Original Article

Optimum Volume of Water to Be Added to the Leishman Stain While Preparing a Blood Smear

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Abstract

Background and Aim: During blood smear preparation, Leishman stain is first added to the slide for fixation. Then, aqueous fluid (e.g., distilled water [DW] and buffered water) is added and mixed with the stain. Different books suggest different amount of aqueous fluid to add with the stain. With this background, the aim of this study was to compare the quality of staining of blood smears stained by Leishman stain with different amount of DW. **Methods:** A total of 4 glass slides, with blood smear were stained by Leishman stain with different amount of DW – half the amount, equal the amount, 1½ times the amount, and double the amount of stain. After washing and drying, these blood films were seen under microscope, and the quality of staining was rated by 10 observers on 10-point scale. Scores for smears were compared by one-way ANOVA in Microsoft Excel 2010. **Results:** The mean score for blood film stained with half DW was 7.5 ± 0.71, double DW was 7.9 ± 0.74, 1½ times DW was 7.7 ± 0.67, and double DW was 7.6 ± 0.52. There was no difference in mean score ($P = 0.58$) among the films when tested by ANOVA. **Conclusion:** There was no difference in quality of staining among smears stained by Leishman stain with different amount of DW. Hence, any volume of DW ranging from half the amount to double the amount of stain may be suggested for staining. However, half the amount may be avoided due to chances of stain precipitation.

Keywords: Blood smear, hematology, Leishman stain, photomicrography, staining and labeling

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Introduction

Romanowsky and Malachowski first described a combination of oxidized methylene blue and eosin for staining blood smear. Then, after 10 years, it was modified by Leishman. He used the same combination to get a precipitate which was then dissolved in methyl alcohol.[1] This Leishman stain is widely used for staining blood smear.[2]

Undergraduate hematology practical curriculum includes preparation and examination of blood smear and differential leukocyte count. For staining the blood smear, students commonly use Leishman stain. The principal steps involved in staining of a blood smear are application of stain for fixation of smear, addition of fluid on the stain for staining, and then washing the smear. Description of the individual step of staining a blood smear differs from book to book.[1,3-7] One of the important distinguishing descriptions is found about the amount of aqueous fluid (e.g. distilled water [DW] and buffered water) to be added to the stain. For this reason, academicians frequently faced the question from students – "How much amount of fluid to add – equal or double the amount of stain?"

This question was the triggering factor for this study. To get a credible answer, this study was aimed to stain blood smears by Leishman stain with different amount of fluid and to compare the quality of staining in those smears.

Materials and Methods

This experiment was conducted in the hematology laboratory of the Postgraduate Department of Physiology, MKCG Medical College, Berhampur, Odisha, during the month of October 2017.

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Preparation of Leishman stain

With the help of laboratory technician, 1 L of methyl alcohol was measured and kept in a conical flask. One and half gram of commercially available Leishman powder (manufactured by Merck Specialties Private Limited, Mumbai, Maharashtra, India) was grinded properly to reduce any large particles. This fine powder was then mixed with the alcohol in the flask. The flask was kept in warm environment of approximately 37°C for 7 days. After that, the stain was filtered with the help of filter paper. Filtration was repeated after 1 day to reduce any procedural errors.

Preparation of blood smears

Twenty glass slides were cleaned properly to make it dust and grease free, if any. Venous blood was collected by venipuncture on antecubital vein in commercially available ethylenediaminetetraacetic acid vial. Then, single drop of blood was added on each slide near one end of the slide. The smear was made according to slide wedge method.[8] Among the 20 slides, 4 slides were selected for staining. The smear was air dried and kept for staining.

Staining procedure

The slides were kept on two glass rods, fixed temporarily on a tray. First, Leishman stain was applied on the slide with dropper to cover the smear. Then, one minute was allowed for fixation of the smear. After that, DW was added to the stain and mixed with gentle air blow on the fluid. Ten minutes were allowed for staining. Then, the slides were washed with tap water to get a pinkish tinged color.

The first slide was stained with 8 drops of stain and 4 drops of DW, 2nd slide was stained with 8 drops of stain and 8 drops of DW, 3rd slide was stained with 8 drops of stain and 12 drops of DW, and 4th slide was stained with 8 drops of stain and 16 drops of DW.

Observation of the slides

Blood films were observed under ×100 objective lens (i.e., oil immersion objective lens) with ×10 eyepiece magnification. Four microscopes were used to focus the slides. The microscopes were arranged randomly to prevent any observer bias. We recruited 10 observers who observed the films and rated the quality of staining in a 10-point scale where 10 denotes "perfectly stained" and 0 denotes "unacceptable quality of staining." Selection of observers was on the basis of convenience. Only verbal consent was obtained from the observers for participation in the study.

Photomicrography

The department where the study was conducted is not equipped with microscope with facility of photomicrography. Hence, we used simple smartphone adapter for digital photomicrography (sSADP) with a smartphone for capturing image through the eyepiece.[9] A set of 4 images from 4 focused blood films were captured and stored. These images were captured with vignette (i.e., black around circular blood film). We cropped the image from the center of the smear with 4:3 ratios for presenting those in this article.

Table 1: Mean scores for the quality of staining of blood films prepared with Leishman stain

	Amount of DW against the amount of Leishman stain				*P*
	Half	Equal	One and half	Double	
Scores	7.5±0.71	7.9±0.74	7.7±0.67	7.6±0.52	0.58

P: *P* value of one-way ANOVA, DW: Distilled water

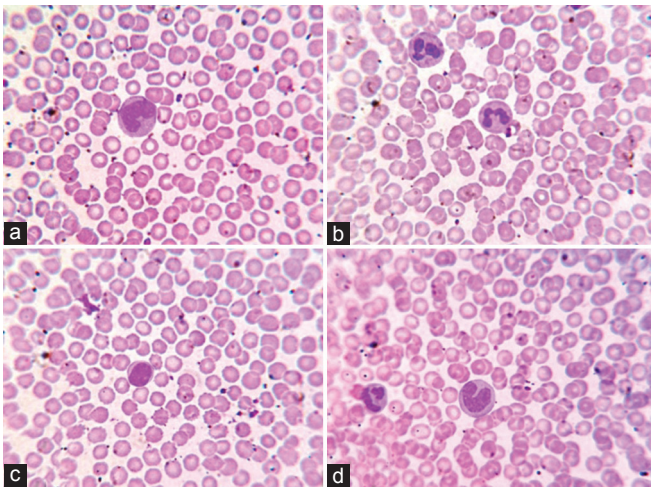

Figure 1: Photomicrographs of stained blood smears captured from microscopes by smartphone camera aided with simple smartphone adapter for digital photomicrography; smears were stained with Leishman stain and different amount of distilled water - (a) half the amount, (b) equal the amount, (c) 1½ times the amount, and (d) double the amount of stain

Statistical analysis of data

Rating of 10 experts was coded and analyzed by one-way ANOVA with $\alpha = 0.05$. Statistical analysis was carried out in Microsoft Excel 2010.[10]

Results

A sample of photomicrographs captured by smartphone camera aided with sSADP from 4 microscopes is shown in Figure 1. Scores for quality of staining of 4 categories of slides are shown in Table 1. There was no statistically significant difference ($P = 0.58$) among the scores for the quality of staining in 4 categories of blood films.

Discussion

It is well-known fact that each batch of Leishman stain comes with an instruction for required time for staining.[11] That may be the reason for different suggested time of staining in different books. We listed the descriptions of Leishman staining provided in different textbooks available in the departmental library in Table 2.

Majority of the practical physiology and hematology books (5 out of 7) indicated that the time may vary according to

Table 2: Procedure of staining of blood smear by Leishman stain, as described in different books

Author, title of the book, edition, year of publication	Time for fixation (min)	Fluid for staining	Amount of fluid	Time for staining (min)	Fluid for washing	Time for washing (min)
Turgeon, Clinical Hematology Theory and Procedures, 5th, 2012[1]	3-10, variable	BW/TW	-	2-5, variable	TW	-
Bain and Lewis, Dacie and Lewis Practical Haematology, 11th, 2011[2]	2	Water	Double	5-7	BW	2, variable
Ghai, A Textbook of Practical Physiology, 8th, 2013[3]	1-2, variable	DW/BW	Equal	6-8, variable	DW	0.5, variable
	0.5	Water	Double	15	TW	0.0166
Jain, Manual of Practical Physiology for MBBS, 5th, 2016[4]	2, variable	BW	Equal	10, variable	TW	2, variable
	2, variable	DW	Double	10, variable	TW	2, variable
Mahapatra and Mahapatra, Essentials of Medical Physiology Practical, 1st, 2011[5]	1-1.5, variable	DW/BW	Double	7-10, variable	TW	-
Pal and Pal, Textbook of Practical Physiology, 4th, 2016[6]	1.5-2, variable	DW	Double	7-10, variable	TW	-
Pramanik, Manual of practical Physiology and MCQs Book, 5th, 2015[7]	1.5-2	BW	-	10	TW	-

BW: Buffered water, DW: Distilled water, TW: Tap water, -: No description available

the different batches of stain [Table 2]. This time can be checked from the literature available with the stain or from the laboratory technician. Hence, there may not be any confusion about it.

During the time period, when Leishman stain is left with aqueous fluid, the stain gets ionized and the actual staining takes place. There are discordant descriptions for the amount of fluid in different textbooks. Hence, there may be controversies among teachers and students about the amount of fluid to add. From the result of this study, it has been established that there is no significant difference in quality of staining ($P = 0.58$) among the blood films [Table 1] stained with different amount of DW. Hence, any amount ranging from half the amount of stain to double the amount can be used for staining blood smears.

This study was conducted in a controlled environment to prevent any excessive evaporation of alcohol of the stain or to prevent drying of slides after application of DW. In a common hematology practical room in medical colleges, this level of precautions may not be possible. Hence, volume of DW half the amount of stain may not be a wise choice for hematology practical classes due to higher chance of drying of the stain and fluid. Hence, it may be a better choice to use DW equal to double the amount of Leishman stain.

A glass slide with ideal smear usually requires 6–10 drops of Leishman stain. Then, if more than double DW is used, there may be chances of spilling out of fluid from the glass slide. For this reason, in this study, we did not test the staining with DW more than double the amount of stain.

Limitations of the study

This study has some limitations. Limited observers rated the quality of the stained slides for only 4 slides. The rating scale had two defined points with 10 denoting "perfectly stained" and 0 denoting "unacceptable quality of staining;" the scoring from 2 to 9 were subjectively decided by each observer. We used DW for staining and tap water for washing as it is practised by students in the institution where we conducted the study. Staining by buffered water, even washing with buffered water, is suggested in literature which we did not practice in this study. Further studies considering these factors would enlighten on this issue in the future.

Conclusion

There is no major difference in quality of blood smears, stained with Leishman stain, with different amount of DW ranging from half to double the amount of stain. However, amount of DW to half the amount of stain may not be suitable for staining as there may be chances of precipitation of stain on the slides if the fluidity is not maintained. Hence, it is recommended that any amount of DW between equal and double the amount of stain may be used according to necessity.

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Conflicts of interest

There are no conflicts of interest.

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