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Sperm oviduct-epithelial-cell-binding assay: Development of an accurate sperm-counting method using flow cytometery and an evaluation of the sources of assay variance.

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ABSTRACT

Survival of sperm within the cow reproductive tract is dependant on the binding of the sperm to the oviductal epithelial cells (OEC) and is a prime determinant of fertility. An assay of sperm survival was set up using the relative binding of sperm to cultured OEC on multi-well plates. A rapid accurate method of counting sperm in the presence of OEC was developed. A precise volume of cell suspension was mixed with a known number of fluorescent micro-beads and the mixture demembranated with detergent, stained with a DNA-binding fluorescence probe and analysed by flow cytometry. Correlations were obtained between flow cytometry and spectrophotometer measurements (R^2 = 0.9815, P<0.001) or manual haemocytometer counts (R^2 = 0.9995, P<0.001). Sperm was loaded into a cell culture well with confluent monolayers of OEC. After incubation sperm cells remaining in suspension in the well were removed. The difference in the number of sperm in the initial sample and that removed from the well indicated the number of sperm bound to OEC. While, there were large between-well variations for the same sperm sample, the assay was still able to distinguish differences between bulls in the binding ability of their sperm. Because a minimum of 10 wells per treatment are needed to statistically detect the difference, coupled with the high effort and time requirement in preparing the culture plates, the assay is impractical for large-scale experimental assays or for routine semen assessment.

Keywords: sperm; oviduct-epithelial cell; flow cytometery; binding assay.

INTRODUCTION

Sperm binding to the cells of the oviductal epithelium (OEC) is one of the major storage mechanisms that control the *in vivo* survival of sperm within the female tract (Lefebvre *et al*., 1995). The duration and extent of this binding has a major influence on the fertility of such sperm (Pollard *et al*., 1991). Sperm treatments including freezing and thawing have a marked effect on sperm–OEC binding due to changes in the sperm membrane resulting from such treatments (Goldman *et al*., 1998). There is also large between-animal variation in sperm-OEC binding, which is thought to be related to individual differences in seminal plasma composition. Recent studies have suggested that the sperm surface has specific receptors for certain lectin complexes on the surface of the OEC and that the binding ability of this receptor is lost as the sperm undergoes the process of capacitation (Lefebvre & Suarez, 1996). The species specificity in sperm–OEC binding is thought to be due to differences in the terminal sugars on the lectin complexes (Ignotz *et al*., 2001). In addition, the protein secretions in the oviduct have marked effects on the viability and motility of sperm (Boquest *et al*., 1999)

While the effects of sperm treatments and other factors affecting sperm binding have been reported, nearly all such reports are based on use of subjective assessment of number of sperm bound to OEC segments or cultures. Very few reports contain quantitative data on actual numbers of sperm that are bound. The aim of this study was to establish a method for the quantitative measurement of sperm–OEC binding to act as an–*in vitro* measure of *in vivo* sperm survival.

METHODS

Oviduct cell collection and preparation of oviduct cell monolayers

Three cows were synchronised with CIDR devices (Macmillan & Peterson 1993), and oviducts were collected following slaughter on the day of standing oestrus. Oviducts were prepared in a laminar flow cabinet after initial dissection from rest of reproductive tract, rinsed in phosphate-buffered saline (PBS) plus 1% antibiotic-antimycotic (Gibco-BRL), and the OECs were recovered by gentle extrusion. The OECs from three animals were pooled so as to reduce between-female effects (Ellington *et al*., 1993). OECs were washed and resuspended in culture medium consisting of Dulbecco's Modified Eagle Medium Nutrient Mixture and Hams F-12 (DMEM/F12, Gibco), +10% Foetal Calf Serum (FCS) + 10µg/ml Epidermal Growth Factor (Sigma) + ITS Supplement [- insulin 5µg/ml, transferrin 5µg /ml, selenium 5ng/ml- Sigma] + 1% antibiotic-antimycotic (Ellington, *et al*., 1991). The OEC suspension was divided into two equal aliquots. One aliquot of freshly extruded cells were placed into 24-well tissue culture plates in 0.5ml aliquots/well in culture media and incubated at 38°C, in 5% CO_2. After 36hrs, unattached cells were removed during media change. Confluent monolayers formed within 4- 6 days and were used for sperm incubation and evaluation (Goldman *et al*., 1998). The remaining aliquot was counted, diluted to give a final concentration of approximately $1x10^6$ ml^{-1} in a freezing medium containing 45% FCS and 10% dimethyl sulfoxide, frozen down and stored in liquid nitrogen until required. Frozen cells were thawed as required. A vial was thawed at 37°C and added to 5ml culture media and centrifuged at 160xG for five minutes and the supernatant removed. This procedure was repeated and cells resuspended in culture media. Cell suspension was plated out as described above for freshly extruded cells.

Semen preparation

An ejaculate of fresh semen was obtained,

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concentration determined and sperm was diluted to 24 x$10^6$$ml^{-1}$ with modified sperm-TALP containing 25mM potassium and no glucose (Goldman *et al*., 1998; Parish *et al*., 1988; Padilla *et al*., 1991). Sufficient frozen–thawed semen was diluted in TALP and loaded onto a 45-90% Percoll gradient and centrifuged at 700xG for 25 minutes. Supernatant was removed and the sperm pellet was resuspended into TALP. Concentration of sperm was determined and sperm was diluted to 24 x$10^6$$ml^{-1}$.

Sperm-Binding Assay

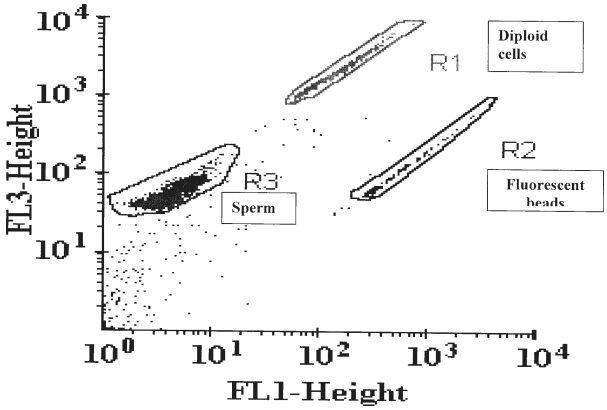
Wells in which monolayers were confluent were used to compare sperm binding properties. Each well was washed with TALP before 0.5ml of 24x 10^6/ml of fresh or frozen sperm was loaded onto each well. Sperm were incubated at 38°C, in 5% CO_2 and were assayed at 3, 24, and 48hr. Each well was microscopically assessed for health of cells, sperm binding and flagella movement. The contents of each well was carefully pipetted up and down five times and transferred to an empty marked tube. A further 500µl of TALP was added to the well and again pipetted 5 times, then pooled with the first aliquot to give a total volume of 1ml. A further 500µl of TALP was added to each well for subsequent incubations. Plates were returned to 38°C, in 5% CO_2 for further incubation. A 100µl aliquot was taken from the 1ml sperm suspension and the sperm concentration was determined.

Determination of sperm concentration in suspension

A flow cytometric technique was adapted from that described by (Everson *et al*. 1993). The 100µl aliquot of sperm suspension was placed in a TruCount'Tube (Becton Dickinson, San Jose, CA, USA) containing a lyophilised pellet of precisely known number of fluorescent micro-beads. A further 400µl of a demembranating and DNA-staining cocktail (0.15% Triton 100 and 26.4µM propidium iodide (PI) in TALP) was added, vortexed gently and incubated in the dark at room temperature for 10 minutes. The number of cells relative to the known number of beads in the suspension was established using a fluorescence-activated cell sorter (FACS) analytical flow cytometer (Becton Dickinson, San Jose, CA, USA) with a built-in air-cooled argon ion laser (excitation 488 nm). Stained spermatozoa were analysed for their fluorescence [green (FL1 - through a 515nm band pass filter) and red (FL3- through a 630nm band pass filter)] (Figure 1). The results were analysed with CellQuest™ Becton Dickinson Software. Distributions of stained cells and fluorescent beads sub-populations were acquired as dot plot cytograms for log FL1 and log FL3. Sub-populations on the cytograms were isolated and quantified using region analysis. Acquisitions were terminated when 1000 events were acquired in a beads gate. Diploid cells and debris were eliminated from analysis by gating-out on Fl-1 versus Fl-3 dot plot (Figure 1). Concentration of sperm in the suspension was calculated as:

$$\frac{\text{(concentration of beads) x (number of events in sperm acquisition gate)}}{\text{(number of events in beads acquisition gate)}}$$

FIGURE 1. A sample dot plot cytogram of the spermatozoa washed from the cell culture wells (contaminated with diploid OE cells) and mixed with fluorescent beads.



For each semen sample, two counting tubes were prepared and three flow-cytometry acquisitions from each tube were performed to give six readings for each sample.

Validation

The method was validated by comparing sperm concentrations of serially diluted semen with concentrations obtained from initial spectrophotometer measurements and dilution factors and from visual haemocytometer counts.

Determination of the level of variation in binding of sperm from the same samples to different wells in cell culture plate

Aliquots of 0.5ml of sperm suspended in TALP medium were applied into 24-cell culture wells containing OEC confluent monolayers and were incubated for 48hr. Sperm concentrations in the sample before application to the OEC wells and in the suspensions obtained at each time point were determined by flow cytometry. The number of sperm bound to the OEC was calculated as a difference between the number of sperm applied to the well and the number of sperm removed.

Statistical Analyses

Correlation coefficients between the concentration values obtained from the different methods of determination were calculated and significance tested. Means and SD values for single wells and whole plate at various incubation times were calculated. Calculations to determine the minimal number of wells necessary to resolve given 10% and 20% difference in sperm binding between treatments with 5% and 10% significance were performed with standard deviation values for the entire plate using a "power analysis spreadsheet" (Guenther, 1981).

RESULTS

Visual Assessment

The visual microscopic inspections of the wells from different treatments showed qualitative differences in the degree of sperm binding to the OEC and also for the duration of that binding. Comparisons of fresh and frozen

FIGURE 2. Correlation between sperm concentration (10^6ml^{-1}) as measured by flow-cytometry versus visual haemocytometer counts (full circles dotted line) and photometric estimations (empty circles solid line).

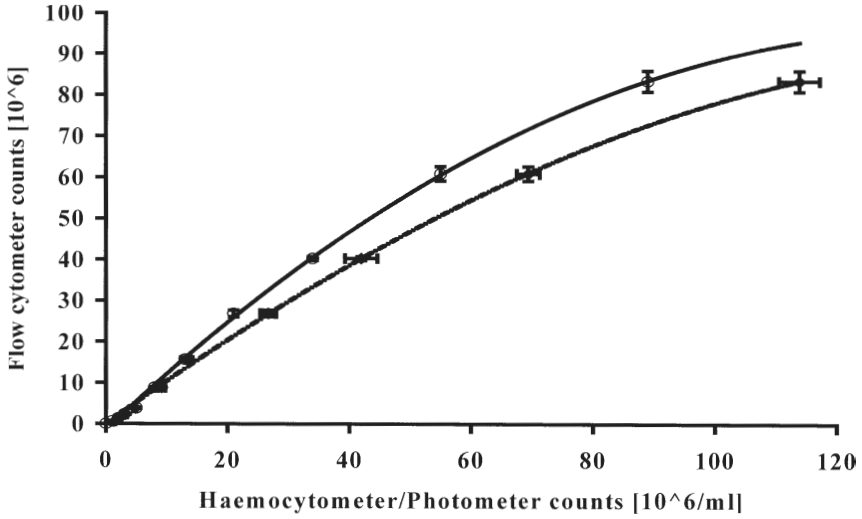


FIGURE 3. The individual well variation in sperm- oviductal epithelia cell binding (10^6 /well) after different periods of incubation

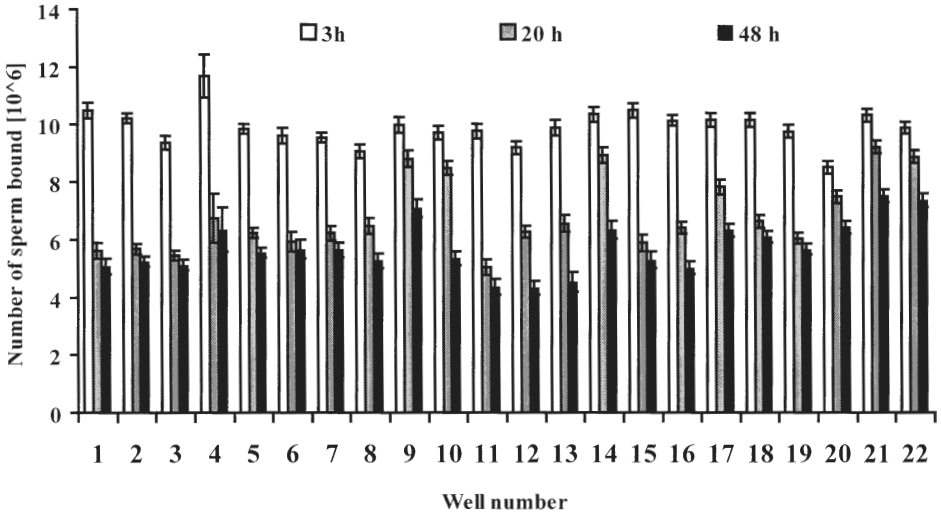
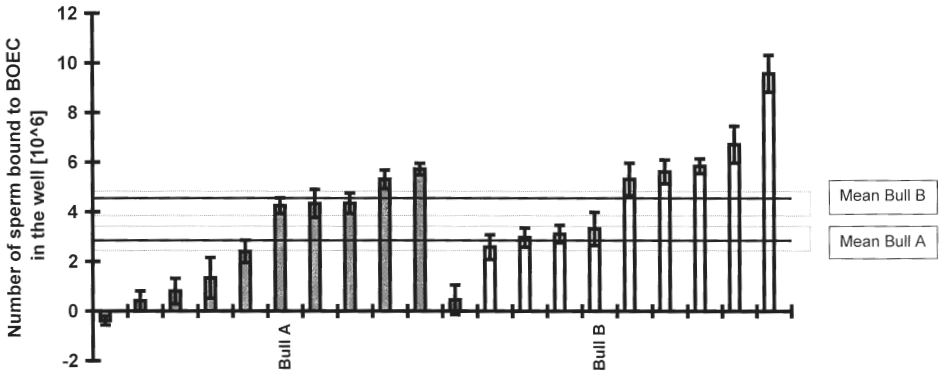


FIGURE 4. Comparison of BOEC binding ability of sperm from two bulls. Lines represent mean values off number of sperm from each bull across all wells, areas marked by dotted line represent standard errors for those means. Columns represent number of sperm from each bull bound to BOEC monolayers in individual wells.



semen from the same bull indicated that the frozen semen showed a lower degree of binding and that frozen sperm had a shorter period of attachment. Variation between bulls in the binding of their frozen semen was observed, with some samples showing little or no binding at all. These inspections also revealed that the majority of the bound sperm were still motile in that they exhibited tail movement. The well washes used to count the unbound sperm also contained small numbers of detached OEC which necessitated to need for the development of

a discriminatory counting procedure.

Sperm counting

Significant (P<0.001) correlations were obtained between measurements by flow cytometry and results derived from initial spectrophotometer readings and dilution ratios (R^2 = 0.9815) and visual haemocytometer counts (R^2 = 0.9995). Smaller standard errors were obtained with flow cytometry counts than with visual haemocytometer counts (Figure 2). The between-well variation and the changes in numbers of sperm bound to OEC over time, from a single sample of fresh bull sperm, is illustrated in Figure 3. The between-well variation actually increased over time and the effect this had on the number of wells per treatment required to determine a statistical difference between sperm samples (or treatments) is shown in Table 1. Using this information, Figure 4 shows the between well variation for two samples of fresh semen from different bulls. Again, large between-well variation was observed but the average sperm-OEC binding at 3h for the two bulls was significantly (P<0.05) different.

TABLE 1: Estimation of the minimal number of wells necessary to statistically resolve differences of 10 and 20 % in sperm – oviductal cell-binding between bulls or treatments at different times of incubation - assuming 5% and 10% significance calculated using the results presented in Figure 3.

Time	3h		24h		48h	
The difference to be resolved	**20%**	**10%**	**20%**	**10%**	**20%**	**10%**
Level of significance						
5%	9	12	23	87	18	66
10%	3	10	19	72	15	55

DISCUSSION

The lower level of binding and the shorter attachment period seen with frozen sperm is confirmatory of many previous findings in bulls and other species (Goldman *et al*., 1998). As observed by Lefebvre & Suarez (1996) in oviductal epithelium explants, sperm attached to confluent BOEC monolayers were not evenly distributed over the entire surface of the bottom of the culture well. They were densely packed in some areas, sparsely in others and were virtually absent in some. This would indicate that the monolayers were composed of several types of oviductal cells with varying ability to bind spermatozoa. Variations in the proportion of areas on the OEC monolayers that bound large number of sperm and those that did not bind sperm could explain the high between-well variation for the same sperm sample. One possible solution to this lack of uniformity is to establish a clonal immortalised cell line derived from the sperm-binding oviductal epithelial cells. Despite the large between-well variability, the assay was still able to distinguish differences between bulls in the binding ability of their fresh sperm. However, because a minimum of 10 wells per treatment are needed to statistically detect the difference, coupled with the high costs and time requirement in preparing the assay culture plates, the assay is impractical for large-scale experimental assays or for routine semen assessment. Other techniques for indirectly determining the binding ability of sperm are currently being investigated. One major concern that has arisen from the results of this project is the veracity of the conclusions drawn in previous publications that have relied on small numbers of wells or OEC explants to compare different sperm samples or sperm treatments.

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