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Acidification of Intracellular pH in Bovine Spermatozoa Suppresses Motility and Extends Viable Life

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ABSTRACT: Intracellular pH (pHi) was determined in ejaculated bovine spermatozoa using a ratiometric absorbance technique under various incubation conditions that drastically altered sperm motility. The pHi was directly correlated with sperm motility. In a medium of Sodium, Potassium, and Magnesium [NKM] that supported active sperm motility, pHi was 6.9. In medium containing weak acids (NKM equilibrated with 100% CO_2 or containing 80 mM 5,5-dimethyl-2,4-oxazolidinedione; DMO), pHi was depressed at least 0.5 pH unit and sperm motility was suppressed. After complete immobilization of sperm was established, removal of the weak acids indicated that suppression of motility was fully reversible for up to 48 hours in CO_2 and up to 24 hours in DMO. This study shows that expression and conservation of sperm motility are inversely related, and that depression of pHi by weak acids can reversibly inhibit sperm motility. These findings may help to explain the mechanisms by which sperm are immobilized within the male reproductive tract, and could be applicable to the design of improved ambient temperature semen extenders.

Key words: Ratiometric absorbance, weak acids, sperm storage, male tract, semen extender.

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Control over the flagellar motility apparatus is exerted at 2 critical stages in spermatozoa. One of these stages occurs when sperm arrive in the upper regions of the female reproductive tract. There, through the process of capacitation (Yanagimachi, 1989), sperm motility is stimulated significantly above basal levels, which apparently assists spermatozoa in penetrating the envelopes surrounding an egg (Suarez and Dai, 1992; Stauss et al, 1995). The other major control point occurs earlier, at ejaculation. In most vertebrate and invertebrate organisms, mature spermatozoa are stored for prolonged periods in a quiescent, virtually immotile state within the male reproductive tract, then rapidly activate when they encounter an egg (ie, just prior to ovulation). This mechanism, which ensures that sperm are fully mature and capable of achieving fertilizing competence close to the time of ovulation, is needed because of the limited fertile life span of ovulated eggs, which is only a few hours. At the time of ejaculation, when sperm are mixed with seminal plasma (internal fertilization) or diluted into water (external fertilization), the flagellar apparatus is rapidly activated and sperm burst into vigorous motility for the first time in their life cycle. However, an apparent consequence of motility activation is a drastic reduction in the fertility and viability of a mature spermatozoon, from several weeks during storage in the male reproductive tract, to only several hours or less following activation.

The cellular mechanisms responsible for holding spermatozoa in an immotile condition during storage within the male reproductive tract are poorly understood in mammals. There is, however, considerable evidence that intracellular pH (pHi) regulates sperm motility and preserves viability during storage in invertebrate species, particularly sea urchins (Nishioka and Cross, 1978; Schackman et al, 1981; Christen et al, 1982; Johnson et al, 1983; Lee et al, 1983). Evidence for such a regulatory role of pHi in vertebrate spermatozoa is less clear-cut, but several studies point to this mechanism. Wong et al (1981) found that the motility of epididymal rat sperm in various media depended on the sodium ion concentration and that the Na^+/H^+-exchange inhibitor, amiloride, would inhibit initiation of motility. These authors concluded that a shift in pHi via a Na^+/H^+-exchange mechanism leads to the initiation of mammalian sperm motility. This hypothesis is supported by the recent work of Saito et al (1996) who postulated that internal alkalization by the Na^+/H^+ exchanger plays a role in the initiation of human sperm motility. Goltz et al (1988) also suggested that mammalian sperm motility might be regulated via changes in pHi because the motility of demembranated, adenosine triphosphate (ATP)-reactivated bull sperm increased as the pH of the medium was raised from 6.6 to 7.1. More recently, Giroux-Widemann et al (1991) demonstrated a similar effect of extracellular pH on the motility of demembranated human spermatozoa. Studies by Carr and

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Acott (1989) using weak acids led them to suggest that high concentrations of a permanent weak acid (lactic acid) combined with the relatively low pH found within the cauda epididymis may be responsible for depressing pHi and producing the quiescence of sperm within the cauda epididymis. However, this hypothesis has been difficult to study experimentally, primarily because of the inability to establish appropriate in vitro conditions that permit prolonged immobilization of sperm cells while maintaining their viability.

Several investigators have examined the pHi of bovine spermatozoa using a variety of techniques (see Jones et al, 1995). However, the reported pHi values vary considerably, which could result from the use of different sources of sperm (caput or caudal epididymal vs ejaculated sperm), use of different techniques to measure pHi, different degrees of viability of sperm at the time of pHi measurement, or to different sperm cell incubation conditions during pHi measurements (Jones et al, 1995). Because of this wide divergence in reported pHi measurements and to provide baseline information for subsequent studies, the experiments presented here were designed to measure the pHi of bovine sperm and to correlate this value with motility and viability (i.e., sustained motility) when sperm cells are stored under conditions that are believed to preserve viability. The hypothesis tested in this study was that decreased pHi leads to suppression of motility, which increases longevity (viability). Ejaculated bull sperm was used because cells are obtainable in large numbers and their motility is easily measured; moreover, data on motility inhibition and activation in this species could prove useful in developing improved semen extenders.

Methods

Semen Collection and Processing

Bovine semen was provided by American Breeders Service (DeForest, WI). Large quantities of sperm cells (>5 mL per ejaculate with $>1 \times 10^9$ sperm/mL) were available on a daily basis from bulls of proven fertility. Semen was collected by artificial vagina and transported in an insulated container via an underground pneumatic system directly into laboratory facilities within 5 minutes after collection. Semen specimens were maintained at room temperature (22°C) except during motility analysis. Sperm concentration (measured spectrophotometrically) and percent motility (subjective estimate) were determined, and samples with low concentration (ie, $<8 \times 10^8$ sperm/mL) or with low motility (<85%) were rejected. Prior to experimentation, semen was washed twice by centrifugation ($250 \times g$ for 10 minutes) to remove seminal plasma, rediluted to a sperm concentration of approximately 1.2 to 1.5×10^9 sperm/mL, and motility re-examined. Any washed semen samples with motility of less than 85% were not used.

Table 1. *Composition of media used for sperm storage*

Component*	Concentration, mM		
	NKM	CO_2-Medium	DMO-Medium
NaCl	120	17.75	50.67
KCl	5	5	5
$MgCl_2$	1	1	1
$NaHCO_3$	. . .	125	. . .
Glucose	65	65	65
MOPSO	20	. . .	20
DMO	. . .	. . .	80
Penicillin-G, IU/mL	100	100	100
PVA, mg/mL	1	1	1
pH	6.8	6.8	6.8
mOsm/L	325	325	325

* MOPSO indicates 3-[*N*-morpholino]-2-hydroxypropanesulfonic acid; DMO, 5,5-dimethyl-2,4-oxazolidinedione (dimethadione).

Sperm Motility Analysis

Subjective estimates of sperm motility were made by diluting 7 μL of a concentrated sperm sample ($\approx 1.2 \times 10^9$ sperm/mL) into 2 mL of medium maintained at 37°C in a water bath, transferring 5 μL to a prewarmed (37°C) siliconized motility chamber (Jones and Bavister, 1988), and observing the motility on a Zeiss standard Universal microscope equipped with a heated stage and dark-field optics. The motility of each sperm sample was rated using a sperm motility index (SMI; Bavister and Andrews, 1988) in which the estimated percent motility (0 to 100) is multiplied by the square of the estimated vigor (1 to 5). Although more precise, objective, computerized sperm motility analyses are currently available, the subjective technique used here provided a much more rapid and less expensive means to evaluate sperm motility that was sensitive enough to detect the large motility differences found in these experiments. However, because these subjective motility estimates were not performed in a blind study, it would be inappropriate to perform statistical analyses of these data. Therefore, only the average sperm motility index was reported for each of the experiments presented here.

Media Preparation

A simple, chemically defined medium, modified from the Sodium, Potassium, and Magnesium [NKM] medium described by Babcock (1983), was used as the basic medium for the experiments presented here (Table 1). The pH was adjusted to 6.8 with 5 N NaOH. The composition of this standard NKM medium was then modified as needed to meet the specific requirements of each experiment.

For Experiment 1 the standard pH 6.8 NKM medium was modified to permit gassing with 100% CO_2 while maintaining an external pH of 6.8. This medium (hereafter referred to as CO_2 medium) was produced by replacing the buffer (20 mM 3-[N-morpholino]-2-hydroxypropanesulfonic acid [MOPSO]) in NKM medium with 125 mM $NaHCO_3$ and reducing the NaCl concentration to 17.75 mM in order to maintain osmolarity at 325 mOsm/L (see Table 1 for complete formulation). The initial pH of this medium was approximately 8.4; however, after bubbling 100% CO_2 through the medium for 20 minutes, the final pH was maintained at approximately 6.8 ± 0.05. Under the con-

ditions used here, the pH of this medium did not increase more than 0.05 pH unit after exposure to air for 15 minutes.

A second modification of the standard NKM medium was prepared by the addition of 80 mM 5,5-dimethyl-2,4-oxazolidinedione (DMO) and reducing the NaCl concentration to 50.67 mM in order to maintain the osmolarity at 325 mOsm/L following adjustment of extracellular pH to 6.8 with NaOH (hereafter referred to as DMO medium, see Table 1). Like CO_2, DMO is a membrane-permeant weak acid that has been used to alter the pHi of a number of different types of cells and also as a pHi indicator (Waddell and Bates, 1969; Gillies, 1981). In addition, DMO is metabolically inert, nontoxic, and has a dissociation constant (pK_a [6.2]) near that of CO_2. Therefore, the DMO medium was used in an attempt to mimic the effects CO_2 observed in Experiment 1.

All media were prepared weekly and stored at 5°C until used.

Intracellular pH Measurements

The advantages and limitations of a number of techniques used to measure pHi in a variety of cell types, in single cells, or cell populations, have been reviewed by others (Roos and Boron, 1981; Nuccitelli, 1982; Kotyk and Slavik, 1989; Jones et al, 1995). Because the hypothesis to be tested involved the relationship between motility and viability of bovine sperm cells, it was necessary to use a technique that would not alter motility, viability, or pHi of cells. A ratiometric absorbance technique was used based on the pH-sensitive fluorochrome 6-carboxyfluorescein (6-CF), as described by Jones et al (1995). The method provides a measure of average pHi for the population of cells examined, which was most appropriate because sperm cell motility and viability are determined on a cell population basis. This pHi probe has a relatively low pK_a, which is important because pHi of sperm cells was measured under conditions that are expected to reduce pHi even further. Of all the probes currently available, 6-CF has a low pK_a (≈6.5; Thomas et al, 1979; Kotyk and Slavik, 1989) and the advantage of being available in a colorless membrane-permeant form (6-carboxyfluorescein diacetate; 6-CFDA), which upon entry into the cell, is converted by nonspecific intracellular esterases into 6-CF by hydrolytic cleavage of the acetate groups (Thomas et al, 1979). 6-CF remains trapped inside the cells because of its high negative charge. In this way, 6-CF can be easily introduced into sperm cells and converted to 6-CF in situ without causing cellular damage (Babcock, 1983; Vijayaraghavan et al, 1985; Schoff and Lardy, 1987; Carr and Acott, 1989; Florman et al, 1989; Parrish et al, 1989). Essentially, a suspension of washed spermatozoa was stirred within a spectrophotometer cuvette and ratiometric measurements were made under different conditions, then pHi was calculated using calibration curves (Jones et al, 1995).

Experimental Protocol

Experiment 1: Determination of pHi of Sperm Cells Stored in NKM Medium ±100% CO_2—Three independent replicate experiments were conducted using the semen from several different bulls. Because each experiment required large volumes of semen (>12 mL), the ejaculates from 2 or 3 individual bulls were pooled for each experiment. The initial motility of the pooled ejaculate was estimated (time = 0 hours) by diluting 7 μL of fresh semen into 2 mL of NKM medium at 37°C. The fresh semen was then divided in equal aliquots, washed in either pH 6.8 NKM medium or pH 6.8 CO_2 medium, diluted to approximately 1.2×10^9 sperm/mL, and the motility examined (elapsed time ≈1 hour). The motility of sperm that had been washed and diluted into NKM medium was examined by diluting 7 μL of semen into 2 mL of NKM medium at 37°C, while the motility of sperm that had been washed and diluted in CO_2 medium was examined by diluting 7 μL of the concentrated semen into both 2 mL of CO_2 medium and 2 mL of NKM medium (activation medium) at 37°C. The concentrated sperm were stored at ambient (22°C) temperature until used for pHi measurements. Sperm samples stored in CO_2 medium were incubated in open culture tubes within a humidity chamber gassed with 100% CO_2. Samples stored in NKM medium were incubated in similar tubes within a humidity chamber that was gassed with air.

As described previously (Jones et al, 1995), pHi measurements were performed using a spectrophotometer operated in multiwavelength mode, set to average 99 readings at each wavelength (570, 494, and 465 nm) and calibrated using a blank containing NKM or CO_2 medium. Prior to loading sperm samples with 6-CFDA for pHi measurements, the volume of concentrated sperm samples was established that would be required to yield an absorbance of approximately 2.25 units at 570 nm following multiple washings and resuspension. This volume was used for subsequent 6-CFDA loading (Jones et al, 1995). Motility of the sperm suspension was again examined immediately prior to loading sperm with 6-CFDA (elapsed time ≈3 hours). A series of aliquots from each storage condition were loaded with 6-CF at 15-minute intervals over a 2- to 3-hour time period. Sperm were loaded with 6-CFDA for 20 minutes by incubating the appropriate volume of semen in 1 mL of the same medium in which they were stored, but containing 50 μM 6-CFDA. Sperm samples stored and loaded in CO_2 medium were incubated in open 1.5-mL microcentrifuge tubes within a humidity chamber gassed with 100% CO_2. Samples stored and loaded in NKM medium were incubated in similar tubes within a humidity chamber gassed with air. Following the incubation period, the cells were centrifuged, washed twice, and resuspended in the same medium that was used for storage. The total absorption at all 3 wavelengths for each sample was then measured following the protocol described earlier; however, the cuvette containing the CO_2 medium was continually gassed with 100% CO_2 prior to and during all absorbance measurements in order to keep the medium saturated with CO_2. The resulting data were exported, stored, and analyzed using a Hewlett-Packard computer. Sperm pHi was then calculated from the absorbance measurements as described previously (Jones et al, 1995).

Intracellular pH measurements were made every 15 minutes over a 2- to 3-hour time interval after which the motility of each sample was examined (elapsed time ≈5–6 hours). At the end of the experiment, the remaining concentrated sperm sample from each storage condition (minimum of 1 mL) was transferred to a culture tube, loosely capped, and stored at ambient (22°C) temperature in a humidity chamber that was either flushed with air (NKM) or with 100% CO_2 (CO_2 medium). The motility of these samples was then examined every 24 hours until no motility was observed after dilution in the NKM activation medium at 37°C.

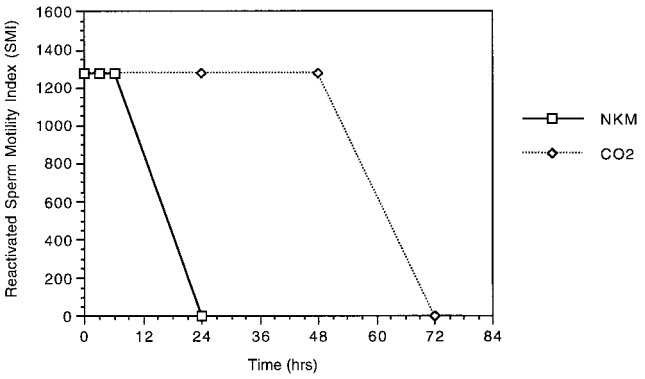

Figure 1. Time course of the decline of sperm motility stored in NKM and reactivation of motility to sperm transiently immobilized with CO_2. Sperm motility index (SMI) of bovine sperm cells diluted into an activation medium (NKM) after storage in NKM or CO_2 media for the time period indicated (see text for range of values plotted). Refer to ''Materials and Methods'' for description of the sperm motility index (SMI).

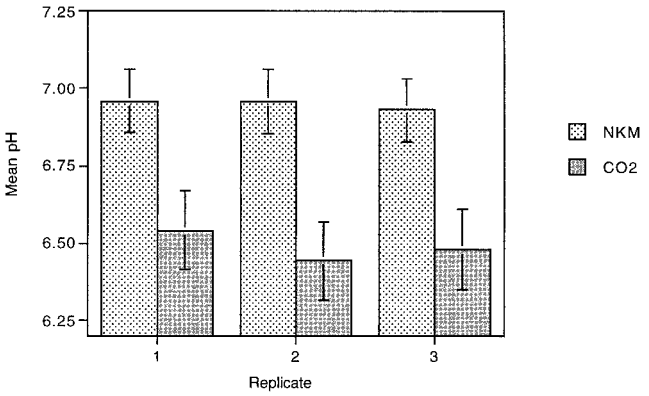

Figure 2. Mean and calculated 95% confidence intervals for the measured pHi of sperm cells washed and stored in NKM medium and CO_2 medium.

Experiment 2: Determine if DMO Can Mimic CO_2 Storage Conditions—Four independent replicate experiments were conducted using the semen from several bulls. As in the previous experiment, these experiments required the use of large volumes of semen (>15 mL); therefore, the ejaculates from 3 or more bulls were pooled for individual experiments. The methods for pHi measurements and motility estimates in this experiment were identical to Experiment 1 except for the addition of a third treatment (80 mM DMO medium).

Statistical Analysis

Details of methods used for statistical analysis of data are provided for each experiment in ''Results.''

Results

Experiment 1: Determination of pHi of Sperm Cells Stored in NKM Medium ± 100% CO_2

The initial estimated motility of sperm samples did not vary between the 3 independent replicate experiments conducted. In addition, motility of semen that were washed and stored in NKM medium remained constant during the first 3 time periods (0, 3, and 6 hours). The sperm motility index (SMI) at each of these time periods ranged between 1040 and 1520 (ie, between 85% and 95% motility with a progressive motility rating of between 3.5 and 4.0). However, after 24 hours of storage in NKM, the motility in all replicates was zero (Figure 1). Essentially, the same result was obtained in another study designed to construct a 6-CF pH standard curve (Jones, 1991). In addition, in a preliminary experiment in which the motility of spermatozoa stored in NKM medium was examined every hour over a 12-hour storage period (data not shown), motility did not vary (SMI range 1040–1520) until after approximately 8 to 10 hours of storage, when it began to decline. After 12 hours of storage the SMI score ranged between 585 and 675, and after 24 hours the score was zero. In this experiment all attempts to restore sperm motility after 24 hours of storage by additions of 2.5 mM caffeine, 1 mM dibutyryl cyclic adenosine monophosphate (cAMP), dilution into NKM medium, or a combination of these failed to restore any motility. Therefore, sperm were classified as nonviable after 24 hours of storage in NKM medium.

The motility pattern for semen that had been washed and stored in CO_2 medium was quite different. Motility of these sperm cells, when diluted into CO_2 medium for motility estimation, showed very low motility (SMI range 120–180) at 3 hours and 6 hours, and no motility (SMI = 0) at 24 hours and subsequent time points. However, when diluted into NKM medium for motility estimation, restoration of motility in sperm that had been stored in CO_2 medium was excellent (SMI range 980–1360) for every time period examined up to 48 hours. After 72 hours of storage, however, motility could no longer be restored by dilution into the NKM activation medium, and therefore, the cells were assumed to be nonviable (Figure 1).

Linear regression analysis of pHi data from each replicate with respect to time indicated that there was no significant ($P > .05$) time effect (ie, the pHi of sperm cells did not change over the 2- to 3-hour time period [3–6 hours] when measurements were performed). Therefore, pHi calculated for all samples measured over the 2- to 3-hour time period from each replicate is presented graphically in Figure 2 as a mean and calculated 95% confidence interval (CI). In addition, one-way analysis of variance (ANOVA) of data for all replicates indicated no significant ($P > .05$) replicate effect; however, the data were analyzed using a block design in order to assure that any undetected replicate effect would not influence the analysis. The data analyzed by two-way ANOVA (blocking by replicate), combining interaction and error sums

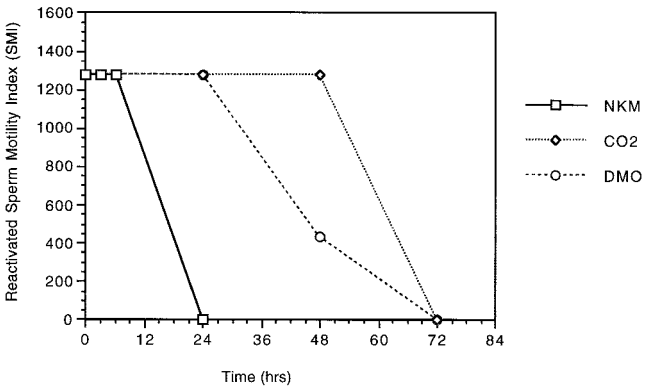

Figure 3. Reactivation of motility to sperm transiently immobilized with CO_2 or DMO. Sperm motility index (SMI) of bovine sperm cells diluted into an activation medium (NKM) after storage in NKM, CO_2, or DMO medium for the time period indicated (see text for range of values plotted). Refer to ''Materials and Methods'' for description of the sperm motility index (SMI).

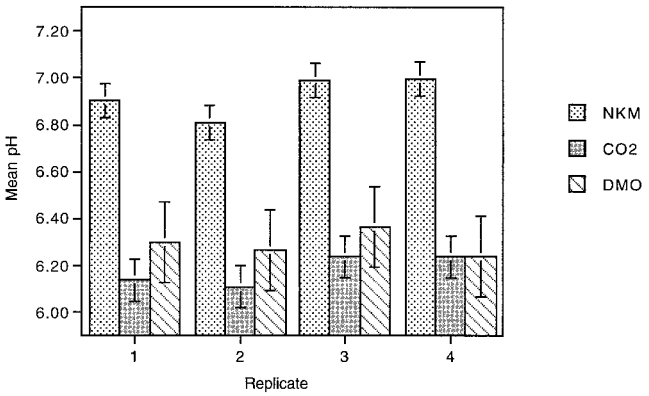

Figure 4. Mean and 95% confidence intervals for the measured pHi of sperm cells suspended in NKM medium, CO_2 medium, or DMO medium.

of squares into a single error term, indicated a significant treatment effect ($P < .005$) with the pHi of sperm stored under 100% CO_2 conditions being 0.5 pH unit lower than the pHi of sperm stored in NKM. The overall calculated pHi of NKM-stored sperm from all replicates was 6.95 $\pm$ 0.051 (mean $\pm$ 95% CI) and the pHi of CO_2-stored sperm was 6.489 $\pm$ 0.051 (mean $\pm$ 95% CI).

Experiment 2: Determine if DMO Can Mimic CO_2 Storage Conditions

As in the previous experiment, the initial estimated motility (time = 0 hour) of sperm samples varied little between replicates (SMI range 980–1360). In addition, motility of sperm that had been washed and stored in NKM medium followed a pattern similar to that in the previous experiment, in which sperm exhibited excellent motility (SMI range 980–1360) during the first 3 time periods (0, 3, and 6 hours) but zero motility after storage for 24 hours (Figures 1 and 3). The motility pattern for sperm washed and stored in CO_2 medium was also similar to that of the previous experiment, in that the motility of sperm stored in CO_2 medium and diluted into CO_2 medium for motility estimation showed very low motility (SMI range 120–180) at 3 hours and 6 hours, and no motility (SMI = 0) at 24 hours and later. When diluted into NKM medium, however, inhibition of motility was completely reversible (SMI range 980–1360) for every time period examined up to 48 hours. After 72 hours of storage in CO_2 medium, motility could not be reactivated by dilution in NKM medium (Figures 1 and 3).

At 3 hours and 6 hours, motility of sperm that had been washed and stored in DMO medium and diluted into DMO medium for motility estimation was slightly less (SMI $\approx$100) than that observed in CO_2 medium. However, at 24 hours, when motility in CO_2 medium had dropped to zero, motility in the DMO medium remained very low but consistent, with an SMI score ranging from 80 to 120. Inhibition of motility during storage in DMO medium was completely reversible during the first 24 hours of storage (SMI range 980–1360, following dilution into NKM medium), only partially reversible after 48 hours of storage (SMI range 315–550), and irreversible after 72 hours of storage (SMI = 0) as shown in Figure 3.

The calculated pHi for sperm samples stored under all 3 conditions (NKM, CO_2, and DMO) for all 4 replicates is presented graphically in Figure 4 as the mean and calculated 95% CI. These data were analyzed using the Fisher LSD method for examination of multiple means. The data, analyzed by two-way ANOVA (blocking by replicate), indicated a significant replicate and treatment effect ($P < .005$). The calculated pHi of NKM-stored sperm was significantly different from the pHi of both CO_2-stored and DMO-stored sperm ($P < .05$); however, the calculated pHi of CO_2-stored sperm was not significantly different ($P > .05$) from the calculated pHi of DMO-stored sperm. The overall average pHi values calculated for spermatozoa stored under each of the storage conditions are listed in Table 2.

In summary, when compared with cells stored in NKM medium, storage of bovine sperm cells in CO_2 medium or DMO medium depressed both pHi and motility, but prolonged their viability.

Table 2. *Calculated mean pHi values for bovine sperm stored in the test media**

Ratio	494/465		
Medium	NKM	CO_2	DMO
Mean *pHi*	6.925	6.181	6.294

* Mean *pHi* values were calculated from ratiometric data. The 95% confidence interval around each mean is $\pm$0.049 pH units. DMO indicates 5,5-dimethyl-2,4-oxazolidinedione (dimethadione).

Discussion

The primary objective of the work presented here was to investigate the relationship between pHi, motility, and viability of mammalian spermatozoa by establishing appropriate in vitro conditions that would permit reversible suppression of sperm motility (ie, maintenance of their viability).

A substantial body of in vivo observational data from both vertebrate and invertebrate organisms indicates the existence of an inverse relationship between sperm motility and the fertilizing life span of spermatozoa. From these observational data it appears that suppression of sperm motility is a highly conserved strategy for the preservation of sperm viability in vivo. However, this apparent relationship has proved difficult to study experimentally, primarily because of the inability to establish appropriate in vitro conditions that reversibly immobilize sperm cells. Prior to the work presented here, these conditions had been established only for sea urchin spermatozoa.

Sea urchin spermatozoa are stored in seminal fluid within the testis of the male for several months in an inactive state that is maintained by low pHi (Schackman et al, 1981; Christen et al, 1982; Johnson et al, 1983; Lee et al, 1983). A large acid extrusion occurs upon release of spermatozoa into seawater at the time of spawning, or into artificial seawater within a laboratory (Nishioka and Cross, 1978; Christen et al, 1982), with much of the acid being released in the form of H^+ (Schackmann et al, 1981). The resulting elevation of pHi initiates sperm motility and dramatically increases their respiration (Schackmann et al, 1981; Christen et al, 1982; Johnson et al, 1983; Lee et al, 1983); however, within a few hours after activation, less than 20% of activated spermatozoa retain their fertilizing competence (Christen et al, 1986). If, however, sperm are diluted into Na^+-free seawater, pHi remains depressed, motility is not initiated, and sperm remain viable for periods of up to 1 week (Christen et al, 1986). The addition of Na^+ (>10 mM) to sea urchin sperm immobilized in Na^+-free seawater initiates motility as well as acid release (Schackmann et al, 1981; Christen et al, 1982; Johnson et al, 1983; Lee et al, 1983), which has been interpreted to indicate the existence of a Na^+/H^+ antiporter mechanism in the plasma membrane that regulates spermatozoon pHi. Therefore, using these in vitro conditions, Christen et al (1986) clearly established a direct relationship between ionic environment, pHi, motility, metabolism (ie, O_2 consumption), and fertilizing life span of the sea urchin spermatozoa, such that:

$$\Delta \text{ ions} \rightarrow \downarrow \text{pHi} \rightarrow \downarrow \text{motility} \rightarrow \downarrow \text{metabolism} \rightarrow \uparrow \text{longevity (motility and fertile life span).}$$

The regulation of cellular events via changes in pHi is certainly not unique to sea urchin sperm. A dramatic transformation from a dormant to an active state is exhibited by a variety of cells that is almost exclusively achieved by a sudden change in pHi (Aronson and Boron, 1986). As a result of the sensitivity of specific enzyme systems to H^+ concentration, a rapid shift of pHi could activate specific cellular processes while leaving others essentially unaffected. For example, glycolysis can be regulated by changes in pHi due to the pH sensitivity of a key regulatory enzyme, phosphofructokinase (PFK; Paetkau and Lardy, 1967). Such an overall governor of cellular activity as pHi would have obvious utility for rapid activation of specific cellular functions, as well as being energy efficient for prolonged storage of cells in an inactive condition. Because the motility apparatus of a spermatozoon uses up to 70% of the energy produced by the cell (Rikmenspoel, 1965), it seems reasonable to assume that reversible cessation or reduction of motility is a device to save unnecessary expenditure of energy, and is a requirement for prolonged viability and fertilizing ability.

Control over mammalian sperm motility is exercised at 2 locations within the reproductive tract (ie, in the cauda epididymis and in the oviduct, but the mechanism of this regulation is much less clear than in sea urchins). Previous studies have indicated a relatively acidic (pH 6.3–6.7) pHi of bovine sperm cells (Florman et al, 1989). Acott and Carr (1984) and Carr and Acott (1989) demonstrated that motility of bovine spermatozoa can be reversibly inhibited by depressing their pHi with a variety of membrane-permeant weak acids. However, after prolonged exposure to conditions used to completely immobilize the spermatozoa (ie, pH 5.5 and 50 mM lactate or pyruvate), it was not possible to reverse motility (Acott and Carr, 1984). Although these experiments suggested a mechanism for reversible suppression of mammalian sperm motility (ie, via depression of pHi), they also seem to disprove the hypothesis that suppression of motility results in prolonged viability. However, it is also possible that the conditions used to depress pHi were not compatible with sustained sperm viability, which detracts from the functional significance of a linkage between pHi and motility. In contrast, in the study described here, sperm motility was reversibly inhibited for at least 48 hours. In this study, a series of experiments was conducted in which motility and viability of spermatozoa diluted into a simple medium (NKM), with and without the addition of membrane-permeant weak acid (CO_2 and DMO), were observed over a 3-day period.

In NKM, motility was not inhibited, and sperm remained viable for fewer than 24 hours. In medium saturated with 100% CO_2, motility was reduced immediately upon dilution and sperm remained completely immobi-

lized during storage. Motility of these sperm cells remained completely reversible for up to 48 hours after immobilization, indicating that there was no change in sperm viability during this period of storage. In addition, the measured pHi of CO_2-immobilized cells was approximately 0.5 pH unit less than the pHi of fully motile cells. In order to determine if the observed effect on sperm motility and viability in these experiments was caused by depression of pHi or some unique feature of CO_2, Experiment 2 was conducted using a metabolically inert membrane-permeant weak acid (DMO) to mimic the pHi environment produced by CO_2. Preliminary experiments indicated that 80 mM DMO reduced the pHi of sperm cells by approximately 0.5 pH unit (ie, equal to the pHi of sperm cells immobilized with CO_2). Under these conditions, motility was significantly inhibited, although not completely immobilized. Motility of these cells remained completely reversible during the first 24 hours of storage but only partially reversible after 48 hours, indicating that the sperm remained viable for at least 24 hours. These observed differences between CO_2-immobilized cells and sperm that were partially immobilized in DMO medium may be due in part to the lack of oxygen available to CO_2-treated sperm cells. Schoff and First (1995) demonstrated that motility of bovine sperm was substantially depressed when cells were exposed to anoxia conditions in the absence of glycolytic substrates for more than 2.5 hours. When diluted into oxygenated medium, the sperm remained immotile but were metabolically active, producing ATP from lactate via oxidative phosphorylation. Treatment of these immobilized sperm with phosphodiesterase inhibitors stimulated a transient peak in cAMP, which was accompanied a resumption of motility. While these authors did not investigate the long-term ($>$2.5 hours) effects of anoxic exposure, they certainly demonstrated that cAMP is required to reactivate sperm motility. It is therefore likely that CO_2-immobilized sperm (ie, anoxic conditions) may also be affected by low cAMP as well as decreased pHi, which was demonstrated in the experiments described here.

We therefore conclude that the sperm-immobilizing effect of CO_2 is due at least in part to its observed depression of pHi (ie, that this is a causal linkage and not merely an association).

The results of these in vitro experiments support the hypothesis that:

$$\downarrow \text{pHi} \rightarrow \downarrow \text{motility} \rightarrow \uparrow \text{longevity.}$$

Although the experiments presented here demonstrate a relationship between pHi, motility, and longevity, additional studies are needed to determine precisely how pHi regulates sperm motility and how depression of motility prolongs viability of spermatozoa stored in vitro. It can be postulated that pHi directly affects the dynein-ATPase activity of the motility apparatus to immobilize the cells and that this suppression of motility reduces metabolism (eg, by increasing the ATP/ADP ratio), resulting in prolonged cell viability. However, the hypothesis that pHi acts directly through dynein-ATPase activity was disputed in a study on the relationship between temperature and pHi effects on fowl sperm motility (Ashizawa et al, 1994). Alternatively, reducing pHi may directly inhibit sperm metabolism (eg, by inactivation of PFK), so that energy production is rate-limiting for sperm motility and other cellular functions. These hypotheses need to be tested in future experiments using in vitro methods such as those reported here.

In addition to investigating the mechanisms that regulate sperm pHi, motility, and viability, the results of experiments presented here may lead to innovative approaches to semen preservation. Lowering the temperature has been the method of choice to reversibly inhibit the metabolic activity of spermatozoa and to prolong their viability in vitro (Blackshaw et al, 1957). However, as indicated by the increased numbers of spermatozoa required for successful fertilization when using fresh vs cold-stored spermatozoa for artificial insemination (Sullivan, 1970; Shannon and Curson, 1984), low temperature is an inefficient mechanism to preserve sperm viability. In addition, current semen preservation technology has proven to be ineffective or economically unfeasible for many agriculturally important species, such as swine and poultry (Sexton and Giesen, 1982; Bakst, 1990; Reed, 1991; Thurston, 1995; Rath et al, 1986). A bovine semen extender developed in the late 1950s (Illini Variable Temperature [IVT] diluent) was proven in field trials to maintain the fertilizing life span of bovine spermatozoa at ambient temperatures for up to 3 days. In addition, it was reported (VanDemark and Sharma, 1957) that spermatozoa became completely immobilized upon dilution into this diluent. The primary difference between this extender and previous, less-effective extenders was the addition of a membrane-permeant weak acid (CO_2) to the IVT extender. Because CO_2 depresses the pHi of other cells (see Gillies, 1981), it was postulated in undertaking the present study that the immobilization of sperm cells in IVT might be due to depression of pHi. Our data are consistent with this hypothesis. Better understanding of the pathways regulating the maintenance of sperm viability in vitro and in vivo may lead to development of new ambient temperature diluents for agriculturally important animals, humans, and endangered species.

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