Distinction of Gray and White Matter for Some Histological Staining Methods in New Zealand Rabbit's Brain

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ABSTRACT

Objective: The aim of this study was to investigate the abilities of some staining methods used in histology to detect neuroglia, cell groups and moieties such as axons and dendrites in the brain, and the ability of detecting the white matter limit.

Materials and Methods: Brain tissue from a 14-month-old New Zealand Rabbit was used in the study. The brain was sliced transversally to make it suitable for histological procedures. For this, the brains were placed on the millimeter paper and sliced into three equal parts. The obtained samples were cut 10µm thickness from same side and cranial to caudal and, slides were stained with six staining methods. Each of these slides was photographed as jpeg format by means of a microscope. The sectional images obtained were transferred to Image J programme to estimate their areas. The Likert scale was used to investigate the adequacy of staining methods to determine the border of gray and white matter and cell groups in the brain. As a result of these procedures, statistical results of obtained data were presented in tables and figures.

Results: As a result of the Likert scale, CT was the highest score whereas MGG was the lowest average score. Considering all structures in the brain, KB, MGG, MMGG and CT stainings for neuroglia cells; KB, MGG and MMGG staining methods for axon, dendrite and Nissl bodies; furthermore for ependymal cells, pia mater and choroid plexus KB, MGG, MMGG, CT, AgNORs and HE staining methods were found to have the highest score. In the distinction of gray and white matter, KB, MG, MMGG and CT staining methods had the highest score, also.

Conclusions: With this study, it is thought that it would help the researchers to determine the boundaries of the anatomical structures of interest in the brain and the selection of histological stains that should be used in the staining of the desired cell groups.

Key Words: Brain, Histological Staining Methods, New Zealand Rabbit

INTRODUCTION

All living organisms are capable of responding to physical and chemical stimuli from their internal and external worlds. This can be achieved by a well-organized nervous system. The nervous system is anatomically divided into two parts as central nervous system (CNS) and peripheral nervous system (PNS). The CNS is composed only of cells. These are categorized into two types: neurons, which receive and transmit impulses, and neuroglia, which support and facilitate the proper functioning of the neurons. In a cross-section of the brain, two different parts appear. They are dark gray mat-

ter and light colored white matter. Gray matter is composed of neuron cell bodies, clusters of which within the CNS are known as nuclei, whereas white matter is recognized by the presence of myelinated axons (Seiferle, 2004; Patestas and Gartner, 2016).

Most diseases of the brain are associated with synaptic loss and gradual deterioration or death of the neural cells (brain atrophy) (Coelho et al., 2018). Volume changing in the brain have been proposed as aids in the diagnosis of Alzheimer disease, autism, hyperactivity disorder, schizophrenia and other types of dementia. Regional brain volume changes or

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atrophy rates have also been proposed as surrogate markers of disease progression for use in clinical examination (Selcuk and Bahar, 2014; Heggland et al., 2015; Aljondi et al., 2019). In the studies, different methods are used to differentiate gray and white matter in the brain or to calculate volume and volume ratios. MRI images is widely used in the diagnosis of brain diseases. However, the same devices can detect different brain volume ratios and cortical thickness, and even different brain volumes between two scanners of the exact same type with identical imaging protocols (Biberacher et al., 2016; Amiri et al., 2019). In addition, although it attempts to define a specific interruption in the rate of pathological complete brain volume loss in the detection of disease in large tissues, the generally accepted thresholds of regional and global pathological brain atrophy have not yet been established (De Stefano et al., 2016; Uher et al., 2019). However, it is important to note that the structure or organ to be examined is clearly differentiated from other structures (Bahar et al., 2013). Models designed in animals, especially considering the rabbits used for modeling because their perinatal development resembles humans, the need for specially developed and expensive computer aided systems and trained technical personnel to carry out these calculations brings additional financial burden to the studies (Basoglu et al., 2007; Kalkan et al., 2007). This is a value factor that increases the costs when considering the number of the test subjects to be used in the study. In these systems, to quantify specific amounts of regional volume, the tissues concerned must be repeatedly exposed to ionizing radiation, causing damage to the tissue as well as ethical dilemmas. Therefore, in experimental studies, researchers frequently use histological and micro-anatomical methods to differentiate between gray and white matter or to calculate volume and volume ratios, to distinguish cell types found in the brain and to determinate atrophy (Bahar et al., 2013; Chuang et al., 2011; Sivapalan and Aitchison, 2014; Bolat, 2018). Many researchers who do not know the histological and micro-anatomical methods have difficulty in deciding the histological dye method to be used because the methods used in the literature searches are not clearly explained.

In this study, it was aimed to investigate the ability of some of the dyes used in morphology to detect the cell groups and parts such as neurons, neuroglia, axons and dendrites in the brain and to investigate the adequacy of detecting the border of gray and white matter.

MATERIALS AND METHODS

Ethical Clearance

The ethical approval for investigation was obtained by Karanoglu Mehmetbey University Faculty of Health Sciences Ethics Committee (protocol number: 2017/07).

Preparation of Cadavers

In this study, these healthy male New Zealand Rabbits aged 14 months were used. During the fixation, abdominal aorta and vena cava caudalis were dissected and plastic catheters were placed and by this way, normal saline solution was given into vena cava caudalis and blood was removed from the vessels. Then, 10% neutral formalin solution was perfused via the vena cava caudalis. The rabbit head was kept in a container containing 10% formaldehyde solution for 20 days to complete the fixation (Bahar and Dayan, 2014).

Histological Process and Tissue Sampling

The brain was sliced transversally to accommodate the histological procedure. For this purpose, these brains were placed on millimeter paper and sliced into six equal parts. Then, these brain samples were dehydrated, cleared, and embedded into the paraffin. Prepared paraffin blocks were cut with a rotary microtome at a thickness of 10µm and six sections were obtained from each block. These sections were stained according to Silver Staining Nucleolus Organiser Regions (Ag-NOR) (Table 1), hematoxylin eosin (H&E) (Table 2), Kluver Barrera (KB) (Table 3), May Grunwald Giemsa (MGG) (Table 4), Modified May Grunwald Giemsa (MMGG) (Bolat et al., 2012), Crosman's Triple Staining (CT) staining procedures (Selcuk and Tıprıdamaz, 2019). The stained sections were imaged with a stereomicroscope (Olympus SZX16) and recorded in jpeg. The brain sections stained with the dyes were given in Figure 1, neurons in Figure 2 and choroid plexus in Figure 3.

Evaluation of the Stained Tissue Samples

Obtained sections were evaluated by blind users who were selected randomly from the senior students of the Faculty of Health Sciences. The survey group was composed of 20 healthy and non-colorblind students. First, they were asked to evaluate the distinction between gray and white matter on images, macroscopically. After, with a light microscope, it was microscopically asked to evaluate the borders of gray and white matter, the abilities to get dyes into neurons, neuroglia, ependymal cells, endothelium, axons, and dendrites according to a Likert scale (1: worst, 5: best).

The point counting method has often used to assess morphological parameters such as area and volume. Using this method, numerical comparison of possible variables in cross-sections can be made (Bas et al., 2009). For this purpose, the grid function of Image J program was used to calculate the surface area of the sections (Figure 4). The distance between the two points was chosen as 1mm on the point area measuring scale used. A different marker was selected for each area of interest and the points falling into the areas were separately counted. The effect of staining procedures on the areas of these sections was statistically analyzed.

Statistical Analysis

Statistical analysis was conducted with SPSS software version 21. Kolmogorov-Smirnov/Shapiro-Wilk's test was applied to the data and found that the data showed normal distribution. One-way ANOVA was used to compare data obtained from the study. Levene test was used to assess the homogeneity of the variances. Data are expressed as means±standard error (SE).

RESULTS

In this study, the gray matter, white matter and brain areas of these histological sections applied different staining methods and obtained from New Zealand Rabbit were given in Table 5. As a result of field measurements on these sections, no statistical difference was found among gray matter, white matter, and brain areas ($P>0.05$).

In order to determine the adequacy of these dyes applied on brain sections of New Zealand Rabbits to distinguish the boundary of gray and white matter the mean, standard error, median, minimum and maximum scores of the Likert scale were given in Table 6. As a result of the Likert scale, CT was the highest score whereas MGG was the lowest average score. In one-way analysis of variance, the adequacy of the six types of dyes used to determine the boundary of gray and white matter alba was statistically significant ($P<0.001$). Statistically, while there was a significant difference between AgNOR and CT stainings, there was no difference among the other staining methods. When H&E and MMGG staining methods were compared to CT, there was a statistically significant difference, but no difference was found among the other staining methods. There was no difference between MGG and AgNOR, H&E and MMGG methods, but there was a statistical difference between KB and CT staining methods. Although CT staining had the highest mean and median value at the end of the scoring, no statistical difference was found between CT and KB staining methods ($P>0.05$).

Microscopic evaluation using the Likert scale was given in Table 7. As a result of the stainings, apart from the distinction of gray and white matter, structures in these brain layers were also observed. Neuron bodies, neuroglia cells, dendrites, axon extensions and Nissl bodies were seen in the gray matter layer of the brain; Myelinated axon extensions and neuroglia cells were detected in the white matter. In addition, the presence of choroid plexus, ependymal cells and pia mater was found in the sections. Considering all these structures, KB, MGG, MMGG and CT stainings for neuroglia cells; KB, MGG and MMGG staining methods for axon, dendrite and Nissl bodies; furthermore for ependymal cells, pia mater and choroid plexus KB, MGG, MMGG, CT, AgNORs and HE staining methods were found to have the

highest score. In the distinction of gray and white matter, KB, MG, MMGG and CT staining methods had the highest score, also.

DISCUSSION

Nerve tissue contains different structures such as neurons and neuroglia cells. Correct characterization of complex arrangements in healthy tissue can help in understanding neurological diseases. Animal models play an important role in the development of neuroscience and, many models have been established to investigate neurocognitive diseases (Eixarch et al., 2012, Ferraris et al., 2018). Although rodent neurocognitive models are well established, translation values are limited especially considering prenatal myelinations, lysencephalic brain structures and low white matter ratio. Alternatively, rabbits may provide a link between small and large animals since their brains develop during the perinatal period and the timing of white matter maturation is comparable to that of humans. Despite these complex brain structures, their cost is low compared to large animals, their suitability to laboratory conditions increases the use of rabbits (Coelho et al., 2018).

Nervous system autism, hyperactivity disorder, schizophrenia, multiple sclerosis, epilepsy, preterm birth, fragile X syndrome, tourette syndrome and many other diseases such as Alzheimer's disease in advanced age in the follow-up and treatment of the disease changes in the volume and surface area are important (Shen et al., 2013). Changes in the surface area of the brain may also be important in some neurological diseases such as epilepsy, schizophrenia, Williams syndrome and cortical developmental malformations (Ronan et al., 2006; Heegland et al., 2015). Volume and surface area are used as important data in the study of brain functions (Selcuk and Bahar, 2014). Therefore, determination of the brain's gray and white matter volume and volume ratios is very important to understand the relationship between tissue atrophy and clinical status. In the diagnosis of diseases affecting the central nervous system, it is very important to know the volume and anatomical structure of the affected anatomical structure (Raznahan et al., 2013; Shen et al., 2013).

In this study, it is aimed to investigate the abilities of some of the dyes used in morphohistology to detect the cell groups and parts such as neurons, neuroglia, axons and dendrites in the brain and the adequacy of detecting the border of gray and white matter. First, the brain tissues were removed from the cavum cranii and, the fixation was performed. After this procedure, the brain tissues were sliced in order to make it suitable for histological procedures and, cross sections were taken by performing routine histological follow-ups and, staining procedures were performed using six histological staining methods.

The preparations were evaluated with a Likert scale after being photographed with a microscope. When the statistical results of the data obtained from the Likert scale were macroscopically compared, it was concluded that CT and KB staining methods were more effective according to the other staining methods in terms of its ability to determine the boundary of gray and white matter. In the distinction of gray and white matter examined with a microscope, although KB, MG, MMGG and CT staining methods had the highest scores, there was no difference among them. As a result of microscopic evaluation, KB, MGG, MMGG and CT staining methods for neuroglia cells; KB, MGG and MMGG staining methods for axon, dendrite and Nissl bodies; for ependymal cells, pia mater and choroid plexus KB, MGG, MMGG, CT, AgNOR and H&E staining methods were the best staining methods.

CONCLUSIONS

Many researchers who do not know the histological and micro-anatomical methods have difficulty in deciding the histological staining methods to be used because these methods used in the literature searches are not clearly explained. With this study, it is thought that it will help the researchers to determine the boundaries of the anatomical structures of interest in the brain and to select the histological staining methods that should be used in the staining of the desired cell groups. Therefore, it is thought that the results of this researches would contribute greatly to the literature.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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Table 1: AgNOR staining procedure (Korek et al., 1991)

Staining Stages			
1	30 minutes in 37°C dark incubator with 2g gelatin 1% formic acid (50ml) + distilled water prepared with 50% silver nitrate solution (100ml)	6	96% alcohol, 3 min.
2	Rinse in distilled water	7	100% alcohol, 3 min.
3	70% alcohol dip once	8	100% alcohol, 3 min.
4	80% alcohol dip once	9	Xylene, 3 min.
5	96% alcohol dip once	10	Xylene, 3 min.

Table 2: Hematoxylin-Eosin staining procedure (Ekicioğlu et al., 2005)

Staining Stages					
1	Xylene, 5 min.	9	Tap water for 5 min.	21	96% alcohol dip once
2	Xylene, 5 min.	10	Transfer to Hematoxylin solution, 5 min.	22	96% alcohol dip once
3	100% alcohol, 3 min.	11	Rinse in distilled water	23	100% alcohol, 2 min.
4	100% alcohol, 3 min.	12	Tap water for 3 min.	24	100% alcohol, 2 min.
5	96% alcohol, 3 min.	13	Eosin solution, 5 min.	25	Xylene, 5 min.
6	80% alcohol, 3 min.	14	Tap water for 5 min.	26	Xylene, 5 min.
7	70% alcohol, 3 min.	15	70% alcohol dip once	27	Xylene, 5 min.
8	Rinse in distilled water	16	80% alcohol dip once		

Table 3: Kluver Barrera staining procedure (Demir et al., 2007)

Staining Stages					
1	Xylene, 5 min.	7	96% alcohol dip once	13	Tap water for 5 min.
2	Xylene, 5 min.	8	Place Luxol fast blue solution in 57°C in the incubator, 20 h.	14	96% alcohol dip once
3	100% alcohol, 3 min.	9	96% alcohol dip once	15	Xylene, 2 min.
4	100% alcohol, 3 min.	10	Tap water for 5 min.	16	Xylene, 2 min.
5	96% alcohol, 3 min.	11	5% Li ₂ CO ₃ solution for 2 min..	17	Xylene, 2 min.
6	96% alcohol dip once	12	70% alcohol dip once		

Table 4: May Grunwald Giemsa staining procedure (Palaskar and Jindal, 2010)

Staining Stages					
1	Xylene, 5 min.	8	Rinse in distilled water	15	100% alcohol dip once.
2	Xylene, 5 min.	9	Tap water for 5 min.	16	100% alcohol dip once
3	100% alcohol, 3 min.	10	Place May Grunwald blue solution for 10 min.	17	Xylol, 2 dk.
4	100% alcohol, 3 min.	11	Rinse in distilled water	18	Xylene, 2 min.
5	96% alcohol, 3 min.	12	Place Giemsa solution for 45 min.	19	Xylene, 2 min.
6	80% alcohol, 3 min.	13	Tap water for 2 min.	20	Xylene, 2 min.
7	70% alcohol, 3 min.	14	96% alcohol dip once		

Table 5: Mean gray matter, white matter and brain areas obtained from transverse sections (Mean±SE)

Staining Method	Gray Matter (mm ²)	White Matter (mm ²)	Brain (mm ²)
AgNOR	51.08±10.67	128.92±14.73	180.00±24.86
H&E	45.33±8.55	121.67±14.37	167.00±22.25
KB	44.33±8.71	118.25±13.92	162.58±21.79
MGG	42.75±8.84	117.50±13.36	160.25±21.44
MMGG	45.68±10.13	119.25±13.50	164.92±23.18
CT	44.67±8.72	118.92±13.15	163.58±21.15

AgNOR: Silver Staining Nucleolus Organiser Regions, **H&E:** Hematoxylin-Eosin, **KB:** Kluver Barrera, **MGG:** May Grunwald Giemsa, **MMGG:** Modified May Grunwald Giemsa, **CT:** Crosman's Triple Staining.

Table 6: One-way analysis of variance in Likert test on images taken for determine whether gray and white matter can be distinguished by staining methods

Staining Method	N	X	SE	Med.	Min.	Max.	F	P
AgNOR	20	2.75	0.28	2.5	1	4	13.648	0.000
H&E	20	3.75	0.19	3	2	5		
KB	20	3.90	0.27	4	1	5		
MGG	20	2.15	0.22	2	1	4		
MMGG	20	3.15	0.25	3	1	5		
CT	20	4.65	0.13	5	3	5		

AgNOR: Silver Staining Nucleolus Organiser Regions, **H&E:** Hematoxylin-Eosin, **KB:** Kluver Barrera, **MGG:** May Grunwald Giemsa, **MMGG:** Modified May Grunwald Giemsa, **CT:** Crosman's Triple Staining.

Table 7: Microscopic evaluation using Likert scale

Staining Method	White Matter				Gray Matter				Differentiation of gray and white matter	Differentiation of axon and dendrite	Appearance of Nissl bodies	Choroid plexus
	Axon	Neuroglia		Neuron	Neuroglia							
		N	C		N	C						
AgNOR	1	4	3	4	3	4	3	1	1	1	1	
H&E	1	2	2	1	2	2	2	1	1	1	1	
KB	5	5	5	5	5	5	5	5	5	5	5	
MGG	5	5	5	5	5	5	5	5	5	4	5	
MMGG	5	5	5	5	5	5	5	5	5	3	5	
CT	4	4	4	4	4	4	4	5	2	2	4	

AgNOR: Silver Staining Nucleolus Organiser Regions, **H&E:** Hematoxylin-Eosin, **KB:** Kluver Barrera, **MGG:** May Grunwald Giemsa, **MMGG:** Modified May Grunwald Giemsa, **CT:** Crosman's Triple Staining, **N:** Nucleus, **C:** Cytoplasm

Figure 1: Stained brain sections (**A:** AgNOR, **B:** H&E, **C:** KB, **D:** MGG, **E:** MMGG, **F:** CT, **Bar:** 1mm). **AgNOR:** Silver Staining Nucleolus Organiser Regions, **H&E:** Hematoxylin-Eosin, **KB:** Kluver Barrera, **MGG:** May Grunwald Giemsa, **MMGG:** Modified May Grunwald Giemsa, **CT:** Crosman's Triple Staining.

Figure 3: Appearance of choroid plexus according to different staining methods (**A:** AgNOR, **B:** H&E, **C:** KB, **D:** MGG, **E:** MMGG, **F:** CT, **Bar:** 20 µm). **AgNOR:** Silver Staining Nucleolus Organiser Regions, **H&E:** Hematoxylin-Eosin, **KB:** Kluver Barrera, **MGG:** May Grunwald Giemsa, **MMGG:** Modified May Grunwald Giemsa, **CT:** Crosman's Triple Staining.

Figure 2: Appearance of neurons according to different staining methods (**A:** AgNOR, **B:** H&E, **C:** KB, **D:** MGG, **E:** MMGG, **F:** CT, **Bar:** 20µm). **AgNOR:** Silver Staining Nucleolus Organiser Regions, **H&E:** Hematoxylin-Eosin, **KB:** Kluver Barrera, **MGG:** May Grunwald Giemsa, **MMGG:** Modified May Grunwald Giemsa, **CT:** Crosman's Triple Staining.

Figure 4: Measurement of the area of brain sections using ImageJ (Area of a point = 1mm², Bar: 1 mm)