New Technique for Assessing Acrosomal Characteristics of Spermatozoa

M. E. WELLS and O. A. AWA
Department of Animal Sciences and Industry, Oklahoma State University, Stillwater 74074

Abstract

A series of exploratory studies calculated to test the efficacy and potential application of a new, differential acrosome stain is presented. The stain, a combination of eosin B and fast green FCF, was effective in revealing acrosomal characteristics of spermatozoa in both fresh and frozen semen samples. The presence or absence of the acrosome could be determined as well as its condition, i.e., smooth and entire, swollen or disintegrating, and degree of attachment to the sperm cell. The staining process is simple and acrosomal evaluation is done with the oil immersion objective of a light microscope. There was a highly significant correlation (P < 0.01) between the incidence of acrosomal abnormalities observed with the Wells-Awa stain and that observed with either the Giemsa stain or bright-field phase microscopy.

Species studies indicate that the stain effectively elucidates the acrosome of bull, ram, boar, stallion, and rabbit spermatozoa. These facts strongly indicate that the Wells-Awa stain may be a valuable addition to routinely used semen evaluation measures.

Introduction

Anomalies of the acrosome cap are classified as primary abnormalities of spermatozoa and have been shown by several investigators to be associated with lowered fertility or complete sterility in bulls and boars (1, 8, 15, 16, 18). Some acrosomal abnormalities are known to be hereditary (6, 8, 10, 19) and several studies (3, 5, 11, 12, 13) have also shown that environment can cause irreversible, destructive changes in the acrosome. Saacke et al. (17) suggested that wide day-to-day variation in the incidence of acrosomal abnormalities was real, indicating that some environments can have an effect on acrosomal integrity.

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1Data in this paper are from thesis submitted by O. A. Awa in partial fulfillment of the requirements for the M.S. degree.

The use of phase contrast, ultraviolet, and electron microscopy has provided valuable details concerning the formation, structure, and function of this important component of spermatozoa. Various stains have also been employed routinely for several years to assist in determining morphological characteristics of spermatozoa. Acridine orange, in conjunction with ultraviolet radiation (2) and the Giemsa stain (9, 17), give good definition of the acrosome. Saacke et al. (17) suggested that the Giemsa stain could be used for screening bulls with acrosomal cap defects. However, the complexities of instrumentation or length, or both, of the aforementioned approaches have tended to limit their usefulness mostly to research laboratories. For routine, commercial use, the evaluation of acrosomal characteristics should be a procedure that can be accomplished with the conventional light microscope and is readily adaptable to laboratory time schedules.

A series of studies in our laboratory has brought about the development of a differential acrosome stain for assessing acrosomal characteristics. This paper reports the staining technique involved, its efficacy in definition of the acrosome, and potential use as a rapid means of acrosomal evaluation with the conventional light microscope.

Experimental Procedures

Staining technique. The differential acrosome stain is prepared by combining one volume of a 1.0% solution of eosin B (88% total dye content), 2.0 volumes of a 1.0% solution of fast green FCF (90% total dye content), and 1.7 volumes of ethyl alcohol.2 These same stains, in different proportions and phosphate buffered, were used by Mayer et al. (14) in a combination which differentially stained live and dead spermatozoa. However, this combination does not stain the acrosome. Exhaustive tests in combining eosin B, fast green FCF, and ethyl alcohol established the aforementioned combina-

2A patent application will be filed pending the favorable outcome of a patentability search.
Fig. 1. Normal bull spermatozoa stained with the Wells-Awa acrosome stain (oil immersion, × 2,425).

Fig. 2. Bull spermatozoa with acrosome abnormalities, one with no acrosome and one with a swollen, disintegrating acrosome (oil immersion, × 2,425).

Fig. 3. Bull spermatozoa with swollen acrosomes and one free acrosome stained with the Wells-Awa acrosome stain (oil immersion, × 2,425).

The Wells-Awa stain has a distinct advantage over other acrosome stains in that the preparation of specimens for acrosomal assessments is neither lengthy nor complex. In this study, the diluting and staining solutions, test tubes, and slides were maintained at 37 C on a slide warming table. Ejaculates were maintained at 30 C in a beaker of water. For fresh ejaculates, with normal concentration, mix one drop of raw semen with ten drops of 2.9% sodium citrate dihydrate in a 5-ml test tube. The dilution rate should be less if the ejaculate is dilute. Mix one drop of the sperm cell suspension with one drop of the prepared stain and let stand for one minute at 37 C. Place one small drop of this mixture on a clean microscope slide, smear with a second slide, and air dry on a 37 C slide warming table. Duplicate slides were prepared and permanent slides, permitting storage and repeated use, were prepared by mounting a coverslip with Diaphane. Very thin smears are necessary; thick smears obliterate the differential character of the stain. The stain has to be prepared immediately before use for optimum results. Stains from two different sources were used, with no apparent difference in staining characteristics. In occasional ejaculates, it was noted that a percentage of the cells would not be stained. Successful staining of such ejaculates was accomplished by mixing the diluted semen and the stain in a 1:2 ratio and allowing this mixture to stand for one to two minutes before smearing on the microscope slides. Using the oil immersion objective of a light microscope, several features of the acrosome can be ascertained. The criteria used in this study included a) presence or absence of the acrosome (Fig. 2), b) degree of attachment to the sperm head, and c) the condition of the acrosome, i.e., whether it was smooth and entire, swollen or irregular (ruffled) in shape, disintegrating or elevated and knobbed, or both, or incomplete (Fig. 2 and 3). A total of 200 sperm cells, 100 per smear, was counted to assign the sperm cells to the various categories. A normal acrosome was defined as being closely adherent to the sperm cell head, smooth and entire in shape (Fig. 1). Typical abnormal conditions observed included absence of the acrosome, swelling or thickening, especially at the anterior tip of the sperm cell, and irregularities in the shape of the acrosome.
Staining efficacy. Acrosomal abnormalities observed with Giemsa-stained smears (17) and bright-field phase microscopy preparations were compared with the values obtained with the Wells-Awa stain. In Experiment 1, simultaneous smears were made with the Giemsa stain and Wells-Awa stain on 23 ejaculates collected over a six-week period from five bulls. In Experiment 2, Wells-Awa-stained smears on 14 ejaculates were compared with simultaneously prepared bright-field phase microscopy specimens. The phase-microscope specimens were prepared by diluting the fresh semen in a drop of distilled water. These ejaculates were collected from five bulls over a four-week period.

An indication of the variation associated with acrosomal determinations with the described procedure was secured by making ten separate preparations and readings on a single ejaculate of each of three bulls, all collected on the same day.

Limited studies were conducted to determine the efficacy of the stain in revealing acrosomal characteristics of bull spermatozoa stored at −196 C and undiluted spermatazoa stored at 5 or 37 C. Exploratory studies were undertaken to determine the efficacy of the Wells-Awa stain for staining the acrosome of other species.

Results and Discussion

A normal cell stained with the Wells-Awa stain is shown in Figure 1. Abnormal acrosomes, either enlarged or ruffled in appearance, are shown in Figure 2. Detached acrosomes, an abnormality in the fresh ejaculate, are easily observed (Fig. 3), being a translucent green in color and having a bathing-cap shape, as described by Blom and Birch-Andersen (4). The capless sperm cells are stained a very pale red color in the anterior portion of the nucleus; the posterior is stained a more intense red. Quite often the diameter of the anterior fraction is somewhat reduced, showing clearly that the acrosome cap has been detached.

Experiment 1. Abnormal acrosome counts on 23 Giemsa-stained ejaculates are compared with Wells-Awa-stained portions of the same ejaculates in Figure 4. These are averaged by collection days, to show that there was considerable variation in the percentage of acrosomal abnormalities among collection days. However, there was a highly significant correlation (P < 0.01) between the values obtained by the two methods (r = .56). It was consistently easier to see and ascertain acrosomal morphology with the Wells-Awa stain than with the Giemsa stain. Staining intensity and definition were more consistent among ejaculates using the Wells-Awa procedure. Capless sperm were typically easier to see in the Wells-Awa preparations.

Experiment 2. Fourteen ejaculates from five bulls were used to compare abnormal acrosome counts, using the Wells-Awa procedure with counts obtained with bright-field phase microscopy.
Table 1. Capless spermatozoa in frozen semen.

<table>
<thead>
<tr>
<th>Bull</th>
<th>Observations per ampule</th>
<th>Capless sperm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.9</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.0</td>
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<td>4</td>
<td>5.2</td>
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<tr>
<td>3</td>
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<tr>
<td></td>
<td>2</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.8</td>
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<td>5</td>
<td>1</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Mean 4.8%  
Standard error 0.27

Table 2. Results of repeated observations on single ejaculates of three bulls.

<table>
<thead>
<tr>
<th>Bull</th>
<th>Abnormal acrosomes (%)</th>
<th>Mean</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10.95</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>11.10</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>23.75</td>
<td>0.33</td>
<td></td>
</tr>
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</table>

Each ejaculate mean is the average of ten separate measurements on a single ejaculate of each bull.

copy. These ejaculates are grouped by bulls in Figure 5. There was wide variation among weekly ejaculates within bulls as well as among bulls. However, the correlation between the values obtained by the two methods ($r = .55$) was highly significant ($P < 0.01$). It should be noted that sperm cells with abnormally shaped acrosomes are much easier to discern with phase-contrast microscopy than are sperm cells with the acrosome missing. No such difficulty is encountered in the Wells-Awa procedure, as both conditions are clearly visible.

The correlations obtained in the above studies, although statistically significant, are not indicative of the reliability of the Wells-Awa procedure in determining acrosomal characteristics of an ejaculate. A measure of reliability was secured by making ten separate determinations of the percentage of acrosomal abnormalities in ejaculates from each of three bulls. The means and standard errors of these determinations are presented in Table 2 and show that close agreement can be expected on successive determinations. Table 2 further illustrates that differences in acrosomal characteristics among bulls can be detected satisfactorily. Consistency in detecting such differences is enhanced by very thin smears of stained sperm cells. On heavily stained slides, the differential character of the stain tends to be obliterated and the state of the acrosome is less distinguishable. Therefore, the authors recommend the smearing technique used in preparation of slides for differential blood cell counts.

Exploratory studies had previously indicated that the percentage of abnormal acrosomes could be drastically increased by incubating an
ejaculate at 37 C. The sensitivity of the Wells-Awa stain in detecting possible progressive acrosomal changes was tested in the following study. One ejaculate from each of three bulls was split in two portions. One portion was incubated, undiluted, at 37 C and sampled at two-hour intervals up to 12 hours of incubation and again at 24 and 48 hours, while the other portion was refrigerated, undiluted, at 5 C for seven days and sampled daily to determine rates of change in the percentage of abnormal acrosomes. The effects of these treatments are compared in Figures 6 and 7. The percentage of acrosomal abnormalities in the incubated fractions rose rapidly from an initial average of 16.2 to 67.1% after 12 hours of incubation and progressed to 85.7% after 24 hours of incubation. The fractions refrigerated at 5 C did not show as rapid an increase, but did rise gradually over the six-day observation period from an average of 16.2 to 67.2% abnormal acrosomes. In addition, Figure 6 shows that there were obvious differences among bulls, in that the 37 C temperature had a greater effect on some ejaculates in the first 12 hours of incubation. Figure 8 shows that changes in the kinds of acrosomal abnormalities can also be followed. In this case, sperm cells were categorized according to whether the acrosome was missing or was abnormal (ruffled or elevated). As is shown in Figure 8, the percentage of capless sperm did not change in the first 12 hours of incubation, whereas the percentage of sperm with ruffled or elevated acrosomes rose rapidly. The elevated acrosomes eventually became detached, as is reflected in a rise in the percentage of capless cells over the next 36 hours. Because of this changing status, the percentage of cells with abnormal acrosomes decreased during the same period of time. Such detected differences establish the sensitivity of the Wells-Awa stain.

These experiments have established that the Wells-Awa acrosome stain is effective in revealing the acrosomal characteristics of ejaculates. This effectiveness, coupled with the simplicity of specimen preparation and observation, strongly indicates that this procedure may well be a significant advance in acrosomal evaluations.

Preliminary studies showed that the stain did not work well on citrate-egg yolk diluted frozen semen. However, successful staining of frozen semen was accomplished by centrifuging the sample for three minutes at 5,000 x g, resuspending the sperm cells in a similar volume of 2.9% sodium citrate, centrifuging for another three minutes, and resuspending again in 2.9% sodium citrate. From this point, the staining process was the same as with the diluted fresh ejaculate. One ampule on each of five bulls was thawed and the sperm cells recovered as just described. The effectiveness of this recovery procedure can be seen in Table 1. Four counts (two in one instance) of capless sperm cells were made per ampule. The mean percentage of capless cells observed was 4.8% with a standard error of 0.27. This again indicates the reliability of the method for determining acrosomal characteristics of a population of sperm cells. In these samples, the acrosome cap was observed to be slightly elevated. This could be due either to the freezing or to the recovery process.

Species application. The Wells-Awa stain and procedure was effective in differentially staining the acrosome of the spermatozoa of the bull, ram, stud, rabbit, and rat. The staining characteristics of the spermatozoa of these species are the same as those described for the bull. Satisfactory staining of the acrosome of boar sperm cells required that the diluted sperm cells be in contact with the stain for five minutes before smearing. The acrosome of the boar covers at least three-fourths of the sperm cell head (see Figure 9). Further species investigations are continuing.

Conclusion

Research continues to indicate that acrosomal abnormalities can have an effect on fertility (13, 16, 17). However, the relationship among kinds and levels of acrosomal abnormalities and fertility is not established. Techniques for acrosome valuation have tended to limit widespread investigation of this important component of sperm cells. This paper presents a method for differentially staining the acrosome. The percentage of abnormalities determined with the

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**Fig. 8.** Changes in distribution of types of acrosomal abnormalities in ejaculates incubated, undiluted, at 37 C. Each line represents the average of three ejaculates, each from a different bull.
Wells-Awa stain was highly correlated (P < 0.01) with those detected with the phase contrast microscope and the Giemsa stain. The Wells-Awa stain is effective in revealing acrosomal differences among bulls, collection days, and weekly ejaculates of bulls, as well as acrosomal changes resulting from an imposed treatment. The stain is effective on the spermatozoa of the bull, boar, ram, stud, rabbit, and the rat. These facts, coupled with the simple staining and evaluation procedure, strongly indicate that this new stain has real utility in the quest for further information on the role of the acrosome in mammalian reproduction.

References