Evaluation of Coomassie blue staining of the acrosome of equine and canine spermatozoa

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Objective—To evaluate Coomassie blue staining of the acrosome of equine and canine spermatozoa.

Sample Population—Spermatozoa of 5 mixed-breed male dogs and 3 Thoroughbred stallions.

Procedure—Various proportions of intact and acrosome-damaged spermatozoa were fixed in 2% phosphate-buffered formaldehyde or 4% paraformaldehyde, smeared onto glass slides, and stained with Coomassie blue stain. Acrosomal status (damaged vs intact) was also assessed by use of flow cytometry after staining with fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (FITC-PSA) and propidium iodide. Comparisons were made between percentages of expected and observed acrosome-intact spermatozoa in different proportions of live and flash-frozen samples; the percentages of acrosome-intact spermatozoa as determined by use of Coomassie blue staining and flow cytometry were also compared.

Results—Strong correlations were found between the expected and observed distributions of acrosome-intact spermatozoa when fixed in 4% paraformaldehyde ($r^2 = 0.93$ and 0.89 for canine and equine spermatozoa, respectively) as well as between Coomassie blue-stained cells and those stained with FITC-PSA and assessed by use of flow cytometry ($r^2 = 0.96$ and 0.97 for canine and equine spermatozoa, respectively). However, in canine samples that were fixed in 2% phosphate-buffered formaldehyde, these correlations were weak.

Conclusions and Clinical Relevance—Staining with Coomassie blue stain was a simple and accurate method to evaluate the acrosome in equine and canine spermatozoa after fixation in 4% paraformaldehyde. This assay should be useful in routine evaluation of semen samples from these species. (Am J Vet Res 2006;67:358–362)
Materials and Methods

In the first experiment, 5 mixed-breed male dogs that ranged in age from 14 to 24 months were used. Three of these dogs were subsequently used for a second experiment. In experiments 1 and 2, 3 Thoroughbred stallions that ranged in age from 5 to 21 years were used. All animal research was conducted under protocols approved by the University of California Animal Use and Care Administration Advisory Committee. Semen was collected into a latex artificial vagina via digital manipulation (dogs) or with an artificial vagina (horses). Only the spermatozoa-rich fraction of the ejaculate was collected, and raw semen was transported back to the laboratory in insulated containers for evaluation and processing within 30 minutes after collection.

Experiment 1—Spermatozoa were flash frozen to disrupt the acrosome and mixed with live spermatozoa in defined proportions (100%, 75%, 50%, 25%, and 0%) to evaluate staining with Coomassie blue stain (modified from the method of Larson and Miller11). The expected and observed proportions of acrosome-intact spermatozoa were compared.

To evaluate whether the method of fixation had an effect on evaluation of the acrosome of canine spermatozoa, approximately 0.25 mL of raw semen (1 ejaculate from each of 5 dogs) was fixed in 0.8 mL of freshly prepared 4% (wt/vol) paraformaldehyde (in PBS solution [pH, 7.4]; stored in aliquots at −20°C until immediately prior to use) and a second aliquot of semen was fixed in 2% PBF (5.4 mL of 37% formaldehyde in 94.6 mL of PBS solution) prior to staining. An additional aliquot of semen (0.5 mL) was flash frozen in liquid nitrogen for 1 minute and thawed in a water bath (37°C) 3 times to provide a population of nonviable, acrosome-damaged spermatozoa. Flash-frozen semen was fixed in either 4% paraformaldehyde or 2% PBF as described. The fixed samples were centrifuged (300 × g for 6 minutes), the supernatant was removed, and the pellet was suspended in 0.5 mL of ammonium acetate (0.1M). At least 200 spermatozoa/slide were evaluated at a concentration of 50 to 100 X 10⁶ spermatozoa/mL as determined by use of hemocytometer counts.

After washing in ammonium acetate, smears of fixed spermatozoa were prepared, air dried, and stained by dipping the slide in Coomassie blue stain. Slides were incubated at 21°C for 90 seconds, rinsed with double-distilled water, and allowed to air dry. Stained smears were evaluated via bright-field microscopy (1,000X magnification) to determine acrosomal status at least 200 spermatozoa/slide were evaluated by a single observer who was not aware of treatment status. The acrosome was defined as intact (uniform dark-blue staining overlying the entire acrosome region), damaged (patchy staining over the acrosome region), or nonintact (total absence of staining or staining only in the equatorial segment).

For evaluation of equine acrosomes by use of Coomassie blue stain, raw semen (1 ejaculate from each of 3 stallions) was fixed in 4% paraformaldehyde (1:1). Phosphate-buffered formaldehyde (2%) was not used because the results with canine spermatozoa indicated a high rate of nonspecific staining of acrosome-damaged spermatozoa after fixation. Raw equine semen was flash frozen and fixed in 4% paraformaldehyde to provide a population of acrosome-damaged spermatozoa. The fixed samples were washed twice in ammonium acetate to remove the fixative, as described. The samples were suspended to a concentration from 50 to 100 X 10⁶ sperm/mL as determined by use of hemocytometer counts. Air-dried smears were prepared as described. The air-dried smears were stained by dipping in Coomassie blue stain (3 times, with incubation at 21°C for 20 seconds each time) followed by dipping in Coomassie blue stain and incubation at 21°C for 2 minutes. Slides were rinsed with double-distilled water, allowed to air dry, and evaluated under immersion oil (1,000X magnification) via bright-field microscopy (minimum of 200 spermatozoa/slide) as described.

Experiment 2—The purpose of the second experiment was to compare acrosomal staining of equine and canine spermatozoa by use of Coomassie blue stain (with bright-field microscopy) with staining by use of FITC-PSA and flow cytometry.4,12,13

For this experiment, 1.0 mL of raw semen (3 ejaculates for each species) was layered onto a 40%/80% discontinuous density gradient6 and centrifuged at 300 × g for 20 minutes to remove seminal plasma and provide an enriched population of normal spermatozoa. The resulting pellet was suspended in 2 mL of a modified Tyrode solution4 and centrifuged at 300 × g for 10 minutes. The supernatant was removed, and the spermatozoal pellet was resuspended in the solution at approximately 200 million spermatozoa/mL. Approximately half of the sample was flash frozen as described, and live and flash-frozen samples were then combined to create the 100%, 75%, 50%, 25%, and 0% live proportions. Aliquots were taken from each of the proportional samples and fixed in 0.4 mL of 4% paraformaldehyde at a final concentration of 25 X 10⁶ spermatozoa/mL. Slides were prepared from the fixed samples, stained with Coomassie blue stain, and analyzed as described.

A separate aliquot of each of the proportional samples was evaluated by use of flow cytometry. Spermatozoa were diluted in modified Tyrode solution to 10⁶ spermatozoa/mL, 2.5 µg of FITC-PSA was added, and samples were incubated for 10 minutes at 37°C. Immediately prior to flow cytometric evaluation, 12µM PI was added to samples. Samples were evaluated with a flow cytometer at an excitation wavelength of 488 nm within 15 minutes of staining. The FITC-PSA was detected with a 530/30 bandpass emission filter, and propidium iodide was detected with a 660/20 bandpass filter. To determine the forward and side scatter of the population of spermatozoa to be analyzed, flash-frozen spermatozoa that were stained with propidium iodide were used to backgate the population of interest. Approximately 10,000 gated events/sample were analyzed.6

Figure 1—Regression analysis of percentages of canine spermatozoa (n = 8 ejaculates from experiments 1 and 2) fixed with 4% paraformaldehyde and stained with Coomassie blue stain for intact acrosomes. Y-axis indicates percentages of observed stained cells; X-axis indicates percentages of expected stained cells (0%, 25%, 50%, 75%, and 100%). The regression equation is y = 2.73 + 0.88x (r² = 0.93).
Statistical analysis—Statistical analyses were based on linear regression analysis. Because a significant (P ≤ 0.05) difference was not detected between the distribution of acrosome-intact, Coomassie blue-stained spermatozoa in experiments 1 and 2, the data were combined for regression analysis against the expected percentages of acrosome-intact spermatozoa in the proportional samples. For purposes of the analysis, flash-frozen samples were considered 0% acrosome intact and the live sample was considered 100% acrosome intact. For experiment 2, the percentage of acrosome-intact spermatozoa as determined by use of the Coomassie blue method was plotted against the percentage of acrosome-intact spermatozoa as determined by use of flow cytometry and analyzed via linear regression. Data are presented as mean ± SEM.

Results

Experiment 1—When canine or equine spermatozoa were fixed in 4% paraformaldehyde and stained with Coomassie blue stain, there was a strong linear relationship between the expected and observed proportions of spermatozoa with intact acrosomes (Figures 1 and 2). In both species, the acrosomes of intact spermatozoa were stained with Coomassie blue stain, whereas spermatozoa damaged by repeated freeze-thaw had patchy or completely absent staining (Figures 3 and 4). Although equine spermatozoa were stained for a longer time, acrosomal staining of equine spermatozoa appeared fainter than that in canine spermatozoa, although the acrosome was clearly delineated. When canine spermatozoa were fixed in 2% PBF, there was a poor relationship between the expected and observed proportions of acrosoke-intact spermatozoa (Figure 5); the observed proportions of stained spermatozoa were less than expected. For this reason, fixation with 2% PBF was not used in the subsequent study with equine spermatozoa.

Experiment 2—Flow cytometric analysis of FITC-PSA–stained equine and canine spermatozoa revealed a strong linear relationship between the proportions of
expected and observed acrosome-intact spermatozoa (for equine, y [observed proportion] = 9.32 + 0.87x [expected proportion; \( r^2 = 0.92 \)]; for canine, y = 0.32 + 0.94x \( r^2 = 0.95 \)). In spermatozoa disrupted by flash freezing, 2.7 ± 1.7% and 6.7 ± 1.9% of canine and equine spermatozoa, respectively, had intact acrosomes as determined by use of FITC-PSA staining and flow cytometric analysis. Conversely, for spermatozoa in the 100% live population, 98.4 ± 0.3% and 91.1 ± 6.8% of canine and equine spermatozoa, respectively, had intact acrosomes (Figure 6). This observation confirmed the characterization of these 2 populations of spermatozoa as used in the proportional analyses of staining with Coomassie blue stain. There was a strong linear relationship between acrosomal status as determined by use of Coomassie blue staining and that determined by use of FITC-PSA staining of each proportional sample for both species (Figures 7 and 8).

Discussion

Results indicated that the acrosomal status of equine and canine spermatozoa fixed in 4% paraformaldehyde can be determined by staining with Coomassie blue stain. The type of fixation was clearly important because canine spermatozoa that were fixed in 2% PBF yielded poor correlation between expected and observed proportions. This may have been caused by the breakdown of formaldehyde and oxidation, which results in formic acid.15 Formic acid production leads to increased background and interaction with protein stains, which causes difficulty in analyzing samples.15-17 In the present study, Coomassie blue staining of canine spermatozoa previously fixed with 2% PBF appeared to result in a higher than expected proportion of acrosome-damaged spermatozoa that were incorrectly identified as acrosome intact because of increased staining. Results of previous studies15,16 indicate that 2% PBF solutions result in a higher level of background staining of the acrosome than fixative solutions made from paraformaldehyde.

In this study, results of Coomassie blue staining of the acrosome were strongly correlated with results of FITC-PSA staining followed by detection with flow cytometry.18,20 The FITC-PSA binds to the acrosomal matrix of spermatozoa that have damaged plasma and outer acrosomal membranes.19 For spermatozoa stained with Coomassie blue stain, the dye binds to sulfite groups in the acrosomal matrix after the plasma and acrosomal membranes have been made permeable during fixation and drying.13 Because bright-field microscopy is often more readily available in clinical situations, use of Coomassie blue stain for detection of the acrosome of equine and canine spermatozoa may be a useful tool in reproductive management of these 2 species.

In the present study, we chose to evaluate proportional distribution of populations of spermatozoa with
intact acrosomes (live spermatozoa) and spermatozoa in which the acrosome had been disrupted by repeated freeze-thaw cycles. It is important to point out that damage to the acrosome induced by repeated freeze-thaw cycles is not the same as acrosomal exocytosis that occurs in the presence of an agonist, such as the zona pellucida or progesterone. We chose the freeze-thaw method to induce acrosomal damage in a predictable manner and allow accurate proportional distribution of spermatozoa with intact and damaged acrosomes across all experiments.

It is important to consider, however, that staining with Coomassie blue as described here does not provide information concerning spermatozoal viability and cannot be used to distinguish between physiologic acrosomal exocytosis and degenerative acrosomal exocytosis, which may occur after cell death. In contrast, flow cytometric evaluation of the dual label, FITC-PSA and PI, allows simultaneous determination of acrosomal integrity as well as spermatozoa viability as indicated by membrane integrity. Nonetheless, determination of acrosomal status by use of Coomassie blue stain may be useful in routine evaluation of canine and equine spermatozoa as well as in the evaluation of spermatozoa from these species after cryopreservation, which disrupts plasma and acrosomal membranes. The addition of a suitable stain for determination of sperm viability along with Coomassie blue stain followed by evaluation via bright-field microscopy would be useful for clinical application of this staining procedure.

References