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A STAINING METHOD FOR THE DIFFERENTIATION OF LIVE AND DEAD SPERMATOZOA [1]

I. APPLICABILITY TO THE STAINING OF RAM SPERMATOZOA

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The increasing importance of artificial insemination during the past few years has emphasized the necessity for improved methods of semen evaluation. A number of methods for semen evaluation are of importance and among these an estimation of sperm motility seems to be the most widely used. However, this gives no criterion for determining the number of live and dead spermatozoa because a few very active sperm may produce the same apparent motility as a larger number of less active sperm. Up to the present time there has been no definite method for determining the relative number of live and dead spermatozoa in semen samples. If such a method could be developed it would be of considerable importance in evaluating fresh and stored semen to be used for artificial insemination, as a criterion for studying methods of processing semen, and as an aid in determining the degree of fertility in males. It is the purpose of this paper to present such a method and to demonstrate its accuracy.

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Several authors have noticed that the protoplasm of dead cells react to external agencies in a different way than the protoplasm of living cells. Walton ('33) stated that the tails of active sperm would coil when the semen was diluted with water, whereas the tails of the inactive sperm were not affected. He suggested this as a method for determining the relative number of live and dead spermatozoa.

Goetz and Goetz ('38) used methylene blue in the differentiation of live and dead yeast cells. They stated that it is known that a number of stains of colloidal character do not affect live microorganisms and that the dye, if not too concentrated, can stay in contact with live cells for a considerable length of time without harmful effects. They further stated that these stains enter the dead cells very rapidly.

PROCEDURE

In investigating the reaction of the sperm of farm animals to various stains it was noted that several stains would enter certain sperm but not others. In every case it was observed that the sperm in which the stain entered were non-motile before and after staining. After considerable investigation the following mixture of stains was observed to give the best results:

Stain A

2% water soluble eosin in M/8 phosphate buffer[3] (pH of 7.3).

Stain B

1 part opal blue[4] (undiluted)
1 part M/8 phosphate buffer[3] (pH 7.4) ⟶ pH 5.7

The staining mixture of one part of A and one part of B was used. This solution was approximately isotonic with semen and had a pH of about 6.7.

The semen used in these studies was collected by use of an artificial vagina from normal mature Rambouillet rams.

[3] Phosphate buffer (pH 7.4): 80.4 cc. M/8 Na_2HPO_4 and 19.6 cc. M/8 KH_2PO_4.

[4] Opal Blue Solution according to Breslau, Dr. G. Grübler and Co., Eimer and Amend, New York.

The ejacula were pooled immediately after collection. Approximately 20 minutes (at room temperature, 25° C.) elapsed between collection and treatment. Three 0.5 cc. samples of the pooled semen were placed in separate vials. One of these samples and 1 cc. of stain mixture in a separate container were immersed for 10 minutes in a water bath at 0° C. The other two samples were treated the same except that one was immersed in a water bath at 25° C. and the other at 40° C. Ten slides were made from each sample of semen at the end of the 10-minute period.

In making these slides one drop of stain was placed on a clean glass slide. A glass stirring rod was dipped in the semen sample and the semen adhering to it was immediately mixed with the stain on the slide. The flat surface of another clean slide was placed over the mixture which caused it to spread out in a thin film between the surfaces of the two slides. The slides were then drawn apart, without applying pressure, and were dried on a warm plate at 40° C.

Motility readings were taken on the remaining semen in each sample immediately after the slides were made.

The slides were studied under oil immersion at a magnification of 970 ×. In each case, 500 sperm were counted and the number of stained and non-stained sperm were tabulated. To obtain a more representative count, several fields on the slide were observed.

RESULTS

An examination of the individual sperm on these slides disclosed that the posterior portion of the sperm head stained with varied intensity from almost red to a dark purple (fig. 1) whereas the anterior part of the head usually stained a light pink but in some cases did not stain at all. The sperm that did not take the dye appeared as a clear outline against a light blue background (fig. 1). Lagerlöf ('34) used the technic of staining the background (with opal blue) in his studies of fertility of bulls.

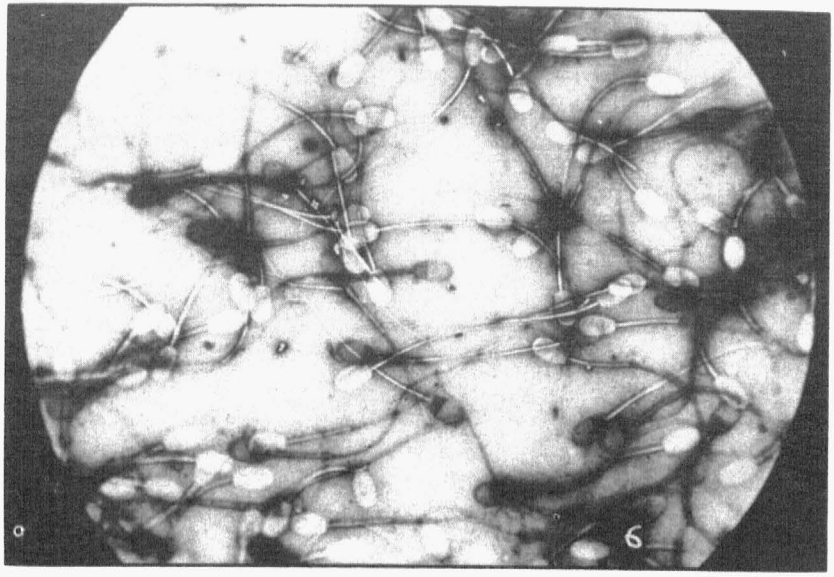

Fig. 1 Ram spermatozoa. Unstained sperm are alive. Sperm that have stained are those that were dead at the time of staining. Opal-blue-eosin × 970.

In testing the accuracy of this method of determining the relative number of live and dead sperm, ten slides at each of the three temperatures were counted by each of two observers, who have had several years experience in the microscopic examination of the sperm of farm animals. By analysis of variance, the influence of the observers doing the counting and of temperature prior to staining upon the percentage of live sperm was determined (table 1).

TABLE 1

Variance due to treatment and observers

SOURCE	D/F	VARIANCE	F-RATIO
Total	59	716.4015	....
Between temp.	2	21,035.1722	6,117.90 [1]
Between men	1	4.8088	1.40
Remainder	56	3.4383	....

[1] Highly significant (P = .01).

There was a highly significant difference (P = .01) between temperature treatments. This indicates that 0° C. and 40° C. produce an injurious effect because 10 minutes at these temperatures brought about the death of a larger percentage of the sperm (table 2). This is supported by the fact that the original high sperm motility of five was lowered to three at 40° C., to one at 0° C., but remained unchanged at 25° C. Since the effects of each of these three temperatures on the sperm were so definite and yet differed so widely, they were selected to test the repeatability of the results of the staining method on a given semen sample. No significant difference was noted between counts made at these temperatures by different observers. This indicates that the method can be used by properly trained individuals with a satisfactory degree of accuracy.

TABLE 2

Means for temperatures and men with necessary difference (P = .01) between means for significance

TEMPERATURE (DEGREES C.)	MEAN	NECESSARY DIFFERENCE [1]
0	9.78	
25	74.09	1.72
40	34.66	
MEN		
A	39.22	1.34
B	39.79	

[1] Necessary difference $= \frac{\sigma}{\sqrt{N}} \cdot \sqrt{2} \cdot t_{(P=.01)}$

The repeatability by one man counting the same sample several times is presented in table 3 in which the mean ± the standard error of the mean for each observer's count at the three temperatures is given.

TABLE 3

The mean ± the standard error of the mean for each observer's count at the different temperatures

TEMPERATURE (DEGREES C.)	A	B
0	8.69 ± .184	10.86 ± .327
25	74.64 ± .505	73.54 ± .779
40	34.34 ± .543	34.97 ± .635

It can be observed that little difference existed between counts on the same sample by the same man.

DISCUSSION

For any method to be of practical use in evaluating the semen of farm animals it must be simple as well as accurate. The method described in this paper meets these requirements. Only a few minutes are required to determine the relative number of live and dead spermatozoa in a semen sample. The accuracy of this method is demonstrated in tables 1, 2, and 3 where it is shown that there is no significant difference in the counts made by two trained men on the same slides, nor is there a significant difference in counts made on several smears of the same semen sample.

It can be demonstrated in numerous ways that only sperm which are non-motile and cannot be reactivated are stained. If a mixture of stain and fresh semen is placed on a slide and observed under a microscope, it can be seen that only non-motile sperm take the stain. If one continues to observe the sperm until the mixture dries, one can also see that none of the non-stained sperm take the stain at the time of drying. Occasionally a non-active sperm is observed which does not stain. However, inactivity of sperm cells does not mean that they are dead. Moore, Mayer and McKenzie ('40) among others, observed that some samples of ram semen which showed no motility after storage could be reactivated by the addition of a buffer. We have observed similar semen samples and have found that smears which were made before and after activation show approximately the same proportion of dead sperm as determined by this staining method.

In addition we treated individual semen samples by methods, which in the experience of this laboratory, have been efficient in killing or irreversibly inactivating all the sperm so treated.

Drying, freezing, heating to 50° C. for 10 minutes or the addition of a toxic substance such as undiluted alcohol were

the methods selected to kill the sperm. No matter which of the above methods was used, all of the sperm was stained.

Storage of ram semen at high or low temperatures results in a progressive increase in the number of sperm which cannot be reactivated either by the addition of a suitable buffer, dilution or temperature change (Moore, Mayer and McKenzie ('40) and unpublished data of the authors). Smears were made of ram semen at different intervals during storage at low temperatures and stained by the method described in this paper. In agreement with the above observations, a progressive increase in the number of stained sperm was noted as the storage time increased.

Apparently, then, our results thus far would indicate that this new staining technique stains dead sperm or sperm which have reached a state of irreversible inactivation and fails to stain live (active and potentionally active) sperm.

The decrease in the number of non-stained sperm (tables 1, 2, 3) at 0° C. is probably due to an injurious effect. That this cooling injures the sperm may be demonstrated by the fact that there is no increase in the number of non-stained sperm when this same semen is rewarmed to 20° C. for 10 minutes. This is in agreement with the work of Chang and Walton ('40) in which they observed that when ram semen was cooled very rapidly to low temperatures the sperm were injured as shown by a lower rate of respiration.

The decrease in the number of non-stained live sperm in samples which were held for 10 minutes at 40° C. is probably also due to an injurious effect. Walton ('33) stated that a temperature of 46° C. would kill sperm immediately.

Since the spermatozoa of farm animals are very sensitive to changes in environment, it is recommended that standardized conditions should be followed in applying this staining method. This makes it possible to eliminate a number of variables in comparing semen samples. On the basis of available information we would recommend that both the stain and semen should be brought to a definite temperature between 20–30° C. and the smears made and dried at this temperature.

(Further adjustments for drying may have to be made in regions of high humidity.)

The staining mixture should be kept in an icebox, when not being used, to keep down bacterial growth and prevent chemical changes which might be injurious to the sperm.

Preliminary observations indicate that this method of staining can also be applied to the semen of man, the rabbit, boar, bull and stallion. Investigations are now in progress to determine the stain mixtures which will give the greatest distinction between the live and dead sperm in the semen of these species.

CONCLUSIONS

1. A method has been described for determining the relative number of live and dead spermatozoa in semen of the ram.

2. A statistical analysis of the accuracy of this method demonstrates that the differences between trained individuals making the counts are insignificant. Furthermore, there is only a slight variation in counts made on different smears of the same semen sample.

3. Data and results herein presented indicate that this new staining technique stains dead sperm or sperm which have reached a state of irreversible inactivation and fails to stain live (active and potentially active) sperm.

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