A METHOD FOR DIFFERENTIAL STAINING OF BOVINE SPERMATOZOA AFTER EXTENSION IN STERILE MILK

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Introduction

A differential staining technique, using eosin B and opal blue, was developed for staining spermatozoa by Lasley *et al.* (11) in 1942. Since then a number of other differential staining techniques have been developed (1, 2, 3, 4, 7, 14, 15, 18).

Extenders have been employed in artificial insemination to increase the volume of semen. Some of the extenders, particularly those opaque in nature, have made viability examinations difficult even with the use of phase contrast microscopy. Sterile milk extender (12) which has been used routinely in the Department of Animal Husbandry, University of Guelph, falls into this category.

The purpose of this paper is to outline a technique for differential staining of bovine spermatozoa after extension in sterile milk. Trypan blue was selected as it has been used as a differential stain for tissue culture cells, leucocytes and human and dog spermatozoa (10, 19, 20, 22). It is an acid dis-azo dye with a molecular weight of 961, and has a solubility of 1% in neutral distilled water at 15° C. with a pH of 9.1. India ink, which is non-toxic to sperm, was selected as a contrast medium.

Materials and Methods

A 1% aqueous solution of trypan blue[1] was prepared, filtered and sterilized. The contrast medium used was India ink.[2]

Staining Method

Add 0.1 ml. trypan blue to a 1 ml. aliquot of extended semen.
Mix thoroughly.
Incubate for 15 minutes at 37° C. in a water bath.
Remix and place one drop of the mixture on a slide previously heated to 37° C.
Add an equal amount of India ink.
Mix gently.
Take a small drop of this mixture and place on a clean slide previously warmed to 37° C. and make a thin smear.
Dry in air or by a warm air current.

Toxicity Test

It was noted that some differential stains tend to be toxic when added to extended semen and stored overnight at 5° C. It was felt advisable to determine the toxicity of trypan blue to spermatozoa. The stain must be in contact with the cells for 15 minutes at 37° C. during the incubation period. An arbitrary period of storage of five days at 5° C. was selected.

Three separate ejaculates obtained from the same bull were used on three different days. From each of 10 vials of glycerolated extended semen, 100 spermatozoa were counted for percentage live cells just after the addition of glycerol. To 10 vials of glycerolated semen 0.1 ml., or three drops of trypan blue, were added. The vials were sealed and kept for five days in storage at 5° C. Ten remaining vials, sealed and stored at 5° C. for five days, served as untreated controls. On the fifth day of storage, the treated vials were removed and incubated at 37° C. for 15 minutes in a water bath. Slides were prepared and 100 cells per slide counted for the percentage of live and dead spermatozoa. These were compared with the controls. Analysis of variance was carried out on the data using arcsin transformation (24).

Osmotic Pressure

Osmotic pressures were taken on samples of unglycerolated milk, glycerolated milk

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[1]British Drug Houses, Toronto, Canada.

[2]Chin Chin Brand, Gunther Wagner, Germany.

and glycerolated milk plus trypan blue using an osmometer.[3]

pH and Tonicity

pH readings were taken on samples of glycerolated milk and glycerolated milk plus trypan blue.

Results

Interpretation of Slides

Dead cells appear blue in a grey to black background depending on the thickness of the smear. Some cells are stained only in the posterior half of the head. These partially stained cells may be counted as dead although they may only be stressed.

Osmotic Pressure

The osmotic pressures, as determined by freezing point depressions, were 292 milliosmols for unglycerolated milk, 2300 milliosmols for glycerolated milk and 2000 milliosmols for glycerolated milk plus trypan.

pH and Tonicity

The pH of trypan blue was 9.1. When 0.1 ml. of trypan blue was added to a 1 ml. aliquot of extended semen, the pH was 6.85. The buffering capacity of the milk extender is such that the small volume of dye would not affect the tonicity.

Toxicity Test

In Series One, 56.67 versus 55.32 approached the statistically significant level. In Series Two, 50.67 versus 50.13, and in Series Three, 58.06 versus 57.93, were not statistically significant. The results are summarized in Table I.

Discussion

Emik and Sidwell (6) using eosin and opal blue reported a relationship between the loss of motility and the staining of spermatozoa which commenced at the base of the head. In our experience, using 1 ml. aliquots of extended semen and 0.1 ml. trypan blue in routine motility examinations, it was observed that color did not appear at the basal portion of the head until 15 minutes after the cessation of observable motility. Cells which had been immobile for a long time stained throughout the entire head. Dott (5) also observed partial staining of spermatozoa using nigrosin-eosin. A similar effect was obtained in this laboratory using trypan blue and India ink. It would appear that cells showing partial staining have been inactivated recently or have been subjected to stresses such as cold shock or old age. Hafez (9) reported that sudden cooling of spermatozoa resulted in loss of motility, disintegration of the cell membrane and changes in cell permeability. White and Wales (23) have postulated that dead cells stain because of a change in permeability which results in a loss of ions and other substances and a disruption of the cell surface.

Blackshaw (3) reported eosin diffuses into cells after a few hours. Trypan blue tends to diffuse into previously unstained cells when on unfixed slides. Madden (13) suggested that a representative number of spermatozoa can be counted before that phenomenon manifests itself.

Mixner and Saroff (17) reported levels of glycerol greater than 4% increased the permeability of the cells and altered the staining reactions of a 0.8% eosin and fast green FCF combination; there was a decrease in the percentage of live cells disproportionately to the percentage motile. Using a solution of either 3% congo red or 0.6% eosin stain with a 5% solution of nigrosin as a background stain, Blackshaw (3) found that glycerol up to 15% did not affect the staining reaction. In our experience, however, 10% glycerol content has not been observed to affect the staining reaction with trypan blue.

Media with a pH of about 6.8 and having the same tonicity as blood or seminal plasma appeared to be ideal for spermatozoa survival (9). Spermatozoa are affected less by hypertonic than by hypotonic conditions in the range of 50 to 150% of normal tonicity. Swanson and Beardon (21) reported a pH range of 6.4 to 8.5 had no deleterious effect on staining reaction using eosin-nigrosin. Dott (5), using eosin-nigrosin, observed higher ranges of pH tended to increase the intensity of the red. He noted no differences in staining

[3]Advanced Instruments Inc., Newton Highlands, Massachusetts.

TABLE I

PER CENT LIVE SPERMATOZOA AND ANALYSIS OF VARIANCE USING ARCSIN TRANSFORMATION

			Per cent Live Spermatozoa	Arcsin Transformation	D.F.	Variance
Series I	No storage	Non-stained	75.5	60.34	9	1.343
	Storage for five days	Non-stained	69.2	56.67	9	1.204
		Stained	67.6	55.32	9	2.421
Series II	No storage	Non-stained	79.7	63.20	9	4.230
	Storage for five days	Non-stained	59.7	50.67	9	7.765
		Stained	58.9	50.13	9	6.332
Series III	No storage	Non-stained	78.0	62.10	9	4.580
	Storage for five days	Non-stained	72.0	58.06	9	9.780
		Stained	71.8	57.93	9	1.789

reaction when the dyes were dissolved in distilled water or citrate buffer as long as the pH range was between six and eight. At this pH, tonicity of the stain had no detrimental effect on the cells.

Seminal plasma (9) has an osmotic pressure similar to blood (i.e. equivalent to 0.9% sodium chloride) which is 290 milliosmols. Since milk and blood are also isosmotic there is no appreciable change in the osmotic pressure when semen is extended in milk diluent. The addition of 10% glycerol to the milk extender which increases the osmotic pressure to 2300 milliosmols had no effect on the fertilizing capacity of bovine spermatozoa extended in this medium. The osmotic pressure of extended glycerolated semen plus the stain was lowered to 2000 milliosmols due to the diluting effect of the stain. It was concluded that the addition of stain would have no deleterious effect due to osmotic variations.

SUMMARY

A technique for differential staining of bovine spermatozoa after extension in sterile milk using trypan blue with a background contrast medium of India ink, has been presented. Effects of pH, tonicity and osmotic pressures were examined. Dead spermatozoa were stained blue against a grey background. Live and actively motile spermatozoa remained unstained. Some cells were observed to exhibit partial staining which was attributed to stress factors.

RÉSUMÉ

On a présenté une technique pour la teinture différentielle de spermatozoïdes bovins ayant passé dans un bain de lait stérile, en employant une coloration bleue sur fond contrastant d'encre de Chine atténuée. On a examiné les effets du pH, de la tonicité et des pressions osmotiques. Pour les spermatozoïdes morts on utilisa une teinture bleue sur fond gris. Les spermatozoïdes vivants et à motilité active ne furent pas affectés par la teinture.

On remarqua certaines cellules partiellement teintes et ce phénomène était attribué à des facteurs de tension.

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ABSTRACT

"Nocardiosis in a dog". R. K. Loveday. J. S. Afr. Vet. Med. Ass. 34: 273. 1963.

A NINE-MONTH-OLD male Alsatian was hospitalized, suspected of suffering from distemper. The temperature was 105° F., the conjunctivae and sclerae were injected and moist rales were heard in both lungs. There was a swollen, partially hairless area at the point of the right shoulder. The pyrexia persisted despite the administration of penicillin and streptomycin. The skin lesion assumed the appearance of a carbuncle and had to be drained. Pus smears showed numerous Gram-positive branching filaments. As the animal's condition became worse, it was destroyed. Post-mortem findings were: broncho-pneumonia and pleurisy and a large, granulomatous, purulent mass extending subcutaneously from the first right rib about two-thirds the way up the neck, and *Spirocerca lupi* in the thoracic oesophagus. Smears showed a growth of numerous non-haemolytic colonies of *Nocardia* spp., smears from 48-hour cultures showed a mixture of filaments, some branched, with rods and cocci. The colonies were rough, discreet, white on Lowenstein-Jensen and serum milk agar, but produced a pink pigment on serum agar and a pink, granular pellicle on 10 per cent serum broth. The organism was not caseolytic, did not liquefy gelatin and caused alkaline peptonisation of litmus milk. It could not be shown to be pathogenic for guinea-pigs.