**: showing the progressive deficit in clinical manifestation according to the gait score analysis. Data expressed as mean values.
Figure 2: showing some of the clinical manifestations appearing on dogs of the second group after induction of the disease: a) crossing the limbs and incoordination between the thoracic and pelvic limbs; b) stepping on the dorsum of the foot; c) No proprioception reflex, walking on the dorsum of the foot and dragging the limb; d) limb crossing and false steps; e) pelvic limb dragging and walking on the dorsum of the foot; f) inability to bear weight with limbs crossing and incoordination. g) and h) non-weight bearing with minimal movements of the limbs and reflexes were nearly absent with marked urinary incontinence.

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 ataxic gait 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 2).

MRI Results

MRI of the spinal cord of the control group showed bilateral hyperintense lesions on T2 weighted images on axial scans and a diffuse hyperintense lesion on sagittal sections as syringomyelia 3 days’ post induction, while after 7 days MRI scan of the spinal cord showed normal intensity on both axial and sagittal scans with no abnormal findings in latter subgroups after 14 or 28 days. The second group 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 subgroup (after 3 days’ post induction) and increased progressively at the 2nd subgroup to greater areas of hypointensity. The 3rd and 4th subgroups showed large areas of hypointensity on T1 weighted images on axial scans referred to as black holes (Figure 3).
Figure 3: showing some of the axial MRI findings of the spinal cord at L1 level where (a-b) represents the control group and (e-h) represents the induction group: a) showing bilateral hyperintense lesions represented by the yellow arrow after 3 days; b) showing very mild hyperintensity of the spinal cord section after 7 days; c-d) showing normal intensity of the spinal cord sections after 14 and 28 days respectively; e) axial section showing hyperintense lesions on both sides of the spinal cord represented by the arrows 3 days’ post induction of the disease; f) larger hyperintense lesions are present after 7 days’ post induction represented by the arrows; g) axial section showing a hypointense lesion in the spinal cord represented by the yellow arrow lateral to hyperintense lesion 14 days’ post induction; h) axial section showing larger hypointense lesion next to hyperintense lesion 28 days’ post induction.

**Histopathological Results**

The histopathological analysis of the control group sections of the spinal cord showed mild gliosis after 3 days with normal axons and myelin sheath while after 7 days’ spinal cord sections were normal and this picture remained at the other subgroups after 14 and 28 days. On the Contrary, 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 with marked astrocytic swelling. 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).
Figure 4: showing some of the histopathological pictures (H&E Stain 200X) of the spinal cord white matter of dogs where (a-d) represents the control group and (e-h) represents the induction group: a,b) represents the lesions after 3 and 7 days respectively showing mild gliosis and normal axon architecture and myelin sheath; c,d) represents the lesions after 14 and 28 days respectively showing normal histopathology of the spinal cord; e) represents the lesions after 3 days' post induction showing severe diffuse microgliosis. f) represents the lesions after 7 days post induction showing Wallerian degeneration, demyelination and appearance of multinucleated foamy gitter cells. g) represents the lesions after 14 days showing multiple vacuolated areas, axonal degeneration and loss of architecture. h) represents the lesions after 28 days post induction showing severe loss of architecture and axonal degeneration. H&E Stain 200X.
Figure 5: showing the Transient Electron Microscopy analysis of the white matter where (a-d) represents the control group and (e-h) represents the induction group; a,b) shows normal compact myelin sheath around axons after 3 and 7 days respectively; c,d) shows normal myelin sheath and axons after 14 and 28 days respectively e) 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 after 3 days. f) showing separation of the axons from the myelin sheath (s) and multiple vacuolations (v) after 7 days; g) showing axonal shrinkage (2) with separation of the myelin sheath (s) and disintegration of the cell processes and vacuolations (v) after 14 days. d) all the axons appeared destructed and disintegrated with large necrotic vacuolations.
Electron microscopy results

Examination of the Induction group samples revealed that the glia cells either astrocytes or oligodendrocytes showed vacuolation with partial disintegration of its cell organelles in their processes after 3 days post induction. 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 subgroup samples showed that after 7 days, 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 subgroup showed that after 14 days marked vacuolation of the processes (v) with partial necrosis or disintegration of the cell organelles (d) of glial cells was noticed in all samples. 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 subgroup showed that the processes of the astroglia and oligodendroglia had marked large sized necrotic vacuolations (v) as well as complete disintegration of the cell organelles(d). The myelin sheath of the nerve fiber were degenerated which appeared with low electron density. The axon of the nerve fiber showed necrotic vacuolation, fragmentation and degeneration. Compared to the control group all samples showed normal axons, myelin sheath and healthy glia cells in all subgroups (Fig. 5).

DISCUSSION

Demyelinating lesions induced by intraspinal injection of gliotoxins have been studied for many years in small animals as rats and mice (Bondan, Custódio, Lallo, Bentubo, & Graça, 2009; Fernandes et al., 2002; Graça & Blakemore, 1986; Kuypers, James, Enzmann, Magnuson, & Whittemore, 2013; Woodruff & Franklin, 1999) 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 in large animals would be extremely useful.

Dogs were chosen as model for many reasons: first; 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.

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 surgical operation using only dental drilling instead of dorsal laminectomy showed technical efficiency with minimal traumatic effect on the spinal cord. And this was supported by the clinical and radiological and histopathological evaluation. Also, no analgesics or anti-inflammatory were given after surgery to avoid any interference on the clinical manifestations of the dogs’ gait.

The results clearly demonstrated that EB injection at the spinal cord of dogs led to decreased locomotor efficiency which began to appear 3 days after injection represented by imbalance, incoordination, walking on the dorsum
of the foot and decreased 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) that referred the symptoms to glial intoxication that started 24 hours post-injection reaching its maximum 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) but this was excluded by our control group results.

The MRI results showed bilateral hyperintensity at the sites of the injection of the spinal cord after 3 days in both control and induced animals and increased to reach all the lateral columns after 7 days in induced animals which confirms that EB intoxication causes inflammation (Lycklama et al., 2003) while the control groups the inflammation regressed and was not noticed till the end of the study. While the later induced subgroups showed the appearance of hypointense lesions which was more attributed to axonal degeneration and demyelination. These findings comes in close 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 is followed by demyelination, axonal swelling and worsen to axonal degeneration, vacuolation and loss of architecture. These findings are similar to those shown in cats and rats (Graça & Blakemore, 1986; Jeffery & Blakemore, 1997) except that the axonal degeneration and vacuolation were extensive with very low macrophage attacks and the remyelination didn’t occur until 28 days.

The electron microscopy pictures were confirmative and explanatory to the histopathologic, 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. the myelin sheath is said to have transformed into lattices of membranous profiles that persisted around axons leading to delayed macrophage attack and clearance of myelin debris. These observations 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 subgroups with large areas of necrotic vacuolation and disintegration of glial cells.

Interestingly, these lesions opposed most of the studies of toxic demyelination in small animals (Blakemore & Franklin, 2008; Crang & Blakemore, 1991; Fushimi & Shirabe, 2002), not only macrophage activation and rapid processing of myelin was very low but also remyelination was not observed until the last subgroup neither by Schwann cells nor oligodendrocytes but rather extensive vacuolation, axonal degeneration and destruction was more prominent.

This was a unique feature of demyelination in dogs, and 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. These points need further investigations to be addressed in the future to fully study this model.

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.

CONFLICT OF INTEREST
The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS
Ashraf A. Shamaa and Omar S. El-Tookhy carried out surgical preparation, the surgical procedures, post-operative care, follow up, clinical and radiological evaluations.
Ahmed N. Abdallah was responsible for the histopathological evaluations. Each author wrote the work draft belonging for him. All authors read and approved the final manuscript.

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REFERENCES


