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RESEARCH ARTICLE

Selective Gray and White Matter Staining of the Horse Spinal Cord

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Summary

The ratio of gray and white matter is an important clinical parameter in the diagnosis of diffuse and compressive diseases of the spinal cord. Although histological methods are used to determine this parameter, there are some difficulties encountered in histological studies related to tissue size. The aim of this study was to evaluate possible modifications to overcome these difficulties. In the study, nine tissue samples taken from the C6 segment of a female Shetland pony and selected by systematic random sampling were used. The dehydration process of the spinal cord of the horse was supported by applying a vacuum. Paraffin blocks were prepared and cut into 10 µm sections to be stained separately with the different staining methods. Six different staining methods, including Modified May - Grunwald - Giemsa (MMGG), were compared and used to image entire slides. The stains, Hematoxylin & eosin (H&E), May-Grunwald-Giemsa (MGG), Masson's trichrome (MT), AgNORs, Kluver Barrera (KB) and MMGG, were evaluated macroscopically and microscopically by participants who were unaware of which staining methods had been used. The staining methods were scored from worst (1) to best (5) using a Likert scale. Vacuum application was found to reduce the difficulties related to inadequate tissue dehydration. MMGG was selected as the best staining method in differentiating gray and white matter in the spinal cord of the horse.

Keywords: *Gray matter ratio, Horse, Imaging, Spinal cord, Stain comparison*

At Omuriliğinin Gri ve Ak Maddesinin Seçici Boyanması

Özet

Medulla spinalis'te yer alan gri ve ak madde oranları diffuz ve kompresif omurilik hastalıklarının teşhisinde önemli klinik parametrelerdendir. Bu parametrelerin tespitinde histolojik metotlar kullanılmasına rağmen, histolojik çalışmalarda doku büyüklüğüne bağlı bazı güçlüklüklerle karşılaşılmaktadır. Bu çalışmanın amacı, bahsedilen zorlukları aşmaya yönelik olası modifikasyonları değerlendirmektir. Çalışmada, Shetland pony ırkına ait C6 segmentinden sistematik rastgele örnekleme kuralına sadık kalınarak elde edilen 9 doku kesiti kullanıldı. Medulla spinalis'in dehidrasyon işlemi sırasında vakum uygulaması yapıldı. Dokuların parafin blokları hazırlandı, dokular 10 µm kalınlığında kesilerek farklı histolojik boyalar ile boyandı. Modifiye May - Grunwald - Giemsa (MMGG)'nın yer aldığı 6 farklı boyama metodunun boyama performansları karşılaştırıldı. Hematoxylin & eosin (H&E), May-Grunwald-Giemsa (MGG), Masson's trichrome (MT), AgNORs, Kluver Barrera (KB) ve MMGG ile boyanan kesitler tek kör grup tarafından makroskopik ve mikroskopik olarak değerlendirildi. Boyaların performansları Likert skalası kullanılarak en kötü (1) en iyi (5) olmak üzere değerlendirildi. Vakum uygulamasının yetersiz doku dehidrasyonundan kaynaklanan problemleri ortadan kaldırdığı görüldü. MMGG, at medulla spinalis'inde gri ve ak madde ayrımında en başarılı boya olarak tespit edildi.

Anahtar sözcükler: *Gri madde oranı, At, Görüntüleme, Medulla spinalis, Boya karşılaştırması*

INTRODUCTION

The part of the central nervous system situated within the vertebral canal, namely, the spinal cord, is composed of 42-43 segments in horses, 8 of which are cervical, 18 thoracic, 6 lumbar, 5 sacral and 5-6 caudal. The spinal cord, enveloped by the meninges, extends from the foramen magnum to the level in-between the first and second sacral vertebrae. In general, the spinal cord is dorso-ventrally compressed and elliptic with two enlargements at the cervical intumescence and the lumbar intumescence, and terminates forming the medullary cone. In histological

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sections, the gray matter, resembling the letter H in shape and composed of nerve and glia cells and blood vessels, is observed to be situated centripetally, whilst the white matter, composed mostly of myelinated axons and blood vessels, is located peripherally. The central canal is located in the center of histological sections. Of all domestic animals, horses have the largest spinal cord, which measures 180-200 cm in length and 250-300 g in weight [1].

The segmental anatomical features of the spinal cord have been demonstrated in the cat [2], monkey [3], dog [4], sheep [5], goat [6], impala [7], horse and cattle [8]. Furthermore, histological and some histomorphometric features of the spinal cord have been ascertained in research conducted in humans [9-11], rats [12], sheep [13], donkeys [14] and horses [15]. However, during the conduct of research on sections pertaining to spinal cord segments of humans, goats, sheep, donkeys and horses, the necessity for full images arose in order to obtain histomorphometric data. For this purpose, several methods were applied, including direct drawings using millimetric paper and a calliper [6,13,14], indirect drawings [15], photograph combining [9], projection [10] and digital tablets [11]. Literature review revealed that different fixation, processing and staining methods were applied for histological tissue processing in the researches referred to above, and yet limited information was provided by the researchers on the methods applied.

The gray and white matter in the central nervous system have different anatomical and cellular properties. Investigation of chronic diseases that affect the central nervous system, such as multiple sclerosis and schizophrenia, using the ratio of gray and white matter is of great importance [16-18]. Imaging techniques, for instance Magnetic Resonance Imaging (MRI) and Diffusion Tensor Imaging (DTI) [19-21], and histological methods are used routinely to determine this parameter [12,22]. Although obtaining data using imaging methods is very rapid and practical, some difficulties (in tissue processing, staining and imaging) are encountered during application of the histological process, owing to the size of tissue samples.

A search of the literature found that a limited number of morphometric and histological studies have been conducted on the spinal cord of the horse. The aim of this study was to overcome difficulties related to tissue processing, to decide which stain is best for the clear differentiation of gray and white matter of the spinal cord of horse, to acquire tissue images as a whole, and to compare the staining performance of various stains macroscopically and microscopically.

MATERIAL and METHODS

Material

A 15-year-old female Shetland pony (230 kg) that was suffering from various orthopedic disorders was sent to the Department of Anatomy from the Equestrian Facility of the Faculty of Veterinary Medicine, Selcuk University for use in this study. The research was approved by the ethical committee of Faculty of Veterinary Medicine, Selcuk University (2011/16). The animal was anesthetized by administration of 10% chloral hydrate (80 mg/kg, IV) [23] and killed under general anesthesia by draining blood from the common carotid artery. Ten percent neutral formaldehyde was administered to the animal through a cannula in the common carotid artery after death. The spinal cord was dissected out from the vertebral column by laminectomy after the cadaver had been kept in a container of 10% neutral formaldehyde for 10 days. Segmentation was performed over the spinal cord following removal of the dura mater and pia mater. The C_6 segment was used in this study; it was 68.8 mm in length and weighed 12.8 g.

Methods

Sampling and Tissue Processing

The C_6 segment was divided into 18 pieces using a tissue slicer prior to histological processing. Each tissue sample was 3.8 mm in length. Systematic random sampling was performed on the separated pieces [24]. A sampling ratio of 1/2 was used, and nine tissue samples were obtained for routine histological processing. The tissue samples were placed on trays, taking into account their cranial-caudal orientation, and dehydrated in an ethanol series. A vacuum was applied during the second application of 96% ethanol (200 mmHg, 30 min), the third application of 100% ethanol (200 mmHg, 1 h), the third application of xylene (200 mmHg, 30 min), and in paraffin (56-58°C, 300 mmHg, the last 2 h), and the samples were blocked in paraffin wax. Samples in paraffin blocks were sectioned consecutively on a rotary microtome at a thickness of 10 µm, and six sections were obtained from each block. The sections were kept in an incubator (37°C) for 24 h, stained according to the order given in *Table 1*, and mounted with Entellan under a glass coverslip.

Table 1. *Section series and stains*
Tablo 1. *Kesit sayısı ve boyalar*

Section Number	Stain	Reference
1	H&E	(11)
2	MMGG	Table 2
3	MGG	(12, 13)
4	MT	(14)
5	AgNORs	(15)
6	KB	(16)

H&E *(Hematoxylin & eosin),* ***MGG*** *(May-Grunwald-Giemsa),* ***MT*** *(Masson's trichrome),* ***KB*** *(Kluver Barrera),* ***MMGG*** *(modified May-Grunwald-Giemsa)*

Preparation of Giemsa Solution

Giemsa stock solution (Merck KGaA, Darmstadt, Germany) was added at 1 drop per ml to 250 mL distilled water using a Pasteur pipette (The solution prepared in this way is not recommended to be used more than two times).

Acquiring Images from Slides Using an Office Scanner

Images of gross biological structures (brain or cerebellum) that are difficult to view under the microscope can be obtained with the help of a standard office scanner [31]. In this study, all slides were scanned as positive image using a standard office flatbed scanner (Hp Scanjet G4010) at 300 dpi for macroscopic evaluation.

Area Calculation and Evaluation of Variations among Stains with Point Counting Frame

The point counting method has been used frequently to assess morphological parameters such as the area, volume, and area or volume ratio [32-34]. This method was used to predict possible variations among stains that could affect measurement of the areas of gray matter and gross section. For this purpose, the grid function of the image analysis software ImageJ was applied to the scanned images. The area per point value was set at 0.4 mm^2 (0.2 × 0.2 mm) and 4 mm^2 (2 × 2 mm) to acquire an optimum CE value [24] for gray matter and the gross section respectively *(Fig. 1)*. The areas of gray and gross section on the scanned images were calculated three times by each of six different operators using the random function of the software. The results were analyzed using ANOVA *(Table 3)*.

Evaluation of the Ability of White and Gray Matter Staining with A Survey

This part of the study was designed as a single blinded experiment. The survey group was composed of 20 healthy and non-colorblind students who were selected randomly

Table 2. *Modified May-Grunwald-Giemsa staining procedure*
Tablo 2. *Modifiye edilmiş May-Grunwald-Giemsa boya prosedürü*

Staining Stages					
1	Xylene, 5 min	8	Rinse in distilled water	15	96% alcohol dip once
2	Xylene, 5 min	9	Tap water for 5 min.	16	96% alcohol dip once
3	100% alcohol, 3 min	10	Rinse in distilled water	17	100% alcohol dip once
4	100% alcohol, 3 min	11	Place in 250 ml May-Grunwald stock solution at room temperature for 10 min	18	100% alcohol dip once
5	96% alcohol, 3 min	12	Rinse in distilled water	19	Xylene 2 min
6	80% alcohol, 3 min	13	Place in Giemsa solution (250 ml) in 56°C incubator for 45 min	20	Xylene 2 min
7	70% alcohol, 3 min	14	Cool at room temperature	21	Xylene 2 min

Table 3. The mean cross-sectional areas of gray matter and gross section and responses of survey participants (median)
Tablo 3. Transversal kesitlerde ortalama gri ve ak madde oranları (mean±SE) ve anket sonuçları (median)

Stain	Gray Matter (mm^2)	Spinal Cord (mm^2)	Median Score
H&E	11.97±0.641	138.20±0.577	1^d
MMGG	11.91±0.255	139.01±0.376	5^a
MGG	11.72±0.513	138.52±0.565	3.5^b
MT	12.00±0.391	138.05±0.425	2^c
AgNORs	11.64±0.444	138.15±0.465	2^{cd}
KB	11.99±0.320	138.52±0.565	4^a

There was no statistical difference among the areas of the structures with different staining methods (P>0.05, ANOVA)
^{a,b,c,d} Different letters in the same column are significantly different (P<0.05, Mann-Whitney U test)

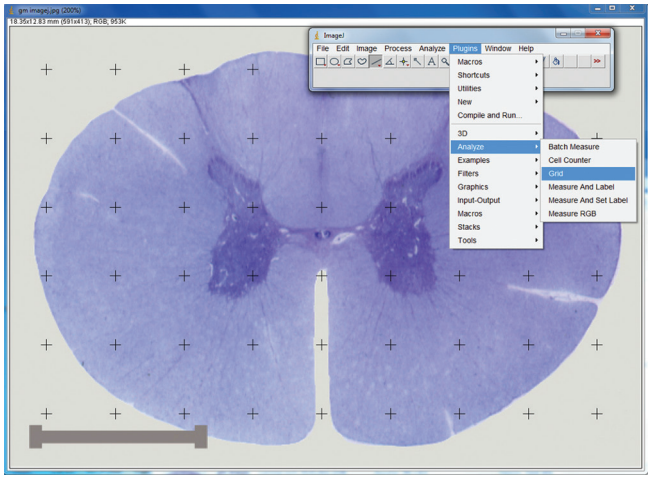

Fig 1. Superimposed point counting frame on cross-section of spinal cord using ImageJ (area per point = 4 mm^2, bar=5 mm)

Şekil 1. Medulla spinalis kesitleri üzerine ImageJ kullanılarak uygulanan noktalı alan ölçüm cetveli (bir noktanın alanı = 4 mm^2, bar=5 mm)

from the senior students of the Faculty of Veterinary Medicine. The students were given a figure *(Fig. 2)* that showed six different stains and were asked to evaluate these stains in terms of the differentiation of gray and white matter according to a Likert scale (1: worst, 5: best). The results were analyzed using the Mann-Whitney U test *(Table 3)*.

Light Microscopic Evaluation of Sections

The ability of the six dyes to stain neurons, glia, ependymal cells, endothelium, axon and dendrites were investigated using a Likert scale and the results are given in *Table 4*.

RESULTS

Collapsed areas were detected particularly in the gray matter region in paraffin blocks of tissues to which vacuum had not been applied during dehydration. Ruptures occurred during the cutting of these blocks with the rotary microtome, and low quality staining was observed especially in that area. This problem was solved by the application of vacuum. As a result of calculations made using the point counting frame superimposed on each slide stained with the different staining methods, the mean cross-sectional surface area of C_6 was found to be 138±0.197 mm^2 and the mean area of gray matter

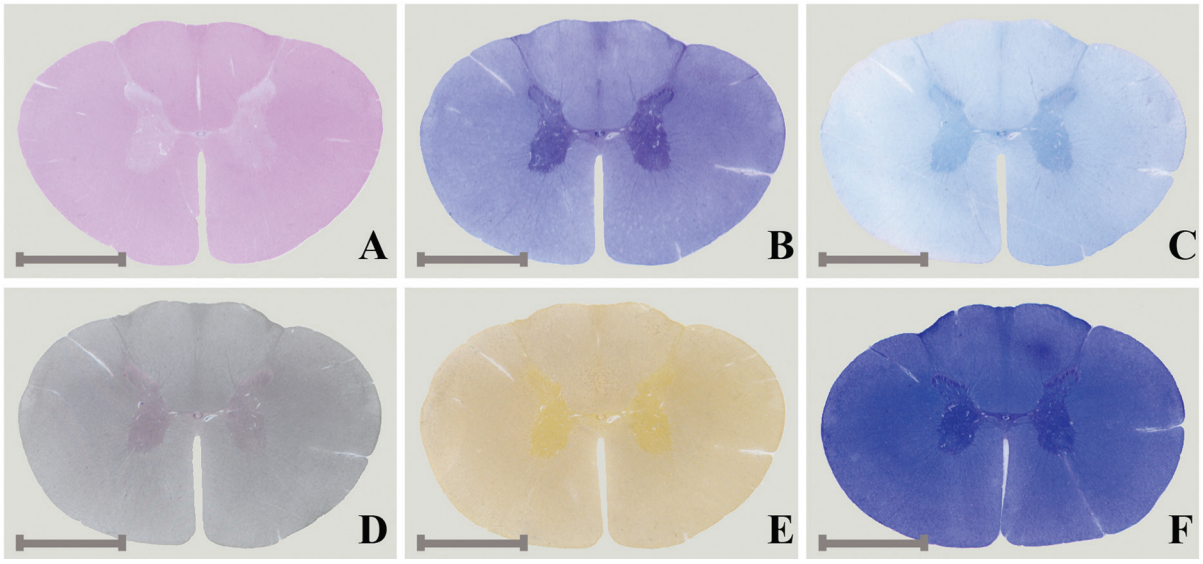

Fig 2. The image used by the survey group to evaluate the performance of stains using a Likert scale (bar= 5mm) (A: H&E, B: MMGG, C: MGG, D: MT, E: AgNORs, F: KB)

Şekil 2. Anket grubu tarafından Likert skalası kullanarak boya performanslarının değerlendirilmesinde kullanılan resim dosyası (bar= 5mm) (A: H&E, B: MMGG, C: MGG, D: MT, E: AgNORs, F: KB)

Table 4. *Microscopic assessment using Likert scale*
Tablo 4. *Likert skalası kullanılarak mikroskopik değerlendirme*

Stains	White Matter					Gray Matter						Ependymal cells		Differantiation of gray and white matter	Differantiation of axon and dendrite	Appearance of Nissl bodies
	Axon	Glia		Endothel		Neuron		Glia		Endothel						
		N	C	N	C	N	C	N	C	N	C	N	C			
H&E	3	4	-	4	-	4	4	4	-	4	-	4	4	1	3	4
MMGG	4	3	-	2	-	2	2	3	-	2	-	1	1	5	2	2
MGG	2	3	-	3	-	3	3	3	-	3	-	2	2	2	3	3
MT	3	4	-	5	-	4	4	4	-	5	-	5	5	2	4	4
AgNORs	3	4	-	5	-	3	2	4	-	5	-	5	3	3	1	1
KB	4	4	-	3	-	5	5	4	-	3	-	4	4	5	5	5

N: *Nucleus,* ***C:*** *Cytoplasm*

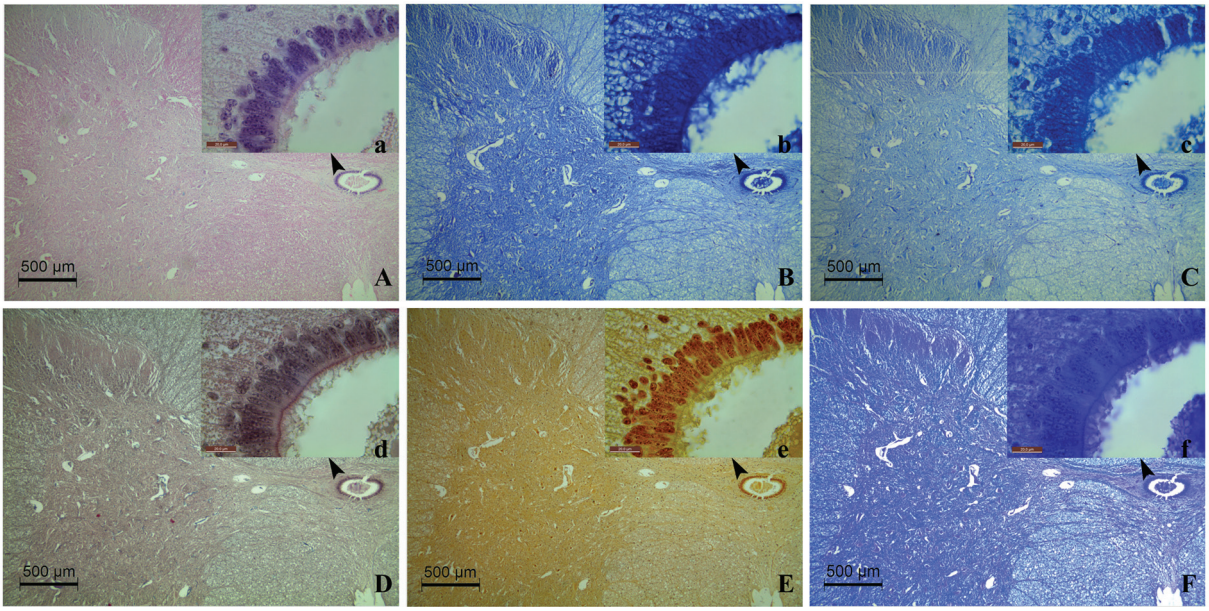

Fig. 3. Cross-sectional images obtained by 4× and 100× objectives under a light microscope (A: H&E, B: MMGG, C: MGG, D: MT, E: AgNORs, F: KB; Upper-case letter: 4×, Lower-case letter: 100×)

Şekil 3. Işık mikroskopu ile 4× ve 100× objektif kullanılarak elde edilen kesit görüntüleri (A: H&E, B: MMGG, C: MGG, D: MT, E: AgNORs, F: KB; Büyük harf: 4×, Küçük harf: 100×)

was estimated to be 11.87±0.170 mm^2. The ratio of gray matter to gross section was determined to be 8.58%. A statistical difference was not detected (P>0.05, *Table 3*) among the areas of gray and white matter calculated from sections made using the six different stains.

The calculated coefficient of error value (CE) for the gray matter was 0.053 and for the spinal cord was 0.048. Statistical analysis of the responses given by the survey group showed that MMGG and KB were thought to be the best staining methods for differentiation of gray and white matter *(Table 3, Fig. 2)*.

DISCUSSION

The application of a vacuum during the dehydration process is not recommended because of the fragile character of central nervous system tissue [25]. In the present study, collapse caused by inadequate dehydration of the surface of paraffin blocks were seen, especially in the gray matter region, as a result of the routinely applied dehydration process without vacuum application. These problems were solved with vacuum application, as described in the section describing sampling and tissue processing. Different staining techniques have been utilized to differentiate gray and white matter macroscopically [35,36] and microscopically [9,37] because of the poor contrast between these structures. Although KB is the staining method used most commonly for the central nervous system, MMGG *(Table 2)* is a preferable staining method to differentiate gray and white matter in the spinal cord of the horse because of its selective quality for this area and rapid reliable results *(Fig. 3, Table 4)*.

The ratio of gray and white matter is used as an important parameter in the diagnosis of several diseases (schizophrenia, multiple sclerosis) in the central nervous system. Although imaging methods such as MRI are used most commonly to obtain this information [16,17,21], its use is limited in large domestic animals. Histological methods are frequently preferred when investigating the spinal cord of these animals [13,15]. However, the entire structure must be displayed to estimate the ratio from histological sections. For this reason, a photographic camera [9,10], Edingerschen Apparatus [15], slide scanner [38] and office scanner [31] have been used to view the entire sections taken from large biological structures. In the current study, stained slides were scanned as JPEG files using a standard flatbed office scanner with a positive image scan option at 300 dpi. Measurement of the scanned images with a point counting frame estimated the ratio of gray matter to be 8.58% in the C_6 segment *(Table 3)*. The ratios of gray matter in C_6 segments were reported as 11.86% of donkey [14], 18.3% of human [10], and 35-40% of rat [12] respectively. Braun [15] reported that the ratio of gray matter was 12.7% in the C_6 segment of horse. It is thought that the dissimilarity between the two results could have resulted from differences in methodology. However, use of an office scanner to obtain images of large structures such as the brain and cerebellum was found to be a rapid method to view the spinal cord of the horse. Under microscopic examination, H&E, AgNORs, MT and KB for glia, MT and AgNORs for endothelial cells, KB for differentiation of axons and dendrites and also Nissl bodies, MT and AgNORs, especially for ependymal cells, were found to be good staining options *(Fig. 3, Table 4)*.

The current study showed that a vacuum can be applied during dehydration of large structures such as the spinal cord of the horse in order to acquire acceptable results. MMGG is a useful staining method for the differentiation of gray and white matter, and it is advised to be used when the spinal cord is examined both macroscopically and microscopically. Use of an office scanner is a cheap and practical method to view and scan large biological tissues. These methods are suggested as tools for use in morphometric studies related to the central nervous system.

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