Progressive model of multiple sclerosis following ethidium bromide injection in dogs’ spinal cord: failure of endogenous remyelination

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This paper evaluated the histopathological picture and pathological pathways of intoxication and apoptosis of oligodendrocytes and astrocytes, oligodendrocyte precursor cell migration and proliferation, and axonal viability of the dogs’ model of Multiple Sclerosis (MS).

Methodology/Principal
Twenty-four dogs divided into 2 groups. All animals received intraspinal injection of 20 µl of 0.1 % Ethidium bromide in the lateral columns using a micro needle syringe attached to a capillarytube. Spinal cord specimens of all animals were examined for routine histopathology, Toluidine blue semithin sections, Immunohistochemical analysis against Bax, Caspase 9, Myelin Basic Proteins, OLig2 and Glial fibrillary acidic proteins. Results showed marked differences in the Dogs’ spinal cord damage and demyelination leading to a more progressive picture of MS model than small animals with high levels of apoptosis as observed by elevated Bax and Caspase 9 markers, a very high astrocytic reaction marked by GFAP and impaired proliferation and differentiation of Olig2 Oligodendrocyte precursor cells into mature oligodendrocytes with extensive axonal degeneration and vacuolation and remyelination was not observed until 28 days post induction. Conclusions/Significance: This model offers unique features, where a progressive form of demyelination and apoptosis and glial scar are different from those of other animal models and access to oligodendrocytes precursor cells was restricted and no spontaneous remyelination was observed. These finding support that dogs provides an alternative model studying progressive forms of MS and new treatment modalities.

Keywords: Demyelination; Dog Model; Ethidium Bromide; Spinal Cord; Multiple Sclerosis.

INTRODUCTION
Multiple sclerosis (MS) is a progressive and recurrent disease affecting the Central Nervous System (CNS) which includes the brain and the spinal cord. It damages the myelin sheath, the material that surrounds and insulates the nerve cells. This damage slows down or blocks messages between the brain and the body, leading to a wide range of symptoms varying from mental, cognitive and muscular disturbances (Compans & Cooper, 2008).

Ethidium bromide (EB) has been extensively used in the mice, rats and cats spinal cord to induce demyelination as a model of progressive MS. EB was also used to induce focal areas of demyelination. EB was selected as the gliotoxic agent in these experiments because the demyelination it induces is delayed from the time of injection (Graça & Blakemore, 1986; Johnson & Rodriguez, 2011; 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, Custódio, Lallo, Bentubo, & Graça, 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 supported their myelin sheaths despite the inhibition of nucleic acid and protein synthesis and although astrocytes retained their structure they would not be able to respond to external stimuli because of inhibition of nucleic acid and protein synthesis.

However, this lesion has not been addressed to a larger animal like dogs, a model with unlimited genetic potential. In our previous publication, we showed that EB induced demyelination in dogs showed unique clinical, radiological and pathological features different from those of rats and cats and these findings support that dogs provide an alternative large model for studying progressive MS (Abdallah, Shamaa, El-tookhy, & El-mottaleb, 2017). Where extensive demyelination and axonal loss without signs of remyelination was observed and animals reached a paraplegic state clinically 4 weeks post induction of MS. All this relates to paper-1 (accepted after publication). Over the past decade, increasing evidence has attributed the failure of axonal regrowth after spinal cord injury to limited intrinsic neuronal plasticity and the local non-permissive microenvironment including myelin-associated growth inhibitors as well as the glial scar (Yuan & He, 2013). As well as the potential presence of an active proliferating pool of endogenous Stem/Progenitor cells to recuperate and regenerate the damaged axons and glial cells (Fushimi and Shirabe, 2002).

In this paper, we focused on the histopathological picture and pathological pathways of intoxication and apoptosis of oligodendrocytes and astrocytes, oligodendrocyte precursor cell migration and proliferation, and axonal viability of the Dog model of MS.

**MATERIALS AND METHODS**

**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 our institutional committee ID: CU/I/S/23/16, can be offered upon request.

**Study Design:**

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

- **Group 1:** Sham operated control where animals underwent the surgical procedures and injected normal saline.
- **Group 2:** Induction of toxic demyelination using Ethidium bromide.

Each group was subdivided into 4 subgroups according to the period of observation 3, 7, 14, 28 days. Experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) ID: CU/S/23/16.

Under general anesthesia, bilateral holes were drilled using a dental drill in the dorsal lamina of the 1st lumbar vertebra. An amount of 20 µl of 0.1 % Ethidium bromide was injected in the lateral columns of the spinal cord using a microneedle syringe attached to a capillary tube (BLAKEMORE, 1982).

Animals were sacrificed at days 3, 7, 14 and 28 post injection, and coronal sections of 1 mm of the spinal cord were taken for histopathology and immunohistochemistry were fixed on neutral buffered formalin (Graça & Blakemore, 1986; Kiernan, 2010).

**Histopathological evaluation (Bancroft, 2008):**

The tissue samples were embedded in paraffin blocks, were sectioned into 10-µm-thick sections. Sections were brought to distilled water then Stained with the alum haematoxylin for 5 mins and rinsed in running tap water. Stain with eosin for 2 mins then dehydrated, cleared and mounted. And sections were examined using light microscopy.

**Semithin sections preparation (Hayat, 1990):**

5 – 10 small pieces 1X1 mm in size were taken immediately after dissection from each spinal cord specimen and Fixed in 5 % cold glutaraldehyde for 24-48 hrs. The specimens were then washed in cacodylate buffer (PH 7.2) for 20 minutes and washing was repeated 3-4 times. Samples were post fixed in 1% O₄S₄ for 2 hrs, then washed in the same buffer four times.

Dehydration by ascending grades of alcohol (30, 50, 70, 90 and 100% for 2 hrs.) of each specimen was operated then embedded in epon – araldite mixture. From the embedded blocks semithin sections (0.5 – 1 microns) by LKB® ultramicrotome were stained using toluidine blue and prepared for examination of the tissue and photographed by SC30 Olympus® camera.
Immunohistochemistry (Gilmore et al., 2009):

Antibodies used for immunohistochemistry were Anti-Bax antibody [E63] (ab32503, Abcam®), Anti-Caspase-9 antibody (ab52298 Abcam®), Anti-GFAP antibody (ab7260, Abcam®), Anti-Myelin Basic Protein antibody (ab40390, Abcam®), Anti-Olig2 antibody (ab136253, Abcam®) with hematoxylin as counter stain.

All sections were deparaffinized, and antigen retrieval was performed in 0.01 Km Tris-EDTA (Sigma-Aldrich; pH 9.0) in a benchtop autoclave for 5 minutes. Sections were pretreated with 10% fetal calf serum (Invitrogen, Dublin, Ireland)/TBS (Sigma- Aldrich) for 20 minutes at room temperature. All antibodies were then incubated on sections overnight at 4°C and detected using peroxidase-labeled anti-rabbit secondary antibodies (Dako) with diaminobenzidine (Dako) as chromogen.

RESULTS

Histopathology:

Histopathological evaluation of the spinal cord of the EB injected dogs revealed pathological deterioration and degeneration over time compared to control ones. The control group animals showed only mild astrogliosis after 3 days and the later subgroups showed normal histopathological picture of the white matter (Figure 1). On the contrary, the induced groups showed a different picture where after 3 days the spinal cord showed signs of inflammation and focal areas of gliosis, mild demyelination with preservation of axonal structure. After 7 days signs of axonal swelling were marked accompanied with demyelination and astrocytic edema with low gliosis. After 14 days, multiple areas of circumscribed vacuolation were observed in all animals with a severe degree of demyelination and marked axonal degeneration and fragmentation with mild attack with macrophages engulfing the degenerated myelin appeared with foamy cytoplasm leaving a clear vacuole. After 28 days, a marked loss of architecture was observed with large vacuolated areas and axonal degeneration and moderate gliosis (Figure 2).

Semithin section analysis:

Toluidine blue stained sections were used to evaluate the extent of demyelination and axonal loss. Semi-thin section of the white matter showed variation in cross section diameters of nerve fibers. All fibers were myelinated with varying staining affinities where the large axons appeared deeply myelinated while the smaller ones appeared faintly myelinated in all animals of the control group. At 3 days post EB injection in the induced group, most of the nerve fibers were myelinated with varying staining affinities with normal axons and after 7 days post induction, some nerve fibers showed vacuolation or splitting of the myelin lamella with shrinking of their axons. The third subgroup after 14 days, Semi-thin sections of the white matter showed the nerve fibers diffusely demyelinated and showed vacuolation or splitting of the myelin lamella as well as shrinkage of the nerve axons. The fourth subgroup, after 28 days, the white matter showed marked deformity of the nerve fibers with extensive demyelination and axonal degeneration, shrinkage, folding, splitting and/or necrotic vacuolation. Some of nerve fibers were completely destructed (Figure 3).

Immunohistochemistry:

Immunostained sections of the induced group’s spinal cord showed mild reaction to Bax antibodies for detection of apoptosis after 3 days compared to the same period of the control group and increased highly after 7 days while it began to decrease again after 14 days and nearly no reactivity was observed after 28 days (Figure 4). Sections stained for the proapoptic marker protein Caspase 9 showed marked reactivity in all groups beginning from 3 days till 28 days in the glia cells and around axons compared to the control group sections which showed no reaction to the marker (Figure 5). The Myelin basic proteins were still present after 3 days and began to decrease as demyelination was ongoing through time to appear only at the peripheries of the white matter and completely disappear after 28 days (Figure 6) on the contrary of the control group which showed normal myelin proteins around axons throughout the study period. Oligodendrocyte progenitor cells were detected using Anti-Olig 2 sections and were only present diffusely at the first group after 3 days and was absent afterward in all animals throughout the 28 days compared to control samples (Figure 7).
Figure 1: showing the histopathological picture of the control group where a) represents the lesions after 3 days showing only mild astrogliosis; b-d) represents the lesions after 7, 14 and 28 days respectively showing normal histopathological picture.

Figure 2: showing the histopathological evaluation of the spinal cord of dogs where a) represents the lesions after 3 days showing mild demyelination with preservation of architecture; b) represents the lesions after 7 days where Wallerian degeneration began to appear with increased demyelination and axonal swelling; c) represents the lesions after 14 days showing severe demyelination with multiple circumscribed vacuolations and axonal degeneration; d) represents the lesions after 28 days showing complete loss of architecture with large vacuolations and axonal degeneration.
Figure 3: showing the light microscopic analysis of the semi-thin sections of the white matter: (a-d) represents the control group showing normal myelin and normal axons with different diameters and staining affinities after 3, 7, 14 and 28 days respectively; (e-h) represents the sections of the induced group where e) represents the white matter of the first subgroup showing variation in myelination affinity, f) the white matter of the second subgroup characterized by separation of the myelin sheath and splitting of its lamellae, g) the white matter of the third subgroup showing demyelination of the axons and separation of the myelin sheath axonal degeneration, h) the white matter of the fourth group showing extensive axonal distortion, demyelination and vacuolation.

Figure 4: showing immunohistochemical analysis of anti Bax stained sections of the spinal cord where (a-d) represents the control group 3, 7, 14 and 28 days respectively showing minimal Bax reaction; (e-h) represents the lesions of the induced group where e) represents the lesions after 3 days showing moderate increase in Bax positively stained cells; f) represents the lesions after 7 days showing marked diffuse increase of Bax reactivity; g) represents the lesions after 14 days showing focal areas of Bax reactivity; h) represents the lesions after 28 days showing mild to no Bax reactivity. Immunoperoxidase 400X.
Figure 5: showing the immunohistochemical analysis of the Caspase 9 stained section where (a-d) represents the control group showing no reaction of Caspase 9 marker after 3, 7, 14 and 28 days respectively; (e-h) represents the induced group where e) represents the lesions after 3 days showing a marked reactivity at most of the glial cells; f) represents the lesions after 7 days showing marked reactivity of the glial cells; g) representing the lesions after 14 days showing persistence of the reactivity at the glial cells and remained till 28 days as represented by h) with marked loss of architecture. Immunoperoxidase 400X.

Figure 6: showing the immunohistochemical analysis of Anti Myelin Basic Protein where (a-d) represents the control group samples showing normal expression of Myelin Basic Protein 3, 7, 14 and 28 days respectively and (e-h) represents the samples of the induced group where e) represents the lesions after 3 days showing high reaction of around axons; f) represents lesions after 7 days, showing faintly stained Myelin at the white matter; g) represents lesions after 14 days showing very mildly stained myelin proteins and restricted to peripheries; h) represents lesions after 28 days, showing very low reaction of Myelin Basic protein. Immunoperoxidase 400X.
Figure 7: showing immunohistochemical analysis against Olig-2 marker where (a-d) represents the samples of the control group showing normal expression of Olig-2 positive cells after 3, 7, 14 and 28 days respectively and (e-f) represents the samples of the induced group where e) represents the lesions after 3 days showing mild reaction at the periphery of the white matter while it was nearly absent after 7 days (f) and 14 days (g) and 28 days (h). Immunoperoxidase 400X.

Figure 8: showing the immunohistochemical analysis against GFAP where (a-d) represents the samples of the control group showing normal expression of GFAP after 3, 7, 14 and 28 days respectively and (e-f) represents the samples of the induced group where e) represents the lesions after 3 days showing high expression of GFAP; f) represents the lesions after 7 days showing increase in reaction and strongly increased after 14 days (g) and 28 days (h). Immunoperoxidase 400X.
Reaction of astrocytes to injury was detected using Anti-Gliai fibrillary acidic protein (GFAP), and sections showed marked increase in the reaction of astrocytes beginning from 3 days to slightly increased after 7 days and markedly increased after 14 and 28 days compared to minimal reaction in the control group samples (Figure 8).

DISCUSSION:

MS is a chronic disease of CNS characterized by inflammation and demyelination the CNS. Major advances have been made in unravelling the mechanisms of the inflammatory and neurodegenerative processes underlying the disease. In order to develop efficient therapies that can halt the disease progression. A number of different animal models of MS contributed not only to a better understanding of MS pathology but also explained fundamental pathophysiological concepts (Dendrou, Fugger, and Friese, 2015). The dog model offers an alternative large animal model of spinal cord injury (SCI) and demyelination where new features of degeneration were observed and new treatment modalities needs to be evaluated.

This study showed a histopathological degeneration that worsens over time characterized by demyelination and axonal loss and large vacuolations or scars began to appear, these findings came in accordance with other animal models like rats and mice (Graça & Blakemore, 1986; Kuypers, James, Enzmann, Magnuson, & Whittemore, 2013) where EB injection in the spinal cord showed demyelination and axonal injury but were more severe and no spontaneous remyelination was observed on the contrary of the other models.

Also, severe axonal destruction and axonal loss were observed. To confirm the demyelination process the Myelin Basic proteins were evaluated and showed a marked decrease over 28 days confirming the death of oligodendrocytes and absence of spontaneous remyelination while studies conducted on mice showed that the damage to the oligodendrocytes and demyelination began at 15 hours post induction and lasted till 7 to 10 days (Sallis et al., 2006) and remyelination began to appear.

Apoptosis, as demonstrated by nuclear DNA fragmentation and caspase activation, was a prominent feature in the spinal cord post injection of EB (Rao, Venkata, & Dasari, 2011). After SCI, some cells at the lesion site die by post-traumatic necrosis, whereas others die by apoptosis. Apoptotic cell death was observed in both neurons and oligodendrocytes and was prominent in the white matter, in which wallerian degeneration was simultaneously observed (Rajewski, Eed, and Ietrich, 2001). Thus, apoptosis of both neurons and oligodendrocytes may contribute greatly to the paraplegic state observed in our first study (Abdallah et al., 2017). The apoptosis factors Bax and Caspase 9 were observed in high reactivity after 3 and 7 days and the Bax decreased till 28 days but the executioner Caspase 9 was very prominent till 28 days explaining the mass destruction that was observed after 28 days. This agreed with a time course analysis in rats revealed that apoptosis occurred as early as 4 hours’ post injury and could be seen in decreasing amounts as late as 3 weeks after SCI (Zhang, Yin, Xu, Wu, and Chen, 2012). After SCI, caspase activation occurs in neurons at the injury site within hours, and in oligodendrocytes adjacent to, and distant from, the injury site over a period of days (Springer E., Azbill D., and Knapp E., 1999). The long-term neurologic deficits after spinal cord trauma may be due in part to widespread apoptosis of neurons and oligodendroglia in regions distant from and relatively unaffected by the initial injury (Martin & Liu, 2002).

It was postulated that the functional recovery after SCI is not simply a significance of the mechanical destruction of tissue, but also ascribed to the appearance of complex secondary events leading to the regenerative failure of injured axons and formation of a glial scar. The glial scar consists mainly of reactive astrocytes that arrest axon regeneration by secretion of chondroitin sulfate proteoglycans which chemically arrest the regrowth (Silver & Miller, 2004). The glial scar formation took place due to astrocyte migration in the area of the lesion. The proliferative action of astrocytes depends on: actin cytoskeleton mediated by the small GTPases, integrity of the intermediate filament including vimentin and glial fibrillary acidic protein (GFAP), cell adhesion (Menet, Prieto, Privat, & Giménez y Ribotta, 2003), and water invasion through the cells’ plasma membrane (Okada et al., 2006). Our results showed marked reaction of astrocytes appearing as a highly proliferating network expressing GFAP especially around the destructed and vacuolated areas after 28 days. The increase in GFAP was attributed to the alterations in the intermediate filament cytoskeleton which have been occupied in astrocyte motility. Investigators had stated that...
active astrocytes, which display abnormal morphology and severely compromised motile behavior (Lepekhin et al., 2001).

On the other hand, the major contributor to the remyelination process, the oligodendrocyte progenitor cells (OPCs) was found to be respond to SCI rapidly by altering their morphologies and accelerating mitosis. Overall, the proliferation rate of OPCs would significantly increase by the end of the first day after injury and remain elevated in the following week (Li and Leung, 2015). Our results showed mild proliferation of Olig2 expressing OPCs after 3 days post induction which declined rapidly at 7 days to be unobserved till 28 days explaining one of the reasons causing the failure of remyelination and this rapid decline in proliferating OPCs might be explained as the result of the formed glial scar from reactive astrocytes that hinder not only the recruitment and proliferation of OPCs but also create a hostile environment for OPCs differentiation and oligodendrocytes maturation (Li and Leung, 2015). Paradoxically, OPCs themselves may also partake in scar formation, suggesting a dual role of OPCs after SCI. Other studies showed that failure of proliferation of OPCs might be the toxic effect of EB itself on OPCs which was observed in mice as a marked decrease of OPCs 22 hours post injection of EB into the spinal cord of rats (Levine & Reynolds, 1999) but the latter study showed that 2 weeks later OPCs were able to proliferate, respond to the injury and remyelinate the axons which was not observed in our study.

Some may attribute this failure of proliferation and maturation of OPCs to the destructive milieu where toxic metabolites, high oxidative stress and inhibitory factors at the site of injury and absence of the appropriate growth factors stimulating the regeneration process (Arnold, Dalton, Schmierer, Pike, and Miller, 2013). And others related the decrease in the OPCs number few days following injury to the rapid differentiation into Type 2 astrocytes rather than oligodendrocytes (Fushimi and Shirabe, 2002) to respond to the high toxic insult causing a rapid depletion of the proliferating progenitor pool which might come closer to our findings.

The current concept that axonal integrity relies heavily on oligodendrocyte support and that the loss of these remyelinating cells would result in axonal degeneration which was observed in our histopathological findings. Besides, myelin sheaths shield axons from their surroundings and limit access to extracellular metabolites. Furthermore, myelinating oligodendrocytes were able to synthesize and deliver ATP to axons through connexons, a kind of gap junctions protein. This increases the conduction speed of action potentials. Oligodendrocytes may also labor as mechanical and trophic supports to axons (Peireira, Cruz-Höfling, Derktigil, & Graça, 1996). As naked or demyelinated axons are more vulnerable to injuries, it is reasonable to expect that, after injury, efficient remyelination is critical not only for cellular replacement but also neuronal cross talk reconstruction and neuronal function recovery.

CONCLUSION

Taken together, all the above-mentioned findings show marked differences in the Dogs’ spinal cord damage and demyelination leading to a more progressive picture of MS model than lab animals where high levels of apoptosis as observed by elevated Bax and Caspase 9 markers, a very high astrocytic reaction marked by GFAP and impaired proliferation and differentiation of Olig2 OPCs into mature oligodendrocytes.

CONFLICT OF INTEREST

There is no conflict of interest here

AUTHOR CONTRIBUTIONS

Ashraf A. Shamaa and Omar S. El-Tookey 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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