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CRITICAL TONICITY DETERMINATION OF SPERM USING FLUORESCENT STAINING AND FLOW CYTOMETRY

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Introduction

The use of cryopreserved, rather than fresh, mammalian semen for artificial insemination confers several important medical and/or economic advantages (Watson, 1990). However, current methods for cryopreservation of both human and bovine spermatozoa result in approximately only a 50% survival rate with thawing, obviously reducing the fertilizing capacity of the semen. This effect is not generally considered of practical importance in cattle, since good fertility can be achieved by insemination with low numbers of functional sperm. Advances in techniques have been largely empirical, as the fundamentals of cryopreservation are only now being intensively studied.

A primary consideration during the cooling process is to avoid intracellular ice crystal formation with its lethal consequences to the cell. Current techniques achieve this by controlling the cooling rate. Initially upon cooling, the sample is undercooled to between -5^0 and -15^{0}C, when ice nucleates in the extracellular media. The intra-cellular water remains liquid and undercooled, putatively due to the plasma membrane blocking intracellular ice formation. As a result of the increased chemical potential of intracellular water, there is a exosmosis of water with subsequent dehydration of the cell (Mazur et al., 1984). If cooling rates are sufficiently slow, intracellular water moves out of the cell to maintain equilibrium. If cooling is too rapid, the cytoplasm will be unable to dehydrate fast enough to maintain equilibrium, resulting in intracellular ice formation once the cell reaches its ice nucleation temperature (Mazur et. al., 1984).

Computation of the time necessary for this dehydration, and hence, the cooling rate, is dependent upon knowledge of the water permeability coefficient (L_p) and its activation energy. One method of L_p determination is to measure the time to cell lysis in a hypotonic solution. To use this approach, however, one must know: 1) the value of the hypotonicity that produces lysis; and 2) the volume of water in the cell at that tonicity relative to the volume of water in the isotonic cell. If the cell behaves as an "ideal osmometer", and electron spin resonance measurements indicate that sperm do (Kleinhans et al., 1990), the relative volume of sperm water at the critical hypotonicity is simply the ratio of the isotonic osmolality to the osmolality of the lytic solution.

The fluorophore, 6-carboxyfluoroscein diacetate (CFDA), which is non-fluorescent, readily crosses the intact plasma membrane. Intracellular esterases hydrolyze CFDA to 6-carboxyfluoroscein, a fluorescent, membrane-impermeable fluorophore. Consequently, spermatozoa with intact plasma membranes fluoresce bright green (Garner et. al., 1986), but those with disrupted membranes do not.

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Therefore, the purpose of this study was to use loss of CFDA fluorescence to determine the osmolality at which 50% of the spermatozoa will swell and lyse (critical tonicity, CT). These data will then be used to determine the L_p and its activation energy for sperm, thus increasing the knowledge available in cellular cryopreservation.

Materials and Methods

Human Samples. Ejaculates were obtained by masturbation from healthy men, aged 20 to 40 years. Criteria used for ejaculates were a minimum concentration of 20 x 10^6 spermatozoa/ml and 40% motility. Semen samples were allowed to liquify for a minimum of 20 minutes, at which time they were subjected to analysis using Cellsoft (Version 3.2/C, CRYO Resources, LTD) as described by Jequier and Crich (1986) and modified by Critser et al. (1988).

Ejaculates were then subjected to a swim-up procedure (Margalloth et al., 1988) for 90 minutes. Following this, samples were centrifuged at 400X G for 7 minutes. The resulting sperm pellet was then resuspended in Mann's Ringer solution (286 mOsm, Mann, 1966). Spermatozoa were then exposed for five minutes at 20^0C to isotonic (286 mOsm) or hypotonic conditions [215, 163, 121, 92, 68, 52, 39, 30, 22, 17, and 3 mOsm, respectively, Mann's Ringer solution diluted with reagent grade water (Milli-Q, Millipore System, Ionpure Technologies Corp.)]. Sperm cells were returned to isotonic conditions by the addition of appropriate concentrations of NaCl solutions.

Bovine Samples. Semen samples were obtained from mature bulls at Purdue University using electro-ejaculation. Ejaculates were diluted 3:1 (v:v) in a TL Hepes buffer solution (Bavister et al., 1983) for transport. Approximately 1 hour later, ejaculates were subjected to analysis using Cellsoft as described by Budworth et al. (1988). Ejaculates were then subjected to all procedures used for human samples.

Fluorescent Stains. The stock solutions for the fluorescent microscope of CFDA (2 mM) were prepared by dissolving 1 mg in 1 ml DMSO (Aldrich Chemical Company, Inc.). The stock solutions for the flow cytometer were prepared by adding .25 mg CFDA in 1 ml DMSO.

Fluorescence Microscopy. Sperm cells were stained by adding 10 μl CFDA stock solution (1 mg/ml DMSO) to each 990 ul of sperm suspension and mixing thoroughly. A minimum of 100 cells per treatment were counted to determine the percentage of cells with intact plasma membranes.

Flow Cytometry. Sperm cells were stained by adding 10 μl CFDA stock solution (.25 mg/1 ml DMSO) to each 990 ul of sperm suspension and mixing thoroughly. Data were obtained using a FACStar Plus Analyzer (Becton Dickinson) and FACStar Plus Research Software. A total of 10,000 spermatozoa/treatment were counted, using a 4 Watt Argon laser operated at 488 nm, and 200 mWatts. Percentage of CDFA fluorescence was measured for each treatment, as well as forward and 90^0 light scatter.

Statistical Analyses. Data were subjected to arsin transformation and normalized to isotonic values [(hypotonic staining %/isotonic staining %) X 100]. Data were analyzed using standard analysis of variance procedures and least significant difference multiple range tests to compare membrane integrity (Steel and Torrie, 1960). Validation of flow cytometry to fluorescence microscopy on human samples was performed by analysis of variance and correlation procedures (Steel and Torrie, 1960). Analyses were performed using SAS procedures (Spector et al., 1985).

Results

Human Samples. The percentages of human spermatozoa with intact plasma membranes, as determined by CFDA staining using either fluorescence microscopy and flow cytometry after exposure to various hypotonicities, are shown in Figure 1. Both techniques indicated a sigmoidal response, in which the percentage of CFDA positive cells decreased ($P<.01$) with decreasing media osmolality. Data show a CT for human spermatozoa of between 68 and 52 mOsm (68 mOsm: 65.3 ± 5.4%; 52 mOsm: 23.2 ± 4.5%, X ± SEM, n=11).

A comparison of the data from fluorescence microscopy and flow cytometry showed great similarity ($r=.97$). Consequently, future data of human spermatozoal membrane integrity trials were collected solely from flow cytometric techniques.

Flow cytometric results of a narrower range of osmolalities, decremented in 5 mOsm steps (95-40 mOsm) are shown in Figure 2. Results indicate that 50% of human sperm lyse when placed in a media of between 60 and 65 mOsm (% CFDA positive at 60 mOsm: 41.76 ± 9.03%; % CFDA positive at 65 mOsm: 54.34 ± 8.86%, n=7).

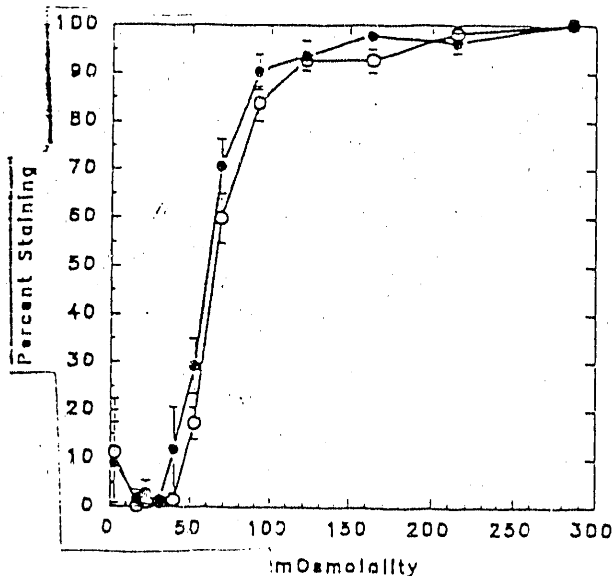

Fig. 1 Percentages of human spermatozoa with intact plasma membranes, as determined by CFDA staining, after exposure to various hypotonicities. Data were obtained using fluorescence microscopy ( ) and flow cytometry ( ). Each point represents the mean of 11 ejaculates ± SEM.

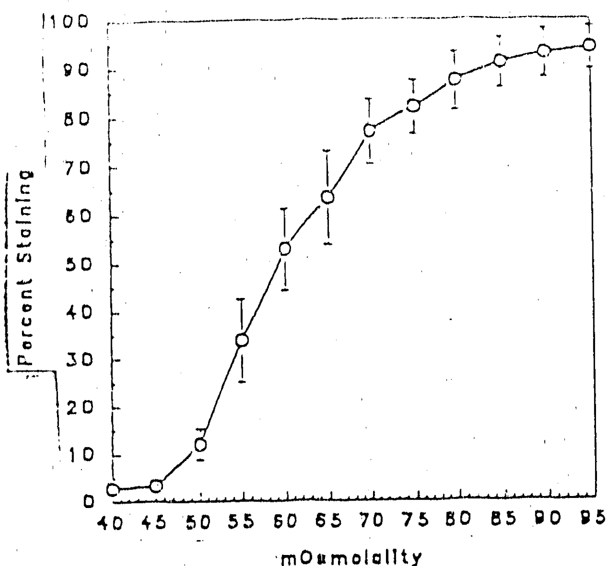

Fig. 2 Percentage of human spermatozoa with intact plasma membranes, as determined by CFDA staining, after exposure to various hypotonicities. Data were obtained using flow cytometry. Each point represents the mean of 7 ejaculates ± SEM.

Bovine Samples. The percentages of bovine spermatozoa with intact plasma membranes, as determined by CFDA staining using fluorescence microscopy after exposure to various hypotonicities, are shown in Figure 3. As with human sperm, CFDA positive bovine sperm showed a CT of between 68 and 52 mOsm (68 mOsm: 76.7 ± 9.2%; 52 mOsm: 43.7 ± 9.9%, n=6).

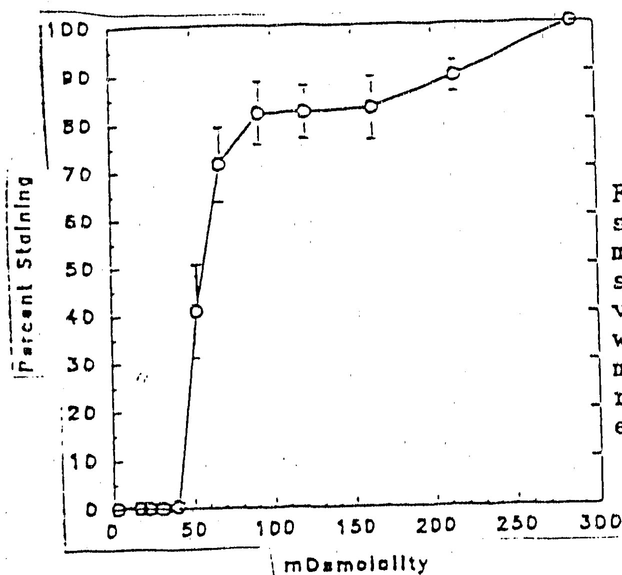

Fig. 3 Percentage of bovine spermatozoa with intact plasma membranes, as determined by CFDA staining, after exposure to various hypotonicities. Data were obtained using fluorescent microscopy. Each point represents the mean of 6 ejaculates ± SEM.

These data indicate that, for both human and bovine spermatozoa exposed to hypotonic conditions at 20°C, the cells are capable of swelling 4.2 to 5.5 times isotonic size before available membrane suface is exceeded and lysis occurs. The CT for both species lies between 68 and 52 mOsm. This approach shows considerable promise for measuring the time to cell lysis at various temperatures, from which data for L_p and its activation energy can be computed.

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