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Blood Smears and the Use of Wright's Stain

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A correctly made blood smear which is properly stained and competently observed has been called the single most valuable laboratory examination in medicine. Not only is it simple to perform, but it also plays a vital role in screening patients, in establishing differential diagnoses, and in following the history of a disease and the effect of treatment. It is, therefore, essential that the preparation of good well-stained films be mastered. The purpose of this paper is to describe the proper techniques used in making blood smears and in staining these smears with Wright's stain. The mechanism of action of Wright's stain is also discussed. It is hoped that this paper will provide an aid to those who wish to employ the blood smear as a diagnostic tool.

Making the Blood Smear

Before any stained smear can be used for a diagnosis, it is imperative that proper technique be used in smear preparation. If it is poorly made, the stained smear will have no value and worse still, may be seriously misleading. There are 2 procedures for making blood smears: the coverglass method and the slide method. Many hematologists feel that a well-made coverglass smear is the more satisfactory. Slides are less desirable for differential counts because the large leukocytes tend to be pushed to the edges of the smear, while the smaller leukocytes remain scattered throughout the smear. However, the coverglass method is a more time-consuming and difficult technique to perform. The slide method is perhaps more applicable to large animals practice where blood smears may be prepared at the farm, while the coverglass method is preferable under controlled conditions in the laboratory. The type of technique used is not as important as the ability to produce well-made smears. One should try both methods and become proficient in the one found easier.

Before either type of smear is made, it is absolutely essential that scrupulously clean glassware be used. The use of disposable slides is the best way to ensure cleanliness. If it is necessary to re-use slides, they should be washed with soap and water, rinsed with hot water followed by a distilled water rinse, and stored in 95% alcohol in a closed container until needed.

The direct smears should be made from freshly drawn venous blood with no anticoagulant or with EDTA anticoagulant. If heparin is used, blood more than 1-2 hours old should not be smeared because after this period of time the anticoagulant causes distortion of the white blood cells.

Coverglass Method

1. Pick up a coverglass with one hand—thumb and index finger on adjacent corners.
2. Hold the coverglass over a large single drop of blood and gently touch the coverglass to the blood (the drop formed by capillary attraction should be about 2-5 mm in diameter).
3. Just before the spreading of the blood is

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complete, grasp the protruding corner of the coverglass on top with the thumb and index finger and separate these glasses in a quick, steady, absolutely horizontal motion. (Care should be taken to avoid any squeezing together of the coverglasses.) The coverglasses must be separated with no deviation from the horizontal.

6. Allow smears to dry.

**Slide Method:**

1. Place a clean, grease-free slide on the tabletop.
2. From a microhematocrit tube or other source, carefully place a small drop of blood (less than 3 mm in diameter) in the middle of the slide approximately 1 cm from the end.
3. With thumb and index finger of one hand hold the 2 edges of the slide on the other end (farthest from the drop).
4. Hold the spreader slide with the thumb on one edge and the other four fingers on the other edge. (This spreader slide can be similar to the slide on the table or may be a special slide with a finely ground edge and a slightly narrower width. However, the spreader slide edge should be perfectly smooth and free from chips.)
5. Place the end of the spreader slide in contact with the slide on the table slightly in front of the drop of blood. (The angle between these 2 slides should be between 25° and 40°.)
6. Draw the spreader slide back toward the drop of blood.
7. As soon as the spreader slide comes in contact with the drop, it will fan out along the width of the edge of the spreader slide. If this does not occur, wiggle the spreader slide a little until it does so. Be careful that the blood does not get in front of the slide.
8. Keeping the spreader slide at a constant angle, and the edge firmly against the slide on the table, push the spreader slide smoothly and rapidly over the entire length of the slide. The spreader should be pushed along with approximately the same speed that one uses to strike a match.
9. Allow the smear to dry.

The most important requirement in preparing a blood smear is that the morphological elements are spread out intact or with as little damage as possible. If the smears are made correctly, the cells will be spread smoothly and evenly with the red blood cells lying flat and with the edges of the cells just touching rather than overlapping. In the slide method, a good smear is one in which there is a gradual transition from the thick area to the thin area and the thin area does not extend to the end of the slide. 1, 3, 5

When producing a smear, the most common source of error is using a drop of blood that is too large in diameter (it should be no greater than 3 mm). 3, 5, 7, 8 The smear can still be made if the drop is too large by touching the spreader slide to the drop so that the width of the end is covered with blood, removing the spreader from the drop, placing the spreader on another portion of the slide, and then continuing as before (from step 7). 3

Another source for error is pushing the spreader slide in a jerky motion and at a poor angle. If the spreader is moved too slowly or the angle used is too small, a thick film will be produced and it will not be readable. If the spreader is moved too rapidly or the angle is too large, a thin film will be produced, resulting in many of the leukocytes becoming aggregated at the edges and in the tail. 3, 6

Other causes of poor films are:
1. Hemolysis of cells due to moist fingers touching the slide.
2. Scratches produced by rubbing moist films immediately after preparation.
3. Holes occurring on the film due to grease on the slide.
4. Holes in the film caused by flies feeding on unfixed slides that are left unprotected. 7

After making the blood smear, the slides should be dried. It is imperative that the slides be dried completely before staining. If the smears are fixed and stained immediately after being prepared, the nuclear structure of the white blood cells might undergo denaturation resulting in disfigurement of the nuclei and peripheral cell outline which can seriously hamper precise differentiation. 7

The method by which drying should be accomplished is disputed among hematologists. One fact that is universally agreed upon is that the drying should be completed as soon as possible after the smear is made. Prolonged drying results in movement of water out of the red blood cells.

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into the plasma leading to crenation of the erythrocytes. Unfortunately, the agreement ends there. Several authors suggest hastening the drying process by waving the slide rapidly in the air. Another author suggests that an advantageous method to facilitate drying is to use table fans which blow unheated air over the slides. This author also emphatically states that drying must be performed without any artificial warming of the slides (not even in sunlight). Still another author states that waving the film over a low gas flame will hasten drying and improve results. This procedure is modified by another individual who advocates the use of slight heating with a gas flame only when aqueous stains are to be applied to the smear. (The smears are passed through the flame 3-4 times to achieve 120°C for a few minutes.) Since the common blood stains are not aqueous stains, this heat fixing of the smear is not absolutely necessary.

One must remember that it takes practice to make good blood smears. Several smears should be made on each sample and the best used for staining. Time spent learning and practicing to make good smears will be well worthwhile.

Staining the Blood Smear

After the smear has been produced properly, it is ready for Wright's staining. The mechanism of Wright's staining is a process in which both chemical and physical factors play a role. During the staining process, the stain in methanol is directly applied to the smear. The alcoholic solution tends to retain the dye, so that it is not absorbed to the protoplasm of the cell. Tissue fixing by the methanol occurs at this time. In order for the stain to act chemically, it is necessary to force the dye out of solution by the addition of buffer which precipitates the stain. Upon precipitation, the stain undergoes partial dissociation into component parts, namely eosin and the azure dyes. The azure dyes have affinity for the components of the cell which are acid in character. Therefore, molecules such as DNA and RNA will readily react with this molecule and will acquire the dye's colorimetric characteristics.

The precise role of eosin in the staining process after partial dissociation from the neutral stain is not agreed upon. One source states that the eosin molecule reacts readily with cell components which are strongly alkaline in a mechanism similar to the azures. Others state that not only does eosin act as a stain, it also plays a role as a fixative. While still other authors insist that eosin acts only as a buffer to allow Bernthsen's methylene violet (a chemical produced from methylene blue oxidation) to stain the appropriate cellular components deeply red. In any event, different cellular chemical components are stained different colors with the use of a single stain.

There are various recipes for Wright's stain application to blood smears. In fact, it is unusual to find 2 different sources who agree exactly upon the staining procedure. These variations in technique occur because of variation in the behavior of the stain and buffer and because of differences in film thickness. Wright's stain does not behave uniformly because it is an alkaline solution in which the concentrations of the dye are continually changing due to the progressive oxidizing action of the alkali. The choice of recipe is immaterial; the essential requirement is consistency in the staining technique.

Before staining, the slide can be fixed in absolute methanol. This will increase the blood cell's affinity for the stain. This step is important if the smears are to be kept for any length of time before staining. Although many authors have stated various staining recipes, the following is the staining schedule for Wright's stain published by the Biological Stain Commission:

1. Place 1 ml. of liquid Wright's stain on the blood film for 1-5 minutes, depending upon its behavior (if it is a new batch of stain, try 5 minutes; then modify as needed).
2. Add 2 ml. of phosphate buffer solution adjusted to about pH 6.5 (distilled water can be used, yet it is not advisable because its pH changes from day to day and the pH at this step is critical in the staining procedure).
3. After allowing this mixture to stand about twice as long as the undiluted stain, flood off the diluted stain with buffer solution until the thin portions of the stained film are pink.

The timing of each procedure may be
modified to give better results from the specific lot of stain used.

4. Stand the slides on end, air dry (or blot very carefully).

If performed correctly, the red blood cells should look yellowish-red and the neutrophils should have dark purple nuclei, reddish-lilac granules, and pale pink cytoplasm.11

Should one forget and leave the stain and buffer solution on too long (20-30 minutes), the cells may become over-stained. In such cases the slide may be saved by soaking it in rubbing alcohol for about 10 minutes and allowing it to dry. It can then be stained in the usual manner.3

There are faster and simpler methods to perform Wright's staining. One method is the Coplin Jar technique where the Wright's stain and buffer are kept in separate coplin jars with screw lids. The slide is simply placed into the jar of Wright's stain for 2-5 minutes and then transferred directly to the jar containing the buffer for 2-5 minutes. The slide is then rinsed with buffer or distilled water and air dried. This method's advantage over the others is that less trouble is encountered with precipitate on the stained film.5

A coplin jar filled with methanol may be used to fix the slides before staining. However, utmost care must be taken to ensure that this methanol remains free from water. It must be covered when not in use and it must be changed regularly. Anhydrous copper sulfate can be added to decrease water uptake by the methanol.5

A number of quick stains are available commercially which require only a few seconds to obtain permanent preparation. Generally these stains can be recommended for use by the busy practitioner. Delicate staining characteristics of specific granules and other fine cytological features appear to be less evident in films stained via these methods.5

The technique of producing a properly made, properly stained blood smear is a vital tool in diagnostic medicine. It is hoped that this discussion has helped those who wish to utilize this laboratory examination.

REFERENCES


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Dr. Dennis J. Carr (ISU '56), a private veterinary practitioner in Montfort, Wisconsin, was elected chairman of the Executive Board of the AVMA for 1978-79. The Executive Board is the governing body of the 29,607-member national professional association.

A native of Iowa, Dr. Carr is active in the AABP, Wisconsin Cattlemen's Association, and Wisconsin Agribusiness Council. He is also president of Carr Farms, Inc. which owns a beef and dairy farm.

Active for many years in local affairs, Dr. Carr has served five 2 year terms on the Montfort Town Board, as 4-H leader, and as president of the Lay Council of St. Thomas Catholic Church of Montfort.

Dr. Lloyd V. Fry (ISU '37) recently retired from his position with Clemson University, as chief of the South Carolina meat-poultry inspection department, with which he had been associated for the past 10 years. He retired from the Army in June '68 as Colonel with 30 years service. Looking forward to this retirement, Dr. Fry and his wife will continue to live in Columbia, S.C.

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