# A Uterine Fluid-Mediated Sperm-Inhibitory System[1, 2]

DONALD C. SMITH AND SEYMOUR J. KLEBANOFF

*USPHS Hospital, Seattle, 98114, and the Departments of Medicine and Obstetrics and Gynecology, University of Washington School of Medicine, Seattle, Washington 98105*

Uterine fluid from estrogen-primed immature or mature ovariectomized rats has sperm-inhibitory activity when combined with $H_2O_2$ and either iodide or thiocyanate ions as measured by a loss of motility and by a decrease in pyruvate oxidation. The uterine fluid can be replaced by lactoperoxidase or myeloperoxidase which suggests that the sperm-inhibitory effect of uterine fluid is due to its peroxidase content. The uterine fluid-mediated sperm-inhibitory system is inhibited by catalase and by a number of low-molecular-weight compounds such as azide, reduced glutathione, cysteine, ergothioneine, and ascorbic acid. Heat-stable, low-molecular-weight inhibitors are found in seminal plasma and, to a lesser degree, in uterine fluid.

Spermatozoa are continually exposed to the secretions of the female genital tract in their passage from the site of ejaculation to the site of fertilization. The influence of the uterine secretions on the spermatozoa is complex. It is probable that uterine fluid supports sperm migration by providing nutrients and a liquid medium and may facilitate fertilization by initiating sperm capacitation. The studies reported here suggest that uterine fluid under certain conditions may also exert a sperm-inhibitory effect.

Peroxidases have bactericidal, fungicidal, and virucidal activity when combined with $H_2O_2$ and an appropriate oxidizable cofactor such as iodide, bromide, chloride, or thiocyanate ions. A peroxidase is present in uterine fluid obtained from estrogen-primed rats and the rat uterine fluid can be utilized as the source of peroxidase for the peroxidase–halide–$H_2O_2$ antimicrobial system (Klebanoff and Smith, 1970a). That this system might also have sperm-inhibitory activity was suggested

by earlier studies on a milk factor toxic to spermatozoa. This factor had properties similar to those of "lactenin," a milk antimicrobial agent; both were inactivated by comparable heat treatment (Thacker and Almquist, 1953; Thacker *et al.*, 1954; Saake *et al.*, 1956) and by the addition of sulfhydryl compounds such as cysteine or glutathione (Johnson *et al.*, 1955; Boyd *et al.*, 1957). Partially purified preparations of "lactenin" exhibited both bactericidal and spermicidal activity (Flipse *et al.*, 1954). The composition of the "lactenin" antimicrobial system has been elucidated; it consists of lactoperoxidase, thiocyanate ions, and $H_2O_2$ (Reiter *et al.*, 1964; Klebanoff and Luebke, 1965; Klebanoff *et al.*, 1966; Mickelson, 1966; Steele and Morrison, 1969). Lactoperoxidase alone is devoid of spermicidal activity (Flipse *et al.*, 1954); however, when combined with thiocyanate ions, it inhibits the penetration of bovine spermatozoa into cervical mucus (Reiter and Gibbon, 1964; Reiter and Oram, 1967). This paper will deal with the sperm-inhibitory effect of the uterine fluid–halide–$H_2O_2$ system. A preliminary report has appeared elsewhere (Smith and Klebanoff, 1970).

## METHODS AND MATERIALS

### Collection and Treatment of Spermatozoa

Bovine semen was collected by artificial vagina on the day of each experiment. The fresh semen sample was immediately diluted with 1–2 vol of 0.125 M sodium phosphate buffer pH 7.4 to make a final sperm concentration of 0.5–1 × 10⁹/ml and kept at 25–30 C until use (approx. 2 hr). The semen specimens were centrifuged at room temperature at 400 g for 20 min, the supernatant removed by aspiration, and the spermatozoa washed three times in $Ca^{2+}$-free Krebs–Ringer phosphate pH 6.5 containing fructose at a final concentration of 10 mg/100 ml. The final pellet was resuspended in the Krebs–Ringer phosphate fructose medium to the desired sperm concentration (4–15 × 10⁸/ml). The second and third centrifugations during the washing procedure were at 400 g for 8 min with approx. 5 ml of suspension in each centrifuge tube. These conditions were vigorously adhered to in order to avoid excessive packing and damage to the cells while assuring adequate removal of seminal plasma. Sperm counts were performed on a Coulter counter.

Seminal plasma was obtained by centrifugation of fresh undiluted bovine semen at 1600 g for 10 min.

### Sperm Motility

The spermatozoa suspensions were incubated with the components indicated in unstoppered 10 × 75-mm test tubes at 37 C in a water bath shaker. At timed intervals, one drop of the sperm suspension was transferred to a clean glass slide, covered with a cover slip and examined with a microscope using a 10× objective. Pipets, slides, cover slips, and microscope stage were maintained at 37 C. Several representative fields were examined and the percentage of motile cells estimated. The degree of motility of the majority of individual sperm was graded as follows: 4—maximum progressive movement across slide; 3—progressive movement across slide; 2—undulating movement, without directional progress; 1—sluggish, nonprogressing movement; 0—occasional, or nondiscernible movement. Two observers independently recorded their observations and the averages were indicated as percentage motile/degree of motility.

### Pyruvate Oxidation

Reaction mixtures (total vol 1.0 ml) containing the components indicated were incubated in unstoppered liquid scintillation vials for 30 min at 37 C in a water bath shaker. Following this preincubation period, catalase (30 μg) and sodium pyruvate-¹⁴C (1 μmole; 0.05 μCi) were added and the vials were immediately

closed with a rubber stopper to which was attached a hanging plastic cup containing 0.1 ml of 10% KOH. Incubation was continued for 30 min at 37 C and 0.2 ml 2N $H_2SO_4$ was added through a No. 22 needle already in place in the rubber stopper. The needle was corked when not in use. The reaction mixture was incubated for an additional 45 min, after which the plastic cup containing KOH was transferred to a liquid scintillation vial containing 20 cc of a toluene–ethanol–PPO–POPCP fluor (Buhler, 1962) and counted in a Tri-Carb liquid scintillation counter. The results are expressed as the number of nmoles of pyruvate oxidized.

Since pyruvate reacts with $H_2O_2$ nonenzymatically to release $CO_2$, it was necessary to destroy the $H_2O_2$ remaining after the preincubation of the spermatozoa with the sperm-inhibitory system by the addition of catalase prior to the measurement of pyruvate oxidation. Catalase served also to terminate the sperm-inhibitory effect of the peroxidase system.

### Other Procedures

Rat uterine fluid was collected from estrogen-primed immature and mature ovariectomized rats as previously described (Klebanoff and Smith, 1970a). Lactoperoxidase (LPO) was prepared by the method of Morrison and Hultquist (1963) and myeloperoxidase (MPO) by the method of Agner (1958). Peroxidase activity was determined by the o-dianisidine method (Klebanoff, 1965). One unit of activity is that causing an increase in absorbancy of 0.001/min at 460 mμ in a Cary M-15 spectrophotometer. The peroxidase activity of rat uterine fluid averaged 200 unit/ml. Catalase (crystalline beef liver 33,150 unit/mg—Worthington Biochemical Corp.) and glucose oxidase (fungal type V—880 unit/ml Sigma Chemical Co.) were dialyzed overnight against water just prior to use. Sodium pyruvate-1-¹⁴C (specific activity 1–5 mCi/mM) was obtained from New England Nuclear Corp. Ultrafiltrates were prepared with a Schleicher & Schuell ultrafiltration apparatus.

## RESULTS

The addition of rat uterine fluid, iodide, and $H_2O_2$ to washed bovine spermatozoa suspended in a $Ca^{2+}$-free Krebs–Ringer phosphate fructose medium pH 6.5 resulted in an inhibition of motility which was complete in 10 min under the conditions employed in Table 1. The spermatozoa remained fully motile for 2 hr when incubated in medium alone. Deletion of $H_2O_2$ from the system abolished the sperm-inhibitory effect; how-

TABLE 1

INHIBITION OF SPERM MOTILITY BY THE PEROXIDASE–HALIDE–$H_2O_2$ SYSTEM[a]

| Supplements | Motility: (% motile/degree of motility) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 10 min | 20 min | 30 min | 60 min | 120 min |
| None | 70/4 | 60/4 | 75/4 | 65/4 | 60/4 | 60/4 |
| Uterine fluid + $H_2O_2$ + iodide | 65/4 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| Uterine fluid + iodide | 65/4 | 70/4 | 65/4 | 70/4 | 70/4 | 65/4 |
| $H_2O_2$ + iodide | 70/4 | 65/4 | 60/3.5 | 60/3.5 | 55/3 | 45/2 |
| Uterine fluid + $H_2O_2$ | 65/4 | 65/3.5 | 55/3 | 60/3 | 55/3 | 45/1.5 |
| Uterine fluid (heated) + $H_2O_2$ + iodide | 75/4 | 50/3 | 50/3 | 45/3 | 50/2.5 | 40/2 |
| LPO + $H_2O_2$ + iodide | 75/4 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| MPO + $H_2O_2$ + iodide | 75/4 | 20/2 | 10/1 | 0/0 | 0/0 | 0/0 |
| Uterine fluid + glucose + GO + iodide | 75/4 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| Uterine fluid + glucose + GO (heated) + iodide | 65/4 | 70/4 | 70/4 | 50/4 | 60/3.5 | 70/4 |
| Uterine fluid + glucose + GO + iodide + catalase | 70/4 | 70/4 | 65/4 | 65/4 | 70/4 | 60/4 |
| Uterine fluid + glucose + GO + iodide + catalase (heated) | 70/4 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| Uterine fluid + $H_2O_2$ + thiocyanate | 70/4 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| Uterine fluid + $H_2O_2$ + bromide | 80/4 | 75/4 | 70/4 | 70/4 | 70/4 | 70/4 |
| Uterine fluid + $H_2O_2$ + chloride | 70/4 | 80/4 | 75/4 | 75/4 | 75/4 | 60/3.5 |

[a] The reaction mixture contained $2 \times 10^8$ spermatozoa in 1.0 ml of $Ca^{2+}$-free Krebs–Ringer phosphate buffer pH 6.5 with fructose (10 mg%) and the supplements as follows: uterine fluid, 0.01 ml.; potassium iodide, 0.1 μmole; $H_2O_2$, 0.1 μmole; LPO, 30 units; MPO, 30 units; glucose, 10 μmoles; glucose oxidase (GO), 0.18 unit; catalase, 30 μg; potassium thiocyanate, 0.1 μmole; potassium bromide, 0.1 μmole; potassium chloride, 0.1 μmole. Reagents were heated at 100 C for 15 min where indicated.

ever, some inhibition of motility was evident on prolonged incubation when either uterine fluid or iodide was deleted. That this latter inhibition is due to the presence of $H_2O_2$ in the reaction mixture was suggested by the comparable inhibition produced by $H_2O_2$ alone. Rat uterine fluid from either estrogen-primed immature or mature ovariectomized rats could be employed; however, uterine fluid heated at 100 C for 15 min was ineffective. The uterine fluid could be replaced by a purified preparation of lactoperoxidase (LPO) or myeloperoxidase (MPO), although MPO was not as effective as LPO under the conditions employed in Table 1. A $H_2O_2$-generating system (glucose + glucose oxidase) could be employed as the source of $H_2O_2$; sperm inhibition was abolished in this instance by heat inactivation of glucose oxidase or by the addition of catalase but not heated catalase. Thiocyanate could substitute for iodide; however, bromide and chloride were

TABLE 2

INHIBITION OF PYRUVATE OXIDATION[a]

| Supplements | Pyruvate oxidation (nmoles ± SD) | % Inhibition | p values |
|---|---|---|---|
| None | 193 ± 29 (12) | — | |
| Uterine fluid + $H_2O_2$ + iodide | 8 ± 7 (12) | 96 | <.001 |
| Uterine fluid + iodide | 205 ± 18 (6) | — | — |
| Uterine fluid + $H_2O_2$ | 171 ± 11 (6) | 12 | <.1 |
| $H_2O_2$ + iodide | 155 ± 26 (12) | 20 | <.01 |
| $H_2O_2$ | 151 ± 30 (8) | 22 | <.01 |
| Uterine fluide + $H_2O_2$ + thiocyanate | 13 ± 11 (6) | 94 | <.001 |
| Uterine fluid + glucose + glucose oxidase + iodide | 11 ± 3 (6) | 95 | <.001 |

[a] The components of the reaction mixture were as described in Table 1. Results are expressed as mean ± SD with the number of determinations in parentheses. p values were determined by Student's t test.

ineffective at pH 6.5 under the conditions employed in Table 1. Table 2 demonstrates the inhibitory effect of the uterine fluid–iodide–$H_2O_2$ system on a second parameter of sperm activity, the conversion of ^{14}C-labeled pyruvate to [14]CO₂. As was observed with sperm motility, iodide could be replaced by thiocyanate and $H_2O_2$ by glucose and glucose oxidase. Table 2 also demonstrates the small sperm-inhibitory effect produced by $H_2O_2$ alone under the conditions employed.

Unwashed spermatozoa were unaffected by the uterine fluid-mediated system under the conditions employed in Table 1 suggesting the presence of inhibitors in seminal plasma. This was found to be the case. Seminal plasma at a final concentration of 30% prevented the inhibition of sperm motility by the uterine fluid–iodide–$H_2O_2$ system under the conditions employed in Table 3. This effect was not observed when the concentration of seminal plasma was decreased to 2.5% whereas concentrations of 5 and 15% produced partial protection. The inhibitors of seminal plasma were unaffected by heating to 100 C for 15 min. Dialysis of the seminal plasma, however, greatly decreased the inhibitory effect, suggesting that the inhibitors were largely of low molecular weight. This was supported by the marked inhibitory effect of a seminal plasma ultrafiltrate. A number of heat-stable, low-molecular-weight substances

decreased the activity of the uterine fluid–iodide–$H_2O_2$ system. These included azide, reduced glutathione, cysteine, ergothioneine, and ascorbic acid (Table 3).

Rat uterine fluid is an essential component of the sperm-inhibitory system. However, previous studies had demonstrated the presence of heat-stable, low-molecular-weight inhibitors of the uterine fluid–iodide–$H_2O_2$ antimicrobial system in uterine fluid heated to inactivate peroxidase (Klebanoff and Smith, 1970a). Heated rat uterine fluid at a final concentration of 40% decreased the sperm-inhibitory effect of the uterine fluid–iodide–$H_2O_2$ system under the conditions employed in Table 4. A weaker sperm-inhibitory system utilizing a lower concentration of $H_2O_2$ was required to demonstrate the inhibition by heated uterine fluid. The heat-stable inhibitor or inhibitors of the uterine fluid–iodide–$H_2O_2$ system were removed by dialysis and were found in the ultrafiltrate of the uterine fluid.

## DISCUSSION

The uterine fluid–iodide–$H_2O_2$ system, previously shown to have bactericidal, fungicidal, and virucidal activity (Klebanoff and Smith, 1970a) also has sperm-inhibitory activity as measured by a decrease in motility and pyruvate oxidation. The uterine fluid contains peroxidase and that the requirement for uter-

TABLE 3

INHIBITION BY SEMINAL PLASMA AND CERTAIN LOW-MOLECULAR-WEIGHT COMPOUNDS[a]

| Inhibitors | Complete system | Motility: (% motile/degree of motility) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 10 min | 20 min | 30 min | 60 min | 120 min |
| — | — | 60/4 | 60/4 | 50/4 | 55/4 | 50/4 | 60/4 |
| — | + | 70/4 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| Seminal plasma | + | 80/4 | 70/3.5 | 65/4 | 60/3.5 | 60/4 | 60/3 |
| Seminal plasma (heated) | + | 80/4 | 70/4 | 70/4 | 70/4 | 70/4 | 70/4 |
| Seminal plasma (dialyzed) | + | 70/4 | 25/1 | 0/0 | 0/0 | 0/0 | 0/0 |
| Seminal plasma (ultrafiltrate) | + | 60/4 | 60/3 | 45/2 | 50/2 | 40/2 | 40/2 |
| Azide | + | 60/4 | 60/4 | 60/4 | 60/4 | 60/3 | 60/3 |
| Glutathione | + | 60/4 | 60/4 | 60/4 | 55/4 | 60/4 | 50/4 |
| Cysteine | + | 60/4 | 60/4 | 50/4 | 50/4 | 55/4 | 60/3 |
| Ergothioneine | + | 60/4 | 60/4 | 60/4 | 55/4 | 60/4 | 50/3 |
| Ascorbic acid | + | 60/4 | 60/4 | 55/4 | 55/4 | 60/4 | 50/3 |

[a] The complete uterine fluid–iodide–$H_2O_2$ system was as described in Table 1. The inhibitors were added as follows: seminal plasma, 0.3 ml; seminal plasma heated at 100 C for 15 min, 0.3 ml; seminal plasma dialyzed for 24 hr against the Krebs–Ringer phosphate fructose (10 mg%) medium, 0.3 ml; ultrafiltrate of seminal plasma, 0.3 ml; azide, 0.01 μmole; reduced glutathione, 0.1 μmole; cysteine, 0.01 μmole; ergothioneine, 0.1 μmole; ascorbic acid, 0.1 μmole. Total vol 1.0 ml.

ine fluid is due to the presence of this enzyme is suggested by its replacement by a purified preparation of lactoperoxidase or myeloperoxidase, by the $H_2O_2$ requirement and by the inhibitory effect of azide, a substance which inhibits peroxidase, as well as other heme-containing enzymes. A peroxidase accumulates in the rat uterus on estrogen stimulation (Lucas et al., 1955). A characteristic of the estrogenic response in the rat uterus is the striking in-

TABLE 4

INHIBITION BY UTERINE FLUID[a]

| Inhibitors | Complete system | Motility: (% motile/degree of motility) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 10 min | 20 min | 30 min | 60 min | 120 min |
| — | — | 60/4 | 60/4 | 70/4 | 60/4 | 50/3 | 50/3 |
| — | + | 60/4 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| Uterine fluid (heated) | + | 60/4 | 45/3.5 | 45/3 | 40/3 | 40/3 | 30/1 |
| Uterine fluid (dialyzed) | + | 60/4 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| Uterine fluid (ultrafiltrate) | + | 65/4 | 50/4 | 50/4 | 55/4 | 55/4 | 50/3 |

[a] The complete system was as described in Table 3 except that 0.05 μmole of $H_2O_2$ was employed instead of 0.1 μmole. The inhibitors were added as follows: heated uterine fluid, 0.4 ml; heated and dialyzed uterine fluid, 0.4 ml; ultrafiltrate of heated uterine fluide, 0.4 ml.

flux of eosinophils into the connective tissue spaces (Rytömaa, 1960; Bjersing and Borglin, 1964; Klebanoff, 1965) where they either lyse, releasing their organelles into the interstitial tissue or are ingested by macrophages (Ross and Klebanoff, 1966). The eosinophil is particularly rich in peroxidase and thus may be the source of the peroxidase in uterine fluid (Rytömaa and Teir, 1961; Paul et al., 1967). Peroxidase activity also has been demonstrated histochemically in the epithelial cells of the estrogen-primed rat endometrium (Brökelmann and Fawcett, 1969).

Iodide and thiocyanate ions were particularly effective as the cofactor in the uterine fluid-mediated sperm-inhibitory system, whereas bromide or chloride were without effect under the conditions employed. Bromide, at relatively high concentrations, did substitute for iodide when the bactericidal activity of the uterine fluid–halide–$H_2O_2$ system was measured (Klebanoff and Smith, 1970a). This may be due to the lower pH employed in the bactericidal studies (pH 5.0), since the uterine fluid-mediated antimicrobial system is more effective at pH 5.0 than at pH 6.5 (Klebanoff and Smith, 1970a). Iodide is present in the rat uterus and its level rises above that of plasma in early pregnancy or following progesterone administration particularly during diestrus suggesting the concentration of iodide by the uterus under these conditions (Brown-Grant, 1965, 1967). The oviduct/plasma ratio for iodide also is greater than one during certain stages of the estrous cycle (metestrus, diestrus) and in early pregnancy (Brown-Grant, 1965).

The requirement for $H_2O_2$ in the uterine fluid-mediated sperm-inhibitory system raises the question of the source of $H_2O_2$ for such a system in situ. The utilization of $H_2O_2$ of either microbial or spermatozoan origin is the subject of a subsequent paper (Klebanoff and Smith, 1970b).

The complex nature of the uterine fluid-mediated sperm-inhibitory system is further emphasized by the demonstration of a heat-stable, dialyzable inhibitor or inhibitors in seminal plasma and to a lesser degree, in uterine fluid. A number of low-molecular-weight compounds decrease the sperm inhibitory effect of the uterine fluid–halide–$H_2O_2$ system and some of these, e.g., ergothioneine and ascorbic acid, may be found in the seminal plasma of some species (see Mann, 1964). The uterine fluid–halide–$H_2O_2$ sperm-inhibitory system is also inhibited by catalase. Although there may be a significant amount of this enzyme in rabbit seminal plasma (Wales et al., 1959), very little, if any, is present in the seminal plasma of a number of other mammalian species (Tosic and Walton, 1950; Wales et al., 1959; Mann, 1964).

The mechanism of sperm inhibition by the uterine fluid-mediated system is unknown. Previous studies have demonstrated that the bactericidal effect of the peroxidase–iodide–$H_2O_2$ system is associated with the iodination of the bacteria (Klebanoff, 1967). Spermatozoa are iodinated by the uterine fluid–iodide–$H_2O_2$ system (unpublished data) and sperm inhibition may be related to this phenomenon.

## ACKNOWLEDGMENTS

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