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The rate at which human sperm are immobilized and killed by mild acidity

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Objective: To determine the rate at which mild acidity immobilizes and kills human sperm and to evaluate an acidic microbicide, BufferGel, for sperm immobilization.

Design: Controlled in vitro study.

Setting: An academic research university and hospital andrology lab.

Patient(s): Eight volunteer male sperm donors.

Intervention(s): Semen samples were treated with hydrochloric acid (HCl) or BufferGel.

Main Outcome Measure(s): Sperm motility was measured by using a computerized automated semen analyzer and video microscopy. Sperm membrane permeability and intracellular pH were measured by using fluorescent techniques.

Result(s): In semen acidified with HCl to pH 4.0, sperm were rapidly immobilized (within 1 min) and were irreversibly immobilized (killed) within 10 minutes. The speed of immobilization and of killing were both linearly proportional to hydrogen ion activity over a pH range of 7.5–4.0. Across the same range, the intracellular pH of human sperm equilibrated to within 0.5 pH units of extracellular pH within 1–2 minutes. BufferGel immobilized sperm significantly faster than HCl from pH 4.0–6.0.

Conclusion(s): Exposure to mild acidity rapidly acidifies the intracellular pH of human sperm and is rapidly spermicidal. BufferGel accelerates acid immobilization of sperm. (Fertil Steril® 2000;73:687–93. ©2000 by American Society for Reproductive Medicine.)

Key Words: Sperm, motility, pH, BufferGel, contraceptive, intracellular pH

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During the reproductive years, a healthy human vagina usually has a pH of 4.0–4.5 (1), and it has long been known that sperm are acid sensitive (2). However, Masters and Johnson (3) showed that ejaculate acts as a potent alkaline buffer, abolishing vaginal acidity within seconds and keeping the vagina neutralized (pH 6.0–7.0) for several hours after intercourse. Moreover, they found that sperm in the vagina remained motile until the vagina became fully reacidified (3). If acidity immobilizes sperm with sufficient rapidity, a product that maintains vaginal acidity despite the arrival of an alkaline ejaculate might provide effective contraception.

BufferGel (ReProtect LLC, Baltimore, MD), currently in phase I clinical safety trials sponsored by the National Institute of Allergy and Infectious Diseases as an anti-HIV microbicide, is a broad-spectrum microbicide with a mechanism of action based on acidification of pathogens. The buffer in BufferGel is Carbopol (B. F. Goodrich, Cleveland, OH), a highly carboxylated polymer composed of lightly cross-linked polyacrylic acid. BufferGel is formulated as a lubricant gel with rheologic properties that make it suitable for use during intercourse.

We examined the speed with which mild acidity both immobilized and irreversibly immobilized ("killed") human sperm. We also observed the speed with which changes in mild extracellular acidity altered the cytoplasmic pH. Finally, we compared the speeds with which sperm were immobilized in semen acidified with either hydrochloric acid (HCl) or BufferGel.

MATERIALS AND METHODS

Semen Collection

Human semen samples from healthy men participating in a donor program approved by

the Review Board on the Use of Human Subjects of the Johns Hopkins University were collected by masturbation after at least 48 hours abstinence and were allowed to liquefy at 37°C for 30 minutes. Samples were included in experiments on the basis of donor availability and on meeting normospermic standards recommended by the World Health Organization (4).

Acidification of Human Semen

Aliquots (250 μL) of semen (five donors) were acidified to pH 4.0–6.0 by using 2–0.2 N HCl or phosphoric acid (H_3PO_4) and were slowly stirred for 2 minutes by using magnetic microstir bars in glass shell vials (Fisher Scientific, Pittsburgh, PA) maintained at 37°C in an aluminum heating block (Barnstead Thermolyne, Dubuque, IA). The pH was monitored throughout each 20-minute experiment, and acid was added to control for the slight upward pH drift of the sample. Separate aliquots were similarly diluted (10%) with either 155 mM or 1.0 M NaCl in control experiments.

The pH of the samples was measured by using a pH microelectrode (MI-414-6 cm pH electrode; Microelectrodes, Inc., Bedford, NH) and a Beckman Instruments Ø11 pH meter (Beckman Instruments, Fullerton, CA). Sodium hydroxide, 1–0.1 N, in distilled water (J.T. Baker, Phillipsburg, NJ) was used to rescue sperm by neutralizing the acidified samples. The osmolality of semen samples was measured by using a Vapro Vapor Pressure Osmometer 5520 (Wescor, Inc., Logan, UT).

Motility Measurements

Acidified semen samples were placed in 20-μm Micro-Cell slides (Conception Technologies, San Diego, CA) and were observed with a microscope. Motility versus time (at 5-minute intervals) was analyzed by using a computerized automated semen analyzer (CASA) with a Cell trak/S program (Motion Analysis, Santa Rosa, CA). The CASA analysis was performed with the following settings: 30 frames/s; minimum motile speed, 8 μm/s; maximum burst speed, 250 μm/s; and ≥100 cells counted. Semen samples that were completely killed by nonoxynol-9 occasionally registered a low level of motility (1%–4%) on CASA. To eliminate this small but misleading CASA artifact, acidified samples were prescreened at each time point and if no motile sperm were observed in three ×100 fields of view (>3,000 sperm), zero motility was recorded. Early time point measurements of <2 minutes of sperm in samples with pH 4.0 were performed by visually analyzing video microscopic recordings.

Sperm neutralization rescue experiments were performed with fresh semen samples (five donors). Aliquots (250 μL) of semen were acidified and, at 5-minute intervals, 50-μL aliquots of the acidified samples were neutralized to pH 7.0–8.0 with sodium hydroxide. Motility was measured 2–5 minutes later by using CASA. Again, samples were visually prescreened for zero motility. To attempt to reproduce the protocol from experiments performed by Makler et al. (5), 450-μL aliquots of semen (four donors) in 13-mm disposable culture tubes (VWR, Bridgeport, NJ) were acidified with 50 μL of HCl and gently vortexed with a Vortex Genie 2 (Fisher Scientific). Motility was observed after 1 minute and 15 minutes, after which the samples were neutralized with sodium hydroxide. Motility was measured 15 minutes after neutralization. In addition, aliquots from one semen sample were checked for motility 1 hour, 4 hours, and 24 hours after neutralization. Samples were maintained at 37°C throughout all of the above experiments.

To compare sperm motility among donors, motility data from each experiment were normalized by using the initial motility of that semen sample. The initial motility was measured five times over 20 minutes and averaged. This average was defined as 100% initial motility and was used to normalize all subsequent motilities, which were expressed as percentages of that sample's original average motility.

BufferGel Experiments

Aliquots of semen at 37°C (five donors) were mixed 1:1 and 3:1 with BufferGel by using glass stir rods for 15–20 seconds, achieving a pH range of 4.5–6.0. The high irregular refractivity of BufferGel made CASA measurements unreliable. Video microscopy observations were performed to determine the time at which sperm were completely immobilized in BufferGel samples (≥100 sperm per observation, ×200 magnification). As a comparison, semen was mixed with 1.0 N HCl to reach similar pH values, and visual observations were performed to determine the time at which sperm were completely (100%) immobilized.

Intracellular pH Measurements

Oregon Green 488 carboxylic acid diacetate (carboxy-DFFDA) and carboxyfluorescein diacetate (5(6)-CFDA) (Molecular Probes, Portland, OR) were used to measure the intracellular pH of sperm (three donors). Sperm were separated from seminal plasma by using a discontinuous 55%/80%-step Percoll gradient (Sigma, St. Louis, MO) centrifuged at 300 × *g* for 20 minutes. They were then labeled with 5(6)-CFDA or carboxy-DFFDA, incubated for 20 minutes at 37°C and 5% carbon dioxide, and washed (centrifuged at 300 × *g* for 10 minutes) in Ham's F-10 nutrient mixture (GIBCO BRL, Grand Island, NY). Fluorescently labeled sperm were resuspended in either Ham's F-10 medium or a high potassium medium for intracellular pH measurements. To obtain a standard curve, Nigericin (Molecular Probes), a K^+/H^+ ionophore widely used in ratiometric experiments (6–9), was used to equalize the intracellular and extracellular pH of sperm in the high potassium medium. The high potassium medium consisted of 20 mM K_2HPO_4 (J. T. Baker), 120 mM KCl (J. T. Baker), 5.5 mM D-glucose (GIBCO BRL), and 10 mM 2-{N-Morpholino}-ethanesulfonic acid (Sigma). Aliquots (250 μL) of labeled sperm were acidified and fluorescence ratio measurements were taken (490 nm/450 nm for 5(6)-CFDA and 490 nm/440 nm for carboxy-DFFDA) as a function of time. Ratiometric mea-

surements were made by using an LS50B fluorometer (Perkin Elmer, Norwalk, CT).

Sperm Membrane Permeability Studies

Aliquots of semen (three donors) were labeled with propidium iodide (Molecular Probes), a membrane-impermeant fluorescent nucleic acid stain. Five hundred-μL aliquots of labeled sperm in semen at 37°C were acidified to pH 4.0–5.0 and nucleic acid staining was observed over time.

Statistical Methods

Comparisons were made by using two-way analysis of variance with pH and time of exposure as the main effects. If the results were significant ($P<.05$), a post hoc multiple-range test (Tukey test) was used to distinguish difference between levels of each effect. Relations were analyzed by using regression analyses to determine the equations of the lines and, in some cases, regression lines were compared. In addition, in some cases, log transformations of data were done before regression analyses. Computations were made by using the Statgraphics Plus statistical program (version 3; Manugistics, Rockville, MD).

RESULTS

Sperm were observed in five samples from five different donors for motility while acidified (immobilization experiments) and for motility in samples that were acidified and then neutralized to "rescue" the sperm (killing experiments). We define killed sperm as sperm that had no motility after neutralization because nonmotile sperm can be expected to be incapable of fertilization. The speed of immobilization and speed of killing both increased with increasing acidity (lower pH) (Figs. 1 and 2). At pH 5.0, sperm were completely immobilized in less than 5 minutes. Video microscopy experiments were performed to observe the more rapid loss of motility at lower pHs. At pH 4.0, sperm were completely immobilized within 30 seconds. Analysis of the data in Figure 1 by measuring the motility half-time (time to reach 50% of original motility) shows that the rates at which sperm are both killed and immobilized in HCl are linearly proportional to hydrogen ion activity over a wide range of pH values (pH 4.0–7.5) (Fig. 2).

In the sperm-killing experiments, all sperm were killed within 10 minutes at pH 4.0. In additional experiments, nine samples from four donors were acidified for 15 minutes and were then neutralized. Their motility was measured 15 minutes after neutralization to ascertain whether killed sperm would regain motility sometime after neutralization. In these experiments, the average pH was 4.36 (range, pH 4.10–4.49), and there was no return of motility 15 minutes after neutralization. In another experiment, aliquots of one sample of semen were acidified to pH 4.0 and pH 4.5. These samples were then neutralized and sperm motility was measured 1 hour, 4 hours, and 24 hours after neutralization. In each case, no motile sperm were observed when the sperm had been acidified for 10 minutes at pH 4.0 or for 15 minutes at pH 4.5.

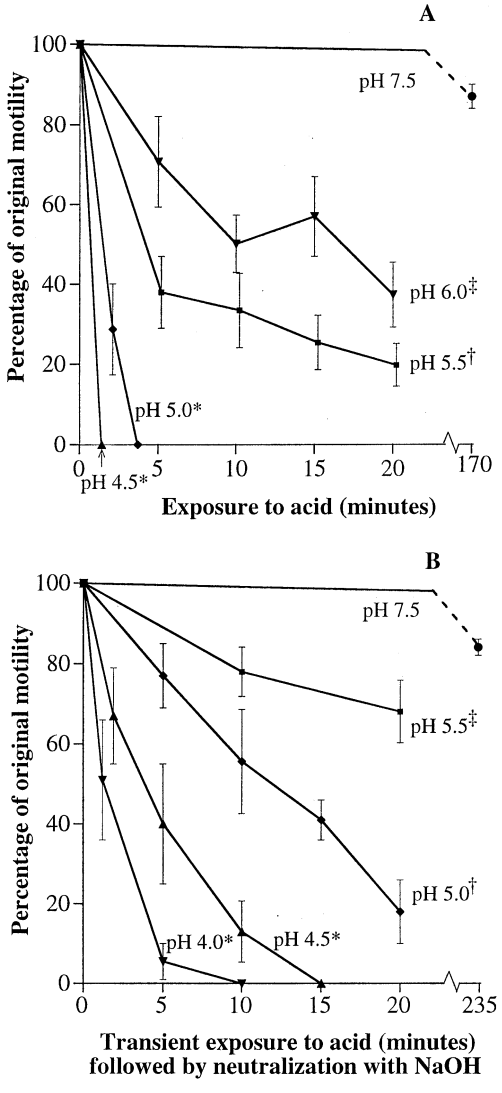
Control experiments were performed to determine the effects of increased Cl^- ions caused by addition of HCl, dilution and stirring of semen, increased osmolality, and time since ejaculation. To determine whether an increase in Cl^- ion activity, as opposed to acidification, caused loss of motility, semen was acidified by using H_3PO_4. Sperm acidified in H_3PO_4 lost motility at rates similar to those for sperm acidified with HCl. For instance, at pH 5.0, the half-time for immobilization was 1.4 minutes with HCl and 2.4 minutes with H_3PO_4; the half-time for killing of sperm was 11 minutes with HCl and 9.0 minutes with H_3PO_4. In these acidification experiments, the sperm were diluted 10% by the addition of dilute acid. Diluting semen by 10% with 155 mM of NaCl did not detectably alter motility. The maximum osmolality during acidification experiments was approximately 475 mmol/kg. To see whether this increase might alter sperm motility, sperm in semen were diluted 10% with 1 N of NaCl to reach a final osmolality of 520 mmol/kg. These sperm did not show a significant decrease in motility; it decreased by an average of only 7.7% compared with normalized controls. All experiments with a single semen sample were completed within 3 hours of ejaculation. During this time, the normalized motility of unacidified samples decreased an average of 15%, which was too small to have significant effect on the observations reported.

Because the irregular refractivity of BufferGel made CASA analysis unreliable, only visual observations were made for semen mixed with BufferGel, and the time to reach complete immobilization was measured (Fig. 3). To make a reliable comparison, aliquots from the same semen samples were acidified with HCl and the time to complete immobilization was also visually determined. At any given pH, BufferGel immobilized sperm in semen significantly faster than did HCl (Fig. 3). When semen was mixed 1:1 with BufferGel, in most cases no sperm were motile at the earliest observation time after semen was first exposed to BufferGel (20–30 seconds). Figure 2 shows the time to reach 50% immobilization with HCl and Figure 3 shows time to 100% immobilization, which took about five times longer.

Two experiments were performed to explore the mechanism by which acid immobilizes sperm. First, we tested for membrane damage of sperm by using propidium iodide, a fluorescent nucleic acid stain that is impermeant to intact cell membranes. When a cell membrane is damaged, propidium iodide can enter the cell and bind to nuclear DNA and becomes an intensely fluorescent red. Although sperm acidified to pH 4.0–5.0 had membrane damage as shown by propidium iodide permeability (Fig. 4), this membrane damage occurred far more slowly than did loss of motility. At pH 4.0, it took >1 hour for all of the sperm to fluoresce red, but it took <1 minute for all the sperm to lose motility and ≤ 10 minutes to become irreversibly immobilized. Furthermore,

FIGURE 1

Immobilization and irreversible immobilization of human sperm by mild acidity. (**A**) Percentage of motility of acidified semen samples plotted versus time (minutes). Semen samples were acidified and their motility was measured by using a computerized automated semen analyzer. Sperm in the samples acidified to both pH 4.0 and 4.5 were immobilized more rapidly than could be observed (1–2 minutes) and are shown as overlapping curves. (**B**) Semen samples were transiently acidified for the time shown, then neutralized to pH 7.0–8.0 before measuring the motility. In **A** and **B**, each data point indicates motility normalized to the original motility for that semen sample. The pH 7.5 data points at 170 and 235 minutes indicate the slow loss of motility of unacidified semen samples over the entire time course of the experiments. Each point represents the mean (±SE) of five samples from five donors. Results of analysis of variance were $P<.001$ for the main effects of time and pH and for their interaction. The Tukey test was used to determine detailed comparison for the pH effects: curves with different superscripts (*, †, ‡) differed significantly ($P<.05$).



Olmsted. Sperm immobilization. Fertil Steril 2000.

most sperm that are immotile soon after ejaculation are not permeable to propidium iodide. Initial motility measurements were 50%–60%, and 75% of the sperm were impermeable to propidium iodide.

The second experiment examined the effect of mild acidity on the intracellular pH of sperm. Two intracellular probes were used to allow observations over a wide pH range (4.0–7.5). Nigericin, a K^+/H^+ ionophore, was used in control experiments to create an in situ standardization curve for intracellular pH measurements (6–9). Results from these experiments show that the cytoplasmic pH of sperm in mild acidic conditions rapidly (within 2 minutes) equilibrated to within 0.5 pH units of extracellular pH (Fig. 5). This is consistent with the speed with which sperm lose their motility. Together, these experiments suggest that the site at which HCl-induced acidity blocks motility is in the cytosol, not the external surface of the plasma membrane.

FIGURE 2

Half-time to immobilize and to irreversibly immobilize sperm acidified with hydrochloric acid as a function of pH. The time to reach 50% motility was calculated for each semen sample at each pH from the experiments plotted in Figure 1. Each point represents the mean (±SE) of the time to reach 50% motility at a given pH. The immobilization time point for pH 4.5 was ≤45 seconds because all samples were completely immobilized as soon as they could be observed (1.5 minutes). On this log-log plot, regression lines were derived from analysis of the mean time to 50% motility at each pH for both immobilization and killing. Both lines have a slope of 1, with $r = 0.996$ and 0.994, respectively. Comparison of regression lines shows a significant difference in y intercept ($P<.001$) but no difference in slope.

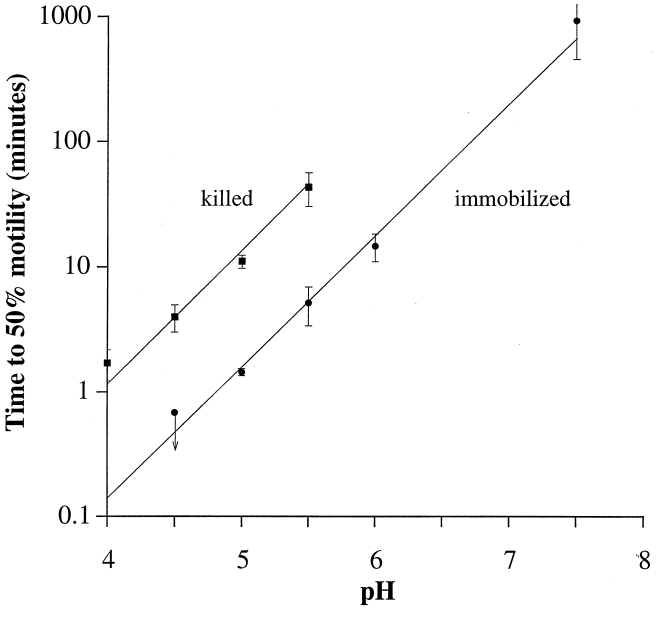


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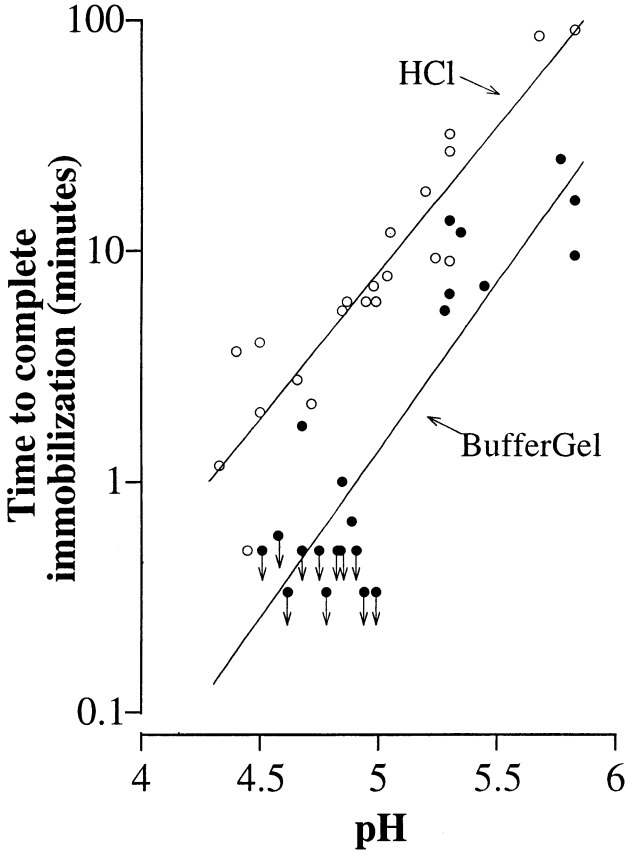
FIGURE 3

Time to 100% immobilization of sperm versus pH for hydrochloric acid–acidified (HCl) (*open circles*) and BufferGel-acidified semen samples (*closed circles*). Semen samples from five donors were acidified with HCl or BufferGel and the time to complete immobilization was measured. Semen was mixed 1:1 with BufferGel, achieving a final pH range of 4.5–5.0, and 3:1 with BufferGel, achieving a final pH range of 5.3–5.7. Hydrochloric acid was mixed with semen samples to achieve pHs similar to those in the BufferGel samples. Samples were observed with a microscope (×200) until no forward or vibrational motion was seen. A minimum of 100 sperm were observed for each time point. Filled circles with arrows indicate that all the sperm observed were immotile as soon as the sample was observed under the microscope (30–45 seconds). Regression lines were drawn using these "maximum" values as data points. Although the slopes of the regression lines do not differ for BufferGel vs. HCl ($P=.39$), the y intercepts differ significantly ($P<.001$). Thus, at any given pH, BufferGel kills sperm significantly faster than HCl.



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DISCUSSION

By using CASA, we quantified the rate at which sperm in semen lose their motility in mildly acidic conditions. Our results confirm and extend the observations by Shedlovsky et al. (2) and Brown-Woodman et al. (10) that sperm are rapidly immobilized by mild acidity. We also found that from pH 7.5 to pH 4.0, sperm in HCl are immobilized and killed at rates linearly proportional to the hydrogen ion concentration. At pH 4.0, this rate is fast enough that all sperm are immobilized in less than 30 seconds.

In addition, our results show that sperm are irreversibly immobilized after 10 minutes at pH 4.0 and after 15 minutes at pH 4.5. This result is contrary to that in a study by Makler et al. (5). In their experiments, sperm were acidified to pH 4.2 (average of 12 samples; no pH range given) for 15 minutes and then neutralized. Fifteen minutes after neutralization, sperm motility returned to approximately 35%. We attempted to duplicate their experimental protocols but did not observe any recovery of motility in samples acidified to pH 4.36 for 15 minutes and observed 15 minutes after neutralization. Thus, our results with both protocols showed irreversible immobilization (killing). There are several possible explanations why Makler et al. (5) might have observed regained motility. To acidify a viscous liquid such as semen, substantial stirring is required to fully equilibrate the acid in the sample. They did not report stirring their samples, nor did they state whether they monitored the pH after mixing. Sperm kept at physiological temperature, 37°C, lose their

FIGURE 4

Percentage of sperm with intact membranes as a function of time and pH. Sperm were incubated with propidium iodide and aliquots were then acidified with hydrochloric acid. The number of sperm with intact membranes was calculated by counting the number of fluorescent sperm and subtracting this value from the total number of sperm in a sample. Each point represents the mean (±SE) of three samples from three donors. Results of analysis of variance were $P<.01$ for both the main effects of time and pH. The Tukey test was used to determine detailed comparison for the pH effects, curves with different superscripts (*, †) differed significantly ($P<.05$).

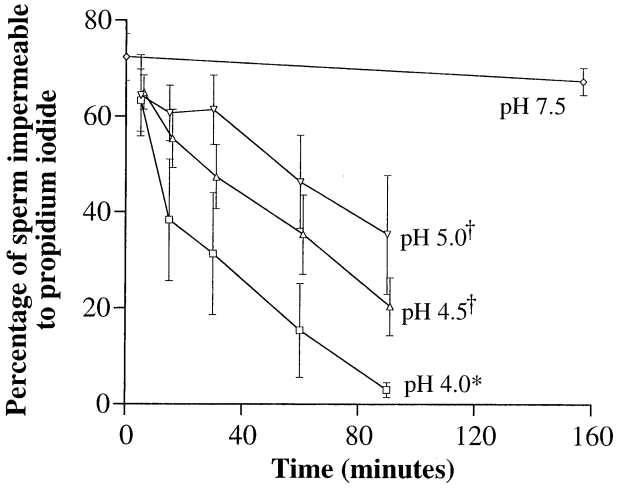

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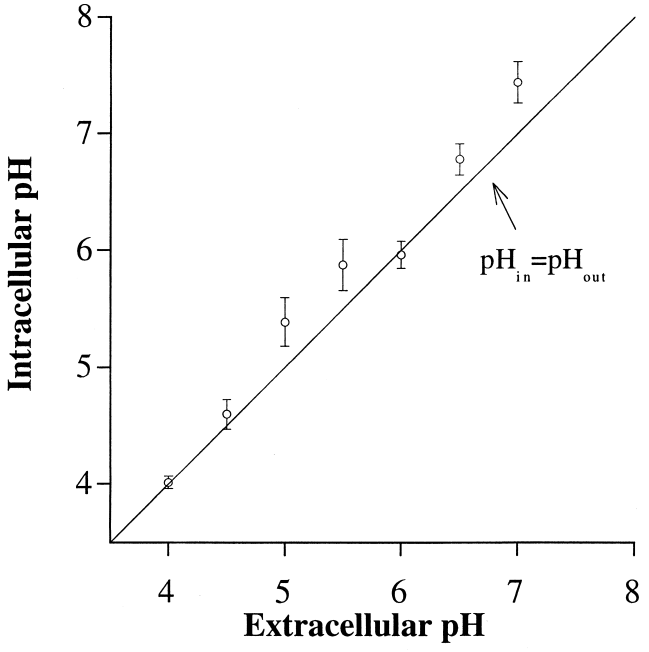
FIGURE 5

Intracellular pH of washed sperm as a function of extracellular pH observed 1–5 minutes after acidifying the external pH. Percoll-separated sperm were incubated with either Oregon green 488 carboxylic acid diacetate or carboxyfluorescein diacetate and then washed. Aliquots of labeled sperm in semen were acidified and fluorescent ratios were obtained. Intracellular pH was calculated by fitting the data to the in situ standardization curve. Each point represents the mean (±SE) of five to eight determinations from three semen donors. Regression analysis shows a high correlation between intracellular and extracellular pH values ($P<.001$). The line indicates where intracellular pH equals extracellular pH.



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motility significantly faster than sperm maintained at 20°C (11). Our experiments were all performed at 37°C to more closely mimic the conditions of sexual intercourse. Makler et al. did not report the temperature at which they performed their experiments.

The spermicides in all vaginal contraceptives now available in the United States are detergents, usually nonoxynol-9. Although detergents rapidly kill sperm by lysing their membranes, detergents have similar effects on all other cell membranes and can therefore cause irritation and cervicovaginal epithelial disruption (12, 13). The normal pH of the vagina, pH 4, is naturally spermicidal and microbicidal. Therefore, if semen did not have a strong alkaline buffer capability, sperm would not survive long in the vagina.

BufferGel, a broad-spectrum microbicide, has a pH of almost 4.0. Our experiments were performed by mixing semen with BufferGel at 1:1 and 3:1 ratios. The expected dose of BufferGel as a vaginal contraceptive is 5 mL. The 1:1 ratio of semen to BufferGel was chosen because this approximates what would occur physiologically for large ejaculates [approximately 90% of all ejaculates are ≤5 mL (14)]. The ratio of semen to BufferGel of 3:1 was chosen to simulate potentially less favorable ratios if uneven distribution and mixing of semen and BufferGel occurred. Our experiments with human semen indicate that BufferGel immobilizes sperm faster than HCl alone. BufferGel also immobilized sperm faster than does Replens (Parke-Davis, Morris Plains, NJ), another polyacrylic acid acidifying vaginal gel, when the agents were mixed with semen at similar ratios (15). The mechanism by which BufferGel accelerates acid immobilization is not yet known and requires further investigation.

Dual-wavelength ratiometry has been used to measure the intracellular pH of sperm of various species (16–18), including humans (19–21), although not in the range of pH that we studied. In the experiments reported here, the speed with which the intracellular pH of sperm decreased to extracellular pH (within about 1 minute) suggests that sperm have minimal ability to maintain their intracellular pH in acidic environments. This is consistent with observations made at pH 7 to 8, at which small increases in extracellular acidification caused immediate (within seconds) intracellular acidification, indicating that sperm do not have effective pH regulatory mechanisms in that pH range (19). These observations, together with our observations of a much slower loss of membrane integrity, suggest that mild extracellular acidity rapidly immobilizes sperm by rapidly acidifying their cytosol.

Masters and Johnson's data show that it takes several hours after ejaculation for the vagina to reacidify from approximately pH 7.0 to pH 5.0 (3). During this time, motile sperm can reach and enter the neutral mucus in the cervix. The rate at which mild acidity immobilizes sperm suggests that a vaginal product that buffers the vagina below pH 5.0 might be effective as a contraceptive. Because BufferGel accelerates the speed with which mild acidity immobilizes sperm, it may be even more effective as a contraceptive than mild acidity alone. Evaluation of its contraceptive efficacy in humans will require postcoital tests and contraceptive trials.

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