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# EFFECT OF TECHNIQUES OF PREPARING SEMEN SMEARS FOR STAINING ON THE MORPHOLOGY OF BULL SPERMATOZOA

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**D**ETERMINATION of the proportion of morphologically abnormal spermatozoa in stained semen smears was formerly considered as one of the best means of estimating the potential fertility of a breeding male (Williams and Savage, 1925; and Moench and Holt, 1931). More recently, the reliability of this method has been questioned (Dougherty and Ewalt, 1941; Herman and Swanson, 1941; and Lasley and Bogart, 1943). Little recent evidence has been obtained to show direct correlation between the proportion of abnormal and relative fertility in the bull (Trimberger and Davis, 1942; and Mercier, 1946) but no proof is yet available that abnormal spermatozoa need no longer be considered in routine semen examination. Direct evidence on this question is needed. Before sound evidence can be obtained easy and precise methods by which the proportion of abnormal spermatozoa in bull semen can be determined routinely in any laboratory need to be developed.

Work in this laboratory revealed that the procedures used in preparing smears for staining might have an influence on the proportion of tailless heads and abnormally-formed spermatozoa observed. Also, evidence was obtained suggesting that in fertile semen the true relationship between abnormal and relative fertility might be obscured by considering the tailless spermatozoa as abnormal (Salisbury *et al.*, 1942; and Mercier, 1944, 1946).

On the other hand, experience of the authors and others has shown that one of the changes accompanying seminal degeneration, whether caused by nutritional deficiencies (Cunningham and Hopkirk, 1935), extreme changes of temperature of the testes (Chang, 1943), or some pathological disturbance (Williams and Savage, 1925), is the appearance of tailless spermatozoa in the semen.

These facts suggested a need for further investigation of the methods of preparing smears for staining in order to differentiate the tailless heads produced in the male genital tract and those artificially made.

Thus, three experiments were carried out to study the effects of the methods used in making, clearing and staining smears on the proportion of tailless heads and on the proportion of abnormally-formed spermatozoa in fresh and stored semen of fertile bulls.

<sup>1</sup> The data contained in this report are from a thesis presented by Ernest Mercier to the Graduate School of Cornell University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1946.

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### Experimental Procedures and Results

The two methods of making smears compared in this paper are referred to as the "pulling" and the "drop" methods. They are methods 1 and 4 described by Salisbury *et al.* (1942). In this study 0.1 ml. of semen was mixed gently with 1.0 ml. of a 3.6 percent solution of  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ , and used for every smear. For semen stored at room temperature sulfanilamide was added at the rate of 300 mg. per 100 ml. of the citrate buffer to prevent bacterial growth (Knodt and Salisbury, 1946).

The smears, when cleared, were immersed in a 1.0 percent solution of chlorazene and then stained by the methods previously described by Salisbury *et al.* (1942), though the order of staining was reversed (Mercier, 1944). Only 100 spermatozoa were examined on each smear (Salisbury and Mercier, 1945), and the morphological abnormalities recorded according to the classification followed by Mercier (1944). All the tailless sperm were recorded separately and only the abnormally-formed tailless heads were included in the number of true abnormal.

The data of each experiment were analyzed by the method of analysis of variance as described by Snedecor (1946).

#### *Experiment 1, Fixing of Smears*

In order to study the effect of fixation by heat or albumin on the results obtained with the two methods of making smears with fresh or stored semen the first experiment was conducted. Four ejaculates were mixed and diluted with citrate buffer containing sulfanilamide. Of the 48 smears made with a part of this sample on the day of collection, 4 of them (two made by each method, one with and the other without albumin) were not heated. Five groups of 4 smears each were heated immediately on a slide warmer at a temperature of  $36.5^\circ\text{C}$ . for a period of 2, 4, 8, 16 and 32 hours, respectively. The remaining 24 smears were given the same heat treatment 24 hours after having been made. The other part of the diluted sample was stored at room temperature for a period of 4 days and used to replicate the first part of the experiment. Five days later, the 96 smears were cleared and stained in a home made 100-slide rack for the purpose of standardizing as much as possible the staining procedure. They were examined three days later. The pertinent data are presented in table 1.

The use of albumin fixative and the heating of smears either immediately or 24 hours after they were made did not influence significantly the proportions of tailless heads and abnormal. Therefore, the data are not presented separately for these two items. Similarly, the length of time smears were heated did not affect the morphology of spermatozoa.

Highly significant differences between methods occurred with respect to

the tailless heads and the abnormals. These results agree with those obtained by Salisbury *et al.* (1942) and by Mercier (1946) and, as discussed later, were believed to be due to a difference in the thickness of the smears. In this study the smears made by the pulling method were much thinner than those made by the drop method, because a part of the 1.0 ml. of diluted semen used for each smear was squeezed out when the upper slide was put on the bottom slide and both pulled apart lengthwise.

More tailless heads were found in smears made with fresh than with

TABLE 1. EFFECT OF METHODS OF MAKING AND HEATING SMEARS ON THE MORPHOLOGY OF SPERMATOZOA IN FRESH AND STORED SEMEN

Hours of heating	Average percent of abnormal spermatozoa											
	Tailless heads				True abnormals				Total			
	Fresh semen		Stored semen		Fresh semen		Stored semen		Fresh semen		Stored semen	
	Pulling	Drop	Pulling	Drop	Pulling	Drop	Pulling	Drop	Pulling	Drop	Pulling	Drop
0	10.25	23.00	9.25	9.25	16.75	27.25	24.00	23.50	25.00	48.75	33.00	32.00
2	11.00	16.50	5.75	17.25	19.00	29.75	20.50	26.75	29.25	44.50	26.00	39.75
4	4.75	20.50	3.50	14.25	11.50	30.50	17.75	28.25	15.75	49.00	21.00	41.75
8	5.00	26.50	7.75	5.25	18.25	35.00	13.50	27.50	23.00	59.25	21.00	31.25
16	4.50	35.75	7.00	13.25	15.75	28.25	18.50	32.75	19.75	62.00	24.75	44.50
32	12.50	27.50	4.25	16.25	10.50	27.50	15.75	25.25	22.50	53.00	20.00	40.50
M.M. <sup>1</sup>	8.00	24.96	6.25	12.58	15.29	29.71	18.33	27.29	22.54	52.75	24.29	38.29
S.M. <sup>1</sup>	16.48		9.42		22.50		22.81		37.64		31.29	
G.M. <sup>1</sup>	12.95 ± 8.22				22.66 ± 6.97				34.47 ± 12.37			

<sup>1</sup> M.M.=mean for each method; S.M.=mean for each kind of semen; G.M.=general mean for each of the three spermatozoa classifications.

stored semen and the difference was highly significant. These results suggest that the dead spermatozoa were more elastic and more resistant to physical treatment than live ones. Moench (1929) found that greater force was required on microneedles to pull body pieces from the heads of dead than from the heads of live human spermatozoa. Death was reported to increase the toughness and elasticity of human sperm cells. The percentage of morphologically abnormal spermatozoa was approximately the same in smears made with either fresh or stored semen.

#### Experiment 2, Clearing of Smears

The second experiment was carried out to compare the two methods with smears of uniform thickness made thin purposely so that half of them might

be stained uncleared. By this means, a comparison of the effect of the clearing process on the proportion of tailless heads and abnormally-formed spermatozoa in smears made with fresh and stored semen was possible.

Forty smears (20 made by each method, 10 to be stained cleared and 10 uncleared) were made with part of a fresh, mixed semen sample composed of two ejaculates. After 8 days of storage at 5° C. the remaining portion of the semen sample was used to replicate these 40 smears. Smears to be cleared

TABLE 2. EFFECT OF METHODS OF MAKING AND CLEARING SMEARS ON THE MORPHOLOGY OF FRESH AND STORED SPERMATOZOA

Smears made with	Percent of abnormal spermatozoa					
	Tailless heads		True abnormalities		Total	
	Pulling	Drop	Pulling	Drop	Pulling	Drop
Fresh semen						
Cleared	11.6	15.8	18.5	16.7	28.3	32.1
Uncleared	3.7	9.1	14.0	13.7	16.7	22.2
Average	7.65	12.45	16.25	15.20	22.50	27.15
Stored semen						
Cleared	8.0	3.4	15.3	16.0	22.8	19.1
Uncleared	3.6	4.1	12.6	13.4	15.7	16.8
Average	5.80	3.75	13.95	14.70	19.25	17.95
M. M. <sup>1</sup>	6.72	8.12	15.10	14.95	20.87	22.55
C. M. <sup>1</sup>	9.70		16.62		25.57	
U. M. <sup>1</sup>	5.12		13.42		17.85	
G. M. <sup>1</sup>	7.42 ± 4.08		15.02 ± 3.78		21.71 ± 4.73	

<sup>1</sup> M. M. = individual mean for each method; C. M. = mean for cleared smears; U. M. = mean for uncleared smears; G. M. = general mean for each of the three spermatozoa classifications.

were cleared simultaneously. Then, both the cleared and uncleared smears were stained together.

The averages for the percent of abnormal spermatozoa for each group of ten smears are given in table 2. More tailless heads and abnormally-formed sperm were found in cleared than in uncleared smears made with both the fresh and the stored semen. The differences were highly significant statistically. Also, the proportion of tailless heads was significantly greater in smears made with fresh than with stored semen.

These results suggest several important points. First, a large proportion

of the tailless heads in normal semen may be produced during the clearing process. Second, fresh spermatozoa are more fragile than stored ones. These two findings are additional evidence that such tailless heads are probably artifacts. Third, there is evidence in this experiment that more morphologically normal spermatozoa than abnormal spermatozoa were removed from semen smears during the clearing with chlorazene, because the proportion of true abnormalities remaining on the slides was significantly higher on the cleared than on the uncleared slides. Morphologically abnormal spermatozoa apparently have a greater adhesive surface than do normal spermatozoa perhaps due to chemical changes in those abnormalities in a stage of decomposition. Thus, the clearing process is believed to be responsible not only for producing a portion of the tailless heads, but for increasing the proportion of true abnormalities observed.

The insignificant difference between the two methods of making smears in respect to tailless heads was in agreement with an experiment conducted by Mercier (1946) in which the smears made by the two methods were uniformly thick. The difference in proportion of morphologically abnormal spermatozoa between the two methods of making smears found in the first experiment was not observed in the second experiment. It appears, therefore, that this difference was a result of the thicker layer of semen on the slides prepared by the drop method.

While no statistical difference was found between the means for the two methods of making smears a significant method x semen interaction was observed, namely, more tailless heads were produced by the drop method with fresh semen whether cleared or uncleared.

### *Experiment 3, Shaking of Semen and Staining of Uncleared Smears*

On the basis of the above results a third experiment was conducted to determine if the shaking of semen which occurred in routine handling before shipment to artificial insemination units had an influence on the proportion of tailless heads. In the experiment the routine amount of shaking was compared with approximately double this amount. All smears were made by the drop method and purposely thin so that they could be stained uncleared. Thirty fresh ejaculates of semen from 12 fertile bulls were used. The results are given in table 3.

These data indicate that moderate handling was not an important cause of tailless heads. Furthermore, when the major cause of the tailless heads was known, namely, the clearing process, and that particular step in the staining procedure was eliminated, plus careful handling at every other step, stained semen smears were made in which the presence of tailless heads was almost eliminated.

While not shown in the table an analysis of variance of the data showed that there were no significant differences between bulls in the proportion of tailless heads produced by the method of making these stained smears. These results show that when the above-mentioned precautions were taken the semen of all bulls studied reacted similarly to the techniques employed. This is the first time in our series of investigations that no significant differences were found between fertile bulls in the proportion of tailless heads contained in their semen.

TABLE 3. THE EFFECT OF SHAKING SEMEN ON THE MORPHOLOGY OF SPERMATOZOA (UNCLEARED SMEARS)

Shaking	Percent of abnormal spermatozoa		
	Tailless heads	True abnormalities	Total
Routine	1.20	6.13	7.07
2×routine	1.13	6.47	7.30
Mean	$1.1167 \pm 1.1155$	$6.25 \pm 1.29$	$7.2 \pm 1.55$

This fact suggests that the preparation of thin smears to be stained un-cleared with aniline gentian violet for one minute, rinsed, dried, and counter-stained for 0.5 minute with Ziehl's carbol fuchsin eliminates the proportion of tailless heads which may be considered as artifacts.

### Summary and Conclusions

In three experiments conducted to determine the effects of the methods used in making, fixing, and clearing smears and the effect of shaking semen on the proportion of tailless heads and on the proportion of abnormally-formed spermatozoa in smears made with fresh and stored semen of fertile bulls it was found that:

1. The "pulling" and "drop" methods of making semen smears gave the same results when the smears were of uniform thickness.
2. Fixing of smears by heat or by albumin did not influence the proportion of tailless heads or true abnormalities.
3. Clearing of smears with 1.0 percent chlorazene was responsible for producing most of the tailless heads and increased the proportion of true abnormalities. Normally-formed tailless heads may be artifacts and, consequently, should be included among the true abnormalities only when the staining procedures do not produce them.
4. Spermatozoa in fresh semen were much more fragile and more subject to breakage during the clearing process than stored spermatozoa.

5. With the semen of fertile bulls the staining of thin, uncleared smears eliminated most of the tailless heads. This method of preparation of smears for staining should eliminate the tailless heads which are artifacts, and should enable the investigator to distinguish those tailless heads which are truly abnormal.

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