The Thai Journal of Veterinary Medicine

Volume 44
Issue 4 *December, 2014*

Article 17

12-1-2014

Comparison of Mulligan's, Alston's and Prussian Blue Reaction's Methods for Staining Dog Brain Slices Prior to Plastination

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Short communication

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Abstract

To follow up our previous investigation of Sudan Red staining in plastinated brain specimen (Vibulchan et al., 2012), the present study aimed to examine staining dyes that can withstand the plastination procedures and be reserved in the specimen thereafter. Three staining methods, Mulligan's method, Alston's method and Prussian blue reaction method were tested in dog brain transverse slices. After the staining procedures the brain slices were dehydrated then forced impregnated using a polymer as described previously. The plastinated brain slices were compared for staining retention and analyzed for shrinkage. Results showed that Alston's method of staining was preferable to dye preservation after plastination procedures. However, the shrinkage occurred with all three staining methods.

Keywords: Alston's method, dog brain, Mulligan's method, plastination, Prussian blue reaction method, shrinkage

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Introduction

Plastination technique was developed by von Hagens et al. (1987) for long-lasting preservation of anatomical specimen and has been widely used in gross anatomy teaching laboratory with great satisfaction. In neuroanatomy study, however, it is difficult to distinguish the white matter from the gray matter, two main components of the brain and spinal cord, in unstained specimens. This is crucial for the study of tracts from nuclei within the brain and spinal cord. Therefore, staining of the specimen with various dyes has been used in order to overcome such obstacle. When combined with the plastination technique, most of the dyes that are selected for staining of the brain and spinal cord cannot tolerate the harsh but essential dehydration and forced impregnation steps and the staining disappeared from the plastinated specimens. In our previous investigation we studied the staining of dog brain specimen using Sudan Red dye prior to plastination and found that the staining somewhat diminished during the dehydration and plastination procedures (Vibulchan et al., 2012). In our present study we investigated the use of Mulligan's solution in three different staining methods in order to find the optimal staining procedure for plastinated brain specimen.

Materials and Methods

Samples: Nine dog brains fixed in 10% formalin for over 12 months were used in this study. The specimens were divided into 3 groups and kept at room temperature. Prior to staining, the brains were transversely sectioned into 4-6 mm slices and all sections from each brain were stained as followed.

Staining methods

Mulligan's method (Gregg, 1975)

The brain slices were stained for 4 min in Mulligan's solution (40 g crystalline phenol, 5 g cupric sulfate, 1.25 ml 0.1N HC1 in 1 l H_2O) at 60-65 °C. After that they were immersed for 10 sec in iced water then 1 min in 0.4% tannic acid (W/V in water) at room temperature. They were then rinsed with running tap water followed by 10-15 sec in 0.08% ferric ammonium sulfate at room temperature before rinsing for 8 h with running tap water.

Alston's method (Alston, 1981)

The brain slices were stained for 20 min in Mulligan's solution at room temperature followed by 20 sec in xylene, 10 sec in 2% sodium hydroxide, and 2 min in 2% potassium ferrocyanide then rinsed with running tap water for 8 h at room temperature.

Prussian blue reaction method (Le Masurier, 1935)

The brain slices were stained for 2 min in Mulligan's solution at 60-65 °C followed by 1 min in ice water, 2 min in 1% ferric chloride, 5 min in running tap water, and 3 min in 1% potassium ferrocyanide, respectively, at room temperature. After the staining the slices were washed for 8 h with running tap water. All stained brain slices were photographed and measured before plastination.

Plastination: The plastination procedure was performed as described by Raoof (2001). The stained brain slices from all three groups were dehydrated in 70%, 90% and 100% ethanol for 24 h each, before transferred to 100% acetone for 48 h, with daily change of fresh acetone. After dehydration, the specimens were impregnated in a mixture of polydimethylsiloxane (Biodur™ S10) and dibutyltindilaurate (Biodur™ S3) 100:1 for 30 d. The impregnated brain slices were wiped clean of excess polymer and placed in a sealed plastic tank containing tetraethoxysilane (Biodur™ S6), which was vaporized for 3 d for curing.

Statistical Analysis: After the plastination was complete, the brain slices were photographed and measured. Shrinkage was calculated and analyzed. Statistical analysis by ANOVA.

Results and Discussion

Results showed that before plastination, all three methods gave clearly distinguishable staining of gray and white matters compared to unstained slices (Figs 1a, d and g). In Mulligan's method (Fig 1b), lipid in white matter's vast myelin was dissolved by phenol to form a jelly coat covering its surface, therefore the white matter was unstained by the aqueous dye and appeared white. The gray matter, on the other hand, reacted with tannic acid thus appeared grayish black (Mulligan, 1931). In Alston's method and Prussian blue reaction method the gray matter was stained brick red (Fig 1e) and greenish blue (Fig 1h), respectively, while the white matter remained white in both groups. Alston (1981) modified the Mulligan's solution by omitting tannic acid and adding potassium ferrocyanide, which reacted with cupric sulfate to form cupric ferrocyanide, which gave the brick red color. In Prussian blue reaction, the color was developed from a chemical reaction between the staining agents and iron molecules present in the brain tissue. The dehydration process did not alter or diminish the staining in all three groups, but did affect Sudan Red staining (Vibulchan et al., 2012). After plastination, however, the gray matter in group 3 appeared markedly darker (Fig 1i), while in the other groups the staining remained similar to pre-plastination. The increase in intensity of the staining may be due to shrinkage of the cells, thus causing the color to appear darker. Some slices showed generally slightly yellowish discoloration, which often occurs in plastinated specimen. Judging from these staining, we appreciated Alston's method of staining over Mulligan's and Prussian blue staining, which is in agreement with Suriyaprapadilok and Withyachumnarnkul (1997). It gave a clearer gray/white matter appearance than Mulligan's method. Prussian blue method, while giving a much darker gray matter staining, was difficult to see the details of nuclei and other components within the gray matter because it was too dark even before plastination. Moreover, the procedure requires heating phenol which is complicated and health-hazardous (Loftspring et al., 2008).

Although it appeared that neither the dehydration nor the impregnation procedures had significant effect on the staining, the process caused

substantial shrinkage of the brain slices (Table 1). When using General Linear Model (GLM) of ANOVA, all three groups showed no significant difference in shrinkage ($p < 0.005$), which seemed to occur during both dehydration and impregnation (Ameko et al., 2012). Sagoo and Adds (2013) suggested that human brain slices at 1 cm thick could well preserve their shape after plastination. Nonetheless, despite the shrinkage, the plastinated specimens from this study are considered appropriate teaching materials for neuroanatomical study. No framing is required to hold the plastinated brain slices. Plastinated brain specimen can also be helpful in teaching neuroanatomy (Weiglein, 1997).

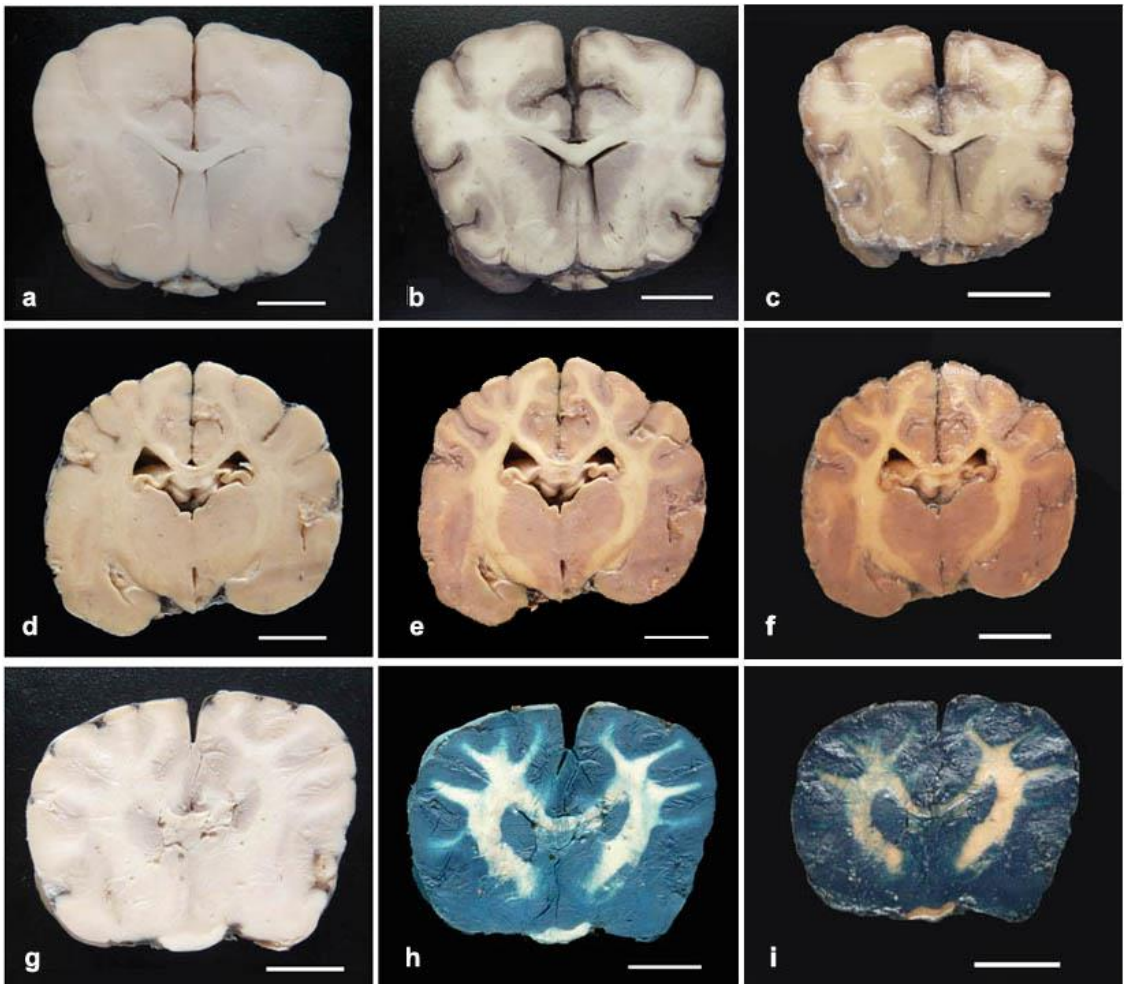

Figure 1 Comparison of brain slices before (a, d and g), after staining (b, e and h) and after plastination (c, f and i). b and c - Mulligan's method; e and f - Alston's method; h and i - Prussian blue reaction method. Bars = 1 cm.

Table 1 Color, number of stained brain slices, and measurement before and after plastination and shrinkage of plastinated brain slices in each method

	Mulligan	**Alston**	**Prussian blue**
Color			
Gray matter	grayish black	brick-red	greenish blue
White matter	white	white	white
Number of brain slices	16	14	16
Width before plastination (cm) (mean±SD)	3.34±1.17	4.21±0.458	4.21±0.276
Width after plastination (cm) (mean±SD)	2.47±0.863	3.1±0.268	3.16±0.212
Shrinkage (cm) (mean±SD)	0.87±0.382	1.11±0.431	1.05± 0.158

Acknowledgements

We would like to gratefully acknowledge Asst. Prof. Chatree khatiworavage for statistic expertise and Mr. Witoon Mabutr and Mrs. Jantima Intarapanya for excellent staining expertise. This study was supported by Chulalongkorn University-Veterinary Science Research Fund RG2/2555.

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บทคัดย่อ

การเปรียบเทียบการย้อมสีชิ้นเนื้อสมองสุนัขด้วยวิธี Mulligan's, Alston's และ Prussian blue reaction ก่อนผ่านการกำซาบด้วยพลาสติก

ปุณณรัตน์ วิบูลย์จันทร์* อรสิริ ชื่นทรวง

การวิจัยนี้เป็นการศึกษาต่อเนื่องจากการวิจัยก่อนหน้านี้ที่ทำการย้อมสีสมองของสุนัขด้วยซูดานเรด แล้วนำไปผ่านกระบวนการกำซาบด้วยพลาสติก (Vibulchan et al., 2012) วัตถุประสงค์ของการวิจัยเพื่อทดสอบหาวิธีการย้อมสีสมองที่มีความคงทนต่อกระบวนการกำซาบด้วยพลาสติก โดยศึกษาวิธีการย้อมสี 3 วิธี ได้แก่ Mulligan's, Alston's และ Prussian blue reaction ในชิ้นเนื้อสมองสุนัขที่ถูกตัดตามขวาง จากนั้นจึงนำชิ้นเนื้อสมองสุนัขที่ถูกย้อมสีไปผ่านขบวนการดึงน้ำออกและกำซาบด้วยสารโพลีเมอร์ตามกระบวนการกำซาบด้วยพลาสติก และทำการเปรียบเทียบการคงอยู่ของสีย้อมภายหลังการกำซาบด้วยพลาสติกและวิเคราะห์การหดตัวของชิ้นเนื้อสมอง จากการเปรียบเทียบผลการทดลองพบว่าวิธีการย้อมสี Alston's เหมาะสำหรับการย้อมสีชิ้นเนื้อสมองก่อนนำไปกำซาบด้วยพลาสติกมากกว่าวิธี Mulligan's และ Prussian blue reaction อย่างไรก็ตามพบการหดตัวของสมองในวิธีการย้อมสีทั้งสามวิธี

คำสำคัญ: Alston's method สมองสุนัข การกำซาบด้วยพลาสติก Mulligan's method Prussian blue reaction method การหดตัว

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