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Effects of Cryopreservation on Bull Sperm Head Morphometry

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ABSTRACT: Artificial insemination using cryopreserved semen is a common management tool of the contemporary livestock producer. However, cryopreservation is detrimental to sperm function and fertility, killing some 50% of the spermatozoa during the process. Prediction of cryopreservation damage from prefreeze samples remains elusive. Computer-automated sperm head morphometry was used in this study to determine the effects of cryopreservation on bovine sperm head morphometry.

Semen was collected from 18 bulls and was divided. One portion was extended to 200 × 10^6 sperm/ml and a microscope slide was prepared, while the remaining portion was cryopreserved in a Tris-citrate-yolk extender. After thawing, the cryopreserved samples were prepared on microscope slides. All slides were air dried and were stained with hematoxylin and rose bengal. The morphometric dimensions for length, width, width/length, area, and perimeter for a minimum of 200 sperm heads were analyzed from each slide by computer-aided sperm head morphometry analysis, and the mean measurements were recorded. Bull sperm heads were significantly ($P < 0.01$) smaller in cryopreserved spermatozoa than in the companion extended samples for length (8.56 ± 0.07 vs. 8.63 ± 0.08 μm), width (4.39 ± 0.05 vs. 4.48 ± 0.05 μm), area (28.42 ± 0.07 vs. 29.14 ± 0.08 μm), and perimeter (23.33 ± 0.21 vs. 23.70 ± 0.23 μm) for all bulls. Width/length was also different (0.513 vs. 0.519). In addition, differences ($P < 0.05$) were found within 14 of 18 bulls for at least four of the morphometric parameters. The percent change in measures after cryopreservation were correlated ($P \leq 0.05$) to the variability of the extended sample. Variations in sperm head measurements were lower ($P \leq 0.05$) in extended samples of the four bulls in which no changes occurred than in extended samples of the remaining 14 bulls. These data suggest that the variability in sperm head measurements of individual bulls, or ejaculates, may be an indicator of sperm cryosurvivability.

Key words: Morphology, spermatozoa.

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The major objective of contemporary cattle breeders is to improve the economic efficiency of producing milk and meat. Breeding for offspring that efficiently increase production of these products is not only a key element in attaining this goal but is also of great economical value. In modern cattle breeding, artificial insemination is the most widely applied tool, thereby facilitating extensive utilization of cryopreserved spermatozoa. To maximally utilize the genetics of desired sires on a commercial basis, attempts are made to package a minimal number of spermatozoa per insemination unit without sacrificing fertility (Foote and Parks, 1993; Shannon and Vishwanath, 1995). Ultimately, the number of motile spermatozoa per insemination is determined by postthaw motility evaluations and nonreturn to estrus rates from a large number of inseminations. The ability to predict postthaw sperm quality and fertility from a routine sperm function assay would be beneficial, considering the extended period of progeny testing (Garner et al, 1994).

Techniques for the successful cryopreservation of spermatozoa have slowly progressed over the past 40 years (Hammerstedt et al, 1990) and are now fairly standardized (Saacke, 1983). The effects of cryopreservation on sperm function and fertility have been widely studied, particularly in bovine. However, current techniques in cryopreservation continue to induce detrimental effects on sperm quality after thawing. For example, a significant decrease in sperm motility is universally accepted as a consequence of sperm cryopreservation in bulls (reviewed in Watson, 1995) and other species (Salamon and Ritar, 1982; Amann and Pickett, 1987) after sperm cryopreservation. This compensable trait can be adjusted for during commercial semen preservation (Sullivan, 1970; Saacke, 1983) to maintain optimal levels of fertility.

The detrimental effects of cryopreservation on various sperm organelles and viability are also known to exist (Watson, 1995). Cryopreservation has been shown to induce the acrosome reaction in spermatozoa (Watson, 1979; Valcárcel et al, 1994; Thomas et al, 1998) and to affect mitochondrial function (Jones and Stewart, 1979;

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Thomas et al, 1998). Fertility of spermatozoa, based on pregnancy rates from artificial insemination, is also compromised after cryopreservation (Ritar and Salamon, 1983; Samper et al, 1991; Valcárcel et al, 1994). Sperm chromatin structure, once believed to be stable during the cryopreservation process (Watson, 1995), is now believed to be altered during cryopreservation (Ackerman and Sod-Moriah, 1968; Karabinus et al, 1991), causing a reduction in surface area (Royere et al, 1988). In addition, cryopreservation appears to reduce the ability of sperm chromatin to decondense (Huret, 1984).

It may be possible that the observed reduction of sperm surface area (determined by microspectrophotometry) may ultimately be manifested in abnormal morphology of the sperm head. Abnormal chromatin structure has been associated with morphological abnormalities of bovine sperm heads (McCosker, 1969). A decrease in the percentage of normal sperm heads in the ejaculate has been correlated with lowered fertility in bulls (Saake and White, 1972), and overcondensation of chromatin appears to be associated with reduced fertility in men (Royere et al, 1991). Therefore, it is reasonable to believe that the adverse effects of cryopreservation on sperm chromatin and head morphology may be responsible for lowered fertility of spermatozoa observed after cryopreservation.

Whereas cryopreservation of spermatozoa has been found to affect chromatin structure and surface area of the sperm head, these changes have not been morphometrically evaluated. Computer-aided sperm head morphometry analysis (ASMA) has recently been developed to assist in accomplishing this objective. Utilizing video microscopy and computer-based digital image processing, ASMA provides quantitative metric information regarding the size and shape of the sperm head, as opposed to qualitative assessment by manual or cytophotometric methods. In general, ASMA systems image spermatozoa through a microscopic field and transfer the image to a frame-grabber board within a computer via a charge-coupled device camera. The frame-grabber board converts the virtual image into a graphic image from which the image-processing software can then perform a number of morphometric measurements (for a complete review of ASMA operation, see Davis et al, 1992). Unlike the variability inherent in subjective, manual methods of sperm morphology assessment (Saacke, 1982), ASMA has been shown to be an accurate and repeatable assay to quantify the morphometric characteristics of sperm heads (Davis et al, 1992). Previous studies utilizing ASMA have indicated that dimensions of sperm heads of subfertile males differ from their fertile counterparts (Katz et al, 1986; Gravance et al, 1996a) and are predictive of *in vitro* fertilization results (Kruger et al, 1995). Gravance et al (1997) found no effect of cryopreservation on head morphometry of cryopreserved spermatozoa when analyzed by ASMA across a population of goat bucks; however, some individual differences were observed. If sperm head morphometry, across a population as a whole, is not affected by freezing and thawing, sperm head dimensions of previously cryopreserved semen samples may be retrospectively analyzed, and the results may be correlated with fertility data of large numbers of inseminations.

The current study was designed 1) to determine the effects of cryopreservation on bull sperm head morphometry, 2) to determine whether the effects vary between individual bulls, and 3) to determine which sperm head morphometric measurements, if any, are associated with changes that occur to the sperm head during cryopreservation and thawing.

Material and Methods

Sample Preparation

Semen samples were collected from 18 bulls of various ages and breeds by artificial vagina. The concentration of spermatozoa in each semen sample was immediately determined by hemacytometer. A 200-μl aliquot of semen was extended to 200 × 10^6 spermatozoa/ml in Tris-citrate buffer, and a microscope slide was prepared by placing 7 μl of the extended semen on the clear end of a frosted slide and dragging the drop across the slide (Zaneveld and Polakoski, 1977). The remainder of each semen sample was extended in Tris-citrate extender containing 10% egg yolk and 4% glycerol, loaded into 0.5-ml plastic straws, and cryopreserved in a programmable freezing unit over a 4.5-minute period. After remaining cryopreserved for a minimum of 24 hours, straws were thawed in a 37°C water bath for 60 seconds, and microscope slides of the cryopreserved samples were prepared as described for extended semen samples.

Morphometric Evaluation of Sperm Heads

Slides were air dried for a minimum of 2 hours and were stained using a modified hematoxylin and rose bengal procedure, originally described for staining of human sperm heads (Davis and Gravance, 1993). Modification from the original procedure included 40 minutes of staining in rose bengal and two additional ethanol rinses (for complete procedure, see Gravance et al, 1996b). Stained sperm samples were permanently mounted to the slide with a coverslip and Permount (Fisher Scientific, Pittsburgh, Pennsylvania). The morphometric dimensions for length (L), width (W), width/length (W/L), area (A), and perimeter (P) were acquired for 250 images (automatically determined by the system to be sperm heads) at 60× objective magnification (Gravance et al, 1996b) using a commercially available ASMA system (CellMorf™, Motion Analysis Corporation, Santa Rosa, California). These morphometric parameters have been shown to properly classify human sperm heads with 95% accuracy (Morruzi et al, 1988). Acquiring 250 images assures that a minimum of 200 properly recognized and measured sperm heads are analyzed after nonsperm images and improperly measured sperm heads are deleted from the analysis (Gravance et al, 1996b). The mean sperm head dimensions and coefficients of variation (CVs) of

Table 1. *Mean sperm head measurements of area, perimeter, length, width, and width/length for extended and cryopreserved semen samples from 18 bulls**

Sample	A (μm)	P (μm)	L (μm)	W (μm)	W/L
EXT	29.14 (5.6)[a]†	23.70 (3.3)[a]	8.63 (3.1)[a]	4.48 (3.6)[a]	0.519 (3.8)[a]
CRYO	28.42 (5.6)[b]	23.33 (3.6)[b]	8.56 (3.5)[b]	4.39 (3.5)[b]	0.513 (4.1)[b]

A, area; P, perimeter; L, length; W, width; W/L, width/length; EXT, extended semen sample; CRYO, cryopreserved semen sample.
* Coefficients of variation (% CV) between bulls are shown in parentheses.
† Values within columns with different superscripts are different between bulls ($P < 0.01$; general linear models analysis of variance, $n = 200$ sperm per sample).

each individual analysis were reported by the system software and recorded. The percent change in mean sperm head measurements between extended and cryopreserved samples was calculated for each bull and recorded. The measurements of each individual sperm head from each slide analysis were saved in an Excel® (Microsoft® Corporation, Redmond, Washington)-compatible database by the software for further statistical analysis.

Statistical Analysis

The effect of cryopreservation on sperm head morphometric dimensions within and between all bulls was analyzed by general linear models analysis of variance using a split plot, randomized complete block design (Statistical Analysis Systems, 1985). Bulls served as the main plot and treatment (i.e., extended or cryopreserved semen) as the subplot. The parameter of sperm head measurements was the block, whereas the parameter of bull times sperm head measurements was used as the error term. Effects were considered significant at $P < 0.01$. The differences between sperm head measurements in extended and cryopreserved samples were compared within individual bulls for all sperm head measurements by the Student's *t*-test (NCSS Statistical Program, Kaysville, Utah). Normal distribution of data was determined by the Kolmogorov-Smirnov normality test (NCSS). The means within analysis CVs were compared between groups by the Mann-Whitney two-sample test. Correlations between sperm head measurements and sample variation (CV) before and after cryopreservation and the percent change in sperm head measurements after cryopreservation were performed by Spearman's rank correlation coefficients (NCSS).

Results

The mean number of properly measured sperm heads in each analysis was 229. There were no differences ($P > 0.10$) in the number of properly analyzed sperm heads between extended and cryopreserved samples (data not shown). Significant treatment effects ($P < 0.01$) of cryopreservation were found within and among bulls on morphometric dimensions of sperm heads in the extended and cryopreserved samples.

Bull sperm heads were smaller ($P < 0.01$) in cryopreserved samples than in the companion extended samples for A, P, L, and W between all bulls. Width/length was also changed. Table 1 shows the mean sperm head measurements and the CVs between bulls for extended and cryopreserved samples. In addition, significant ($P < 0.01$) within-bull effects were found for all sperm head measurements between the extended and cryopreserved samples. No significant differences ($P > 0.10$) in the means within analysis CVs were found between the extended and cryopreserved samples for L (4.5 vs. 4.6%), W (5.6 vs. 5.7%), A (7.2 vs. 6.8%), P (4.8 vs. 4.6%), and W/L (6.5 vs. 6.5%), either within or among bulls.

In 14 of the 18 bulls, differences ($P < 0.05$) were observed in at least four of the five morphometric parameters between extended and cryopreserved semen samples. The percent change in sperm head measurements from extended and cryopreserved semen of the four bulls that showed no differences (ND) and the 14 bulls where differences occurred (DIF) were different for A (0.1 vs. −5.9%, $P = 0.04$), P (−0.1 vs. 4.6%, $P = 0.04$), W (0.4 vs. −4.3%, $P = 0.01$), and W/L (1.0 vs. −1.2%, $P = 0.05$). No significant differences in any sperm head measurements were detected in extended or cryopreserved samples when the ND group of bulls was compared to the DIF group. However, the variability (percent CV) of sperm head measurements in extended semen samples was lower ($P < 0.05$) in the ND bulls than in the DIF group for A (6.9 vs. 7.7%), P (4.0 vs. 5.1%), and L (4.5 vs. 4.6%).

The percent difference in individual parameters of head measurements of spermatozoa from extended and cryopreserved samples for all bulls was correlated ($P \leq 0.05$) with the CVs of the corresponding measurements for A ($r = -0.484$), P ($r = -0.616$), L ($r = -0.543$), and W/L ($r = -0.483$) of the initial extended sample.

Discussion

In the present study, cryopreservation of bull spermatozoa had a significant effect on the morphometry of sperm heads across a limited population of 18 bulls. Sperm head measurements of cryopreserved samples were significantly lower than those of the extended samples for all morphometric measurements across all bulls. These results contradict those previously reported for the effects

of cryopreservation on head morphometry of goat spermatozoa (Gravance et al, 1997). Gravance et al (1997) found no overall effect of cryopreservation on goat sperm head morphometry; however, effects within a limited number of bucks were observed. Because differences in sperm head measurements were found across the population of bulls, utilizing sperm head morphometry analysis of cryopreserved semen in the retrospective study of fertility from ejaculated samples cannot be performed. It will now be necessary to perform the cumbersome task of performing prospective studies comparing sperm head morphometry and fertility from inseminations of freshly extended semen. Previous work has shown that sperm head morphometry is not affected by simple extension of bull semen in a Tris-citrate buffer (Gravance et al, 1997); therefore, such studies are possible.

One possible explanation for the contrasting results in the effects of cryopreservation on head morphometry acquired for goat and bull spermatozoa may be due to the cryopreservation methods. Cryopreservation methods are known to have a large effect on the postthaw motility (Olar et al, 1989; Watson, 1995), viability (Garner et al, 1988), and acrosomal status (Thomas et al, 1998) of spermatozoa. The fertility of the semen sample is also affected by cryopreservation methods (Foote and Parks, 1993). Methods of cryopreservation also appear to have varying effects on sperm chromatin structure (Karabinus et al, 1991). A number of steps of the cryopreservation protocol could affect the extent of damage incurred by the spermatozoa, including glycerol levels, chilling and freezing rates, and thawing methods. Whether modification of these protocols would reduce the morphometric alterations to sperm heads is unclear.

Similar to the results of cryopreservation of goat spermatozoa (Gravance et al, 1997), it appears that the effect of cryopreservation on bull sperm heads varies among individuals. However, in contrast to the previous findings of Gravance et al (1997), the majority of the bulls in this study incurred changes in the morphometric parameters of the sperm head. The average percent change across all bulls ranged from 1.1% for W/L to 5.6% for A. Although the average changes in dimensions were less than 6% for all measurements, they were still found to be significantly different utilizing the precision of ASMA. In addition, analyzing the data within and between bulls using individual sperm head data as experimental units (n = 3,600 per group) makes the power of analysis of variance quite discerning. This method of analysis was able to detect the very small changes in morphometric dimensions of cryopreserved sperm heads.

Whereas no significant differences in the morphometric measurements of the ND and DIF populations were detected from freshly extended or cryopreserved samples, the variability (CV) of sperm head dimensions was significantly lower in the samples that showed no changes in measurements. The negative correlations indicate that, as the variability of the samples increased, the morphometric dimensions decreased in a correlated fashion. It is not known from this study whether the lower variability is associated with bull fertility; however, previous studies of sperm head morphometry indicated that the variability of sperm head measurements is indicative of fertility. Williams and Savage (1930), using projected images of spermatozoa and performing manual measurements, found the variability in sperm head length to be associated with stallion fertility. Katz et al (1986), utilizing ASMA, also found that the variability in human sperm head morphometry was related to fertility. In humans, the individual variation in fertility after artificial insemination with cryopreserved semen is quite high (Mahadevan and Trounson, 1984). If the data presented for bull sperm morphometry apply to humans, it may be possible that the variation within a fresh sample may be indicative of subsequent fertility after cryopreservation. Alternatively, the changes in sperm head morphometry may be an indicator of the potential fertility of a semen sample after cryopreservation. Whether the variability of sperm head morphometric measurements is indicative of bull fertility remains to be studied.

A number of possible causes for the differences in sperm head dimensions between extended and cryopreserved samples of individual bulls exist. One hypothesis may be an increase in the concentration of spermatozoa in which acrosomal exocytosis occurred during cryopreservation and thawing. An increase in the concentration of acrosome-reacted spermatozoa has been found after cryopreservation (Jones and Stewart, 1979; Mahadevan and Trounson, 1984; Thomas et al, 1998). Because one component of the staining system utilized in this study is the acrosome-specific rose bengal (Talbot and Chacon, 1981), this particular region would no longer have been recognized by the ASMA system. However, the thickness of the acrosomal membrane and contents are not known; therefore, it is unclear whether the 1 to 6% difference in dimensions can be explained by exocytosis of the acrosomal matrix. If these results can be explained by the loss of the acrosome, ASMA may have promise as a simple and objective assay for detecting acrosome-reacted populations of spermatozoa.

The difference in morphometric dimensions between extended and cryopreserved spermatozoa observed in this study may also be explained by changes in the sperm chromatin structure. Royere et al (1988) found that the surface area of sperm heads tended to decrease after cryopreservation and thawing. This decrease in surface area was attributed to changes in sperm chromatin structure, and it was hypothesized that cryopreservation induces overcondensation (Royere et al, 1988) of the sperm chro-

matin. Additional studies have also associated abnormal chromatin structure with abnormal sperm head morphology (McCosker, 1969; Gledhill et al, 1971) and reduced fertility (McCosker, 1969). If detection of slight but significant differences in sperm head morphometry due to structural abnormalities of chromatin structure is possible, ASMA possesses further utility in routinely detecting these nuclear anomalies. In this respect, ASMA would not only be a benefit to the bull semen industry but would assist in the screening of fertile donors for human artificial insemination with cryopreserved semen (Royere et al, 1991). Whether sperm heads with reduced morphometric dimensions are associated with abnormal sperm chromatin structure and reduced fertility warrants further investigation.

In summary, morphometric dimensions of the heads of cryopreserved bovine spermatozoa were significantly smaller than those found in extended samples across a population of bulls. The impact of the effects was variable across bulls, with only 20% of the bulls showing no significant change in morphometric dimensions. The reason that differences in sperm head dimensions occurred is not readily apparent but could be attributable to acrosomal exocytosis or nuclear overcondensation. The variability (CV) of the sperm head measurements from extended samples was lower in samples where no differences in measurements occurred after cryopreservation. The lower variability found in these samples was correlated with significantly smaller changes in sperm head dimensions after cryopreservation. It appears that the variability of the sample prior to cryopreservation may be predictive of subsequent changes. Whether these changes are associated with fertility, however, remains to be studied.

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