FERTILITY AND STERILITY

Vol. 39, No. 4, April 1983
Printed in U.S.A.

Human sperm penetration of zona-free hamster eggs after storage of the semen for 48 hours at 2° C to 5° C*

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The motility of human spermatozoa and their ability to penetrate zona-free hamster eggs were examined after dilution of the semen with TES-Tris (TEST) yolk buffer and storage for 48 hours at 2° C to 5° C. Semen samples from 10 fertile donors and 19 infertility patients were studied. More than 65% of the spermatozoa which were initially motile in the TEST yolk buffer remained active after storage. During storage, the mean swimming speed of the sperm declined to approximately 60% of the prestorage value. The percentage of zona-free hamster eggs that were penetrated by spermatozoa from patients and donors increased significantly following 48 hours of storage at 2° C to 5° C. Normal semen and abnormal semen were equally preserved by this storage method. This procedure may be used to ship semen samples by commercial transportation to specialized laboratories for testing. Low temperature storage in the TEST yolk buffer appears to enhance the fertilizing capacity of human spermatozoa in vitro. *Fertil Steril 39:536, 1983*

In 1976, Yanagimachi and his colleagues[1] discovered an exceptional property of oocytes from the Golden hamster. In this species, the principal block to interspecies fertilization is located at the zona pellucida, an outer investing layer that is easily removed by enzymic digestion. Very little species specificity remained when the zona was removed, and human spermatozoa readily fused with zona-free hamster vitelli.[2] There was almost immediate clinical interest in the hamster egg assay, and subsequent reports have demonstrated a significant association between human male infertility and the failure of fusion between spermatozoa and zona-free hamster eggs in vitro.[3-12] The possibility that the assay may detect fertilization dysfunction of human sperm has encouraged its application not only in the clinical evaluation of male fertility but also in contraceptive development and in surveillance of occupational and environmental hazards to male fertility potential.

The zona-free hamster egg assay is a highly specialized test that at the present time can only be performed and interpreted in a relatively small number of research centers. We undertook the present experiments to evaluate a means for short-term storage of human semen that would permit its shipment to a distant laboratory for testing with the hamster egg assay. In previous studies of bovine spermatozoa, the low temperature preservation of semen was accomplished with an egg yolk extender containing the buffers

Received September 28, 1982; revised and accepted December 20, 1982.

*Supported by National Institutes of Health grant HD 15149, Environmental Protection Agency grant R 809089, and National Institute for Occupational Safety and Health grant OH 01148.

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N-Tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) and hydroxymethyl aminomethane (Tris).[13] The TES and Tris (TEST) yolk buffer has recently been shown to support the motility of human spermatozoa during storage at 5° C for 24 to 96 hours.[14]

A storage temperature of 2° C to 5° C (ice water) and a transport interval of 24 to 48 hours are logistically feasible for commercially available transportation services. In this communication, we report experiments on the physiology of spermatozoa from fertile donors and infertility patients that were stored under such conditions. We have measured sperm motility in the fresh semen, and we have assessed the ability of these sperm to penetrate zona-free hamster eggs in vitro. These results have been compared with those obtained using an aliquot of the same specimen which was stored for 48 hours at 2° C to 5° C in the TEST yolk buffer. We found that the fertilizing capacity of human spermatozoa was always maintained under these conditions; and in many cases, the percentage of penetrated hamster eggs significantly increased after semen storage.

MATERIALS AND METHODS

PREPARATION OF THE TEST YOLK BUFFER

The buffer was prepared according to Graham et al.[13] The following chemicals were dissolved in 50 ml of water twice distilled in glass: TES, 2.16285 gm; Tris, 0.51346 gm; dextrose, 0.10000 gm; streptomycin sulfate, 0.01250 gm; and penicillin-G, 0.00750 gm. TES, Tris, and streptomycin were obtained from Sigma Chemical Company, St. Louis, MO; dextrose from Fisher Chemical Company, Pittsburgh, PA; and penicillin-G from U.S. Biochemical Corporation, Cleveland, OH. The yolk from freshly laid chicken eggs was then added to make a 20% egg yolk solution in the buffer. The yolk buffer was centrifuged at 840 × g for 10 minutes, and the pellet was discarded. The pH of the supernatant was adjusted to 7.35 to 7.45 by addition of Tris, and the osmolality was adjusted to the range of 290 to 320 mOsm. The TEST yolk buffer was either used immediately or was stored at −4° C for up to 1 month.

PREPARATION OF SPERM SUSPENSIONS

Semen specimens, collected by masturbation, were obtained from 10 fertile donors and from 19 patients attending our infertility clinic. The volume of each specimen was measured, and the sperm concentration was determined by hemocytometer counts. Videotapes of the semen were analyzed for sperm motility assessment (percentage of motile cells and mean swimming speed),[15] and the morphologic characteristics of immobilized living sperm were also assessed by videomicrography.[16] All of the donors had normal semen, i.e., ≥ 2 ml, ≥ 20 × 10^6 sperm/ml, ≥ 50% motility, a mean swimming speed of ≥ 25 μm/second, and ≥ 50% normal heads. Eleven of the infertility patients also had normal semen, but 8 patients had abnormalities in one or more of the parameters evaluated.

All semen specimens were tested within 2 hours of collection. Each specimen was divided into two aliquots. The first aliquot (1 ml) was diluted with 1 ml of the TEST yolk buffer, and the resulting mixture was transferred to a 2-ml cryopreservation tube (Vanguard International, Neptune, NJ). The tube was placed inside a plastic specimen container (Scientific Products, McGraw Park, IL) filled with 120 ml of water at room temperature (25° C). The water-filled container was then placed inside a Styrofoam chest containing crushed ice (Fig. 1), where it remained for 48 hours. At the end of the storage period, the water-filled specimen container was removed from the ice bath and transferred directly to a 37° C incubator for 1 hour. Spermatozoa were prepared for incubation with zona-free hamster eggs by injecting the previously stored semen into a 15-ml

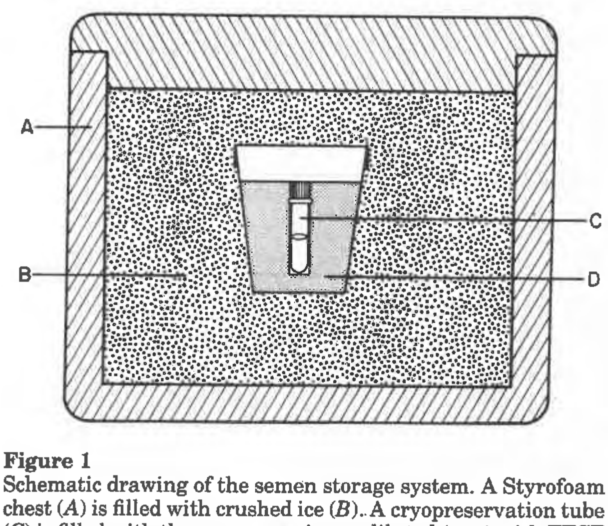

Figure 1
Schematic drawing of the semen storage system. A Styrofoam chest (*A*) is filled with crushed ice (*B*). A cryopreservation tube (*C*) is filled with the semen specimen, diluted 1 to 1 with TEST yolk buffer. The sealed tube of diluted semen is placed within a water-filled plastic container at room temperature. The sealed container is then buried within the ice bath (*D*) and is left undisturbed until the end of the storage period.

Table 1. *The Motility of Human Spermatozoa Before and After 48 Hours of Storage in TEST Yolk Buffer at 2° C to 5° C*[a]

Group	% Motility		% Retention of motility	Swimming speed		% Retention of swimming speed
	Prestorage	Poststorage		Prestorage	Poststorage	
				μm/sec		
Fertile donors	68 ± 4	48 ± 5	70 ± 6	54 ± 6	31 ± 3	61 ± 6
Patients: normal semen	59 ± 4	44 ± 3	76 ± 4	48 ± 3	29 ± 3	64 ± 8
Patients: abnormal semen	47 ± 4	31 ± 4	66 ± 6	42 ± 3	24 ± 3	59 ± 5

[a]Values are mean ± standard error of the mean among specimens.

plastic centrifuge tube (Falcon Plastics, Oxnard, CA) beneath 6 ml of Biggers, Whitten and Whittingham (BWW) culture medium.[17] The BWW medium was modified according to Yanagimachi et al.[18] and contained 3 mg/ml bovine serum albumin (Fraction V, Sigma). The tube was inclined at a 20-degree angle in a 37° C incubator with an atmosphere of 5% CO_2 in air. Sixty to 90 minutes were allowed for upward migration of motile spermatozoa from the stored semen into the BWW medium. The BWW layer was carefully removed and centrifuged for 5 minutes at 500 × *g*. The sperm pellet was washed twice by dilution with BWW medium and recentrifugation. The concentration of motile sperm in the final suspension was determined and adjusted to 5 to 10 × 10^6 motile sperm/ml.

The second, control aliquot of the original fresh semen sample was prepared for incubation with zona-free hamster eggs by our standard laboratory procedures. Whole semen was injected under BWW medium as described above, and the suspension of motile sperm was washed twice by dilution and centrifugation. The final sperm suspension (0.2 to 0.5 ml) was incubated for 18 to 24 hours in the CO_2 incubator at 37° C. At the end of incubation, the concentration of motile spermatozoa was determined and adjusted to 5 to 10 × 10^6 motile sperm/ml.

EVALUATION OF SPERM MOTILITY

Sperm motility was analyzed by videomicrography (1) in the original semen specimen, (2) immediately after dilution with TEST yolk buffer, and (3) after 48 hours of storage at 2° C to 5° C. Sperm movement in each suspension was recorded on videotape at 37° C as described by Katz and Overstreet.[15] We analyzed 50 spermatozoa to determine the percentage of motility and measured the swimming speeds of 25 spermatozoa to obtain a mean swimming speed. For each of the three groups of men, we used the mean values for percentage of motility and swimming speed in TEST yolk buffer before and after storage to calculate a percentage retention of motility and swimming speed during storage (percentage retention = poststorage value ÷ prestorage value × 100).

EVALUATION OF SPERM INTERACTION WITH ZONA-FREE HAMSTER EGGS

Zona-free hamster eggs were obtained by standard methods for superovulation, ovum recovery, and removal of the zona pellucida.[1] Twenty-five to 50 zona-free hamster eggs were transferred in BWW medium to 30 × 10 mm polystyrene Petri dishes (Falcon) containing mineral oil and 150 μl of the sperm suspension (i.e., either stored sperm or control sperm). Following 3 to 4 hours of incubation at 37° C in the CO_2 incubator, the hamster eggs were mounted between a glass slide and a coverslip and examined with phase-contrast microscopy.[19] The number of decondensing sperm heads with associated tails was counted in each egg.

RESULTS

THE TEST YOLK BUFFER SUSTAINED THE MOTILITY OF SPERMATOZOA DURING 48 HOURS OF STORAGE

Comparison of sperm motility in the TEST yolk buffer before and after storage indicated that 66% to 76% of the spermatozoa that were initially motile remained active after storage (Table 1). The mean swimming speed declined to approximately 60% of the levels prior to storage (Table 1). When the effect of storage on sperm motility was compared in the group of fertile donors and the two groups of infertility patients (Table 1), analysis of variance showed no difference between the three groups in the retention of either motility or swimming speed.

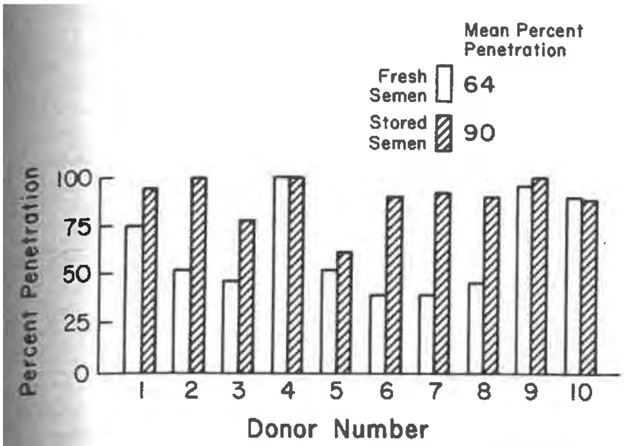

Figure 2
Penetration of zona-free hamster eggs by spermatozoa from fertile donors. Results with fresh semen are compared with semen stored for 48 hours at 2° C to 5° C.

SPERM PENETRATION INTO ZONA-FREE HAMSTER EGGS WAS ENHANCED AFTER 48 HOURS OF STORAGE IN TEST YOLK BUFFER

For each group of men, the percentage of zona-free hamster eggs penetrated by spermatozoa was significantly increased after 48 hours of sperm storage in TEST yolk buffer at 2° C to 5° C (paired t-test after transformation to angles, $P \leq 0.05$). Figures 2 to 4 show the results for individual men within each group. Clearly, the increase in the percentage of penetration differed from man to man, and in some individual cases it was not significant (as assessed by chi-square analysis). For all three groups of men, significantly more spermatozoa penetrated the hamster eggs after sperm storage in comparison with spermatozoa tested prior to storage (Table 2).

DISCUSSION

The results of these experiments clearly demonstrate that human sperm may be stored for up to 48 hours at 2° C to 5° C without adversely affecting their capacity to fuse with zona-free hamster eggs. This study further suggests that normal semen and semen of poor quality are equally well preserved by this method. Since commercial transportation within the United States can routinely deliver parcels within a 24- to 48-hour period, it appears feasible at this time to ship semen samples to specialized laboratories for testing. Such testing may be appropriate for diagnosis of male infertility when the semen quality is otherwise normal and in field studies of occupational or environmental hazards to male fertility, especially of men who are not concurrently attempting a pregnancy.

A better understanding of the "optimum" in vitro conditions for fusion between human spermatozoa and zona-free hamster eggs is badly needed. The assumption that optimum conditions already exist in the currently employed clinical tests is a serious misconception. There are a number of studies that suggest that a normal range has been established for the percentage of zona-free hamster eggs penetrated by human spermatozoa in vitro. The lower limit of this range has been frequently set at 10% to 15% penetration,[4, 7, 10, 12] and it appears to be the practice of some laboratories to interpret any result below this range as an indication of male infertility.[8, 9] In addition to the fundamental biologic principles that challenge this logic,[20] the present results suggest that alteration of the assay conditions

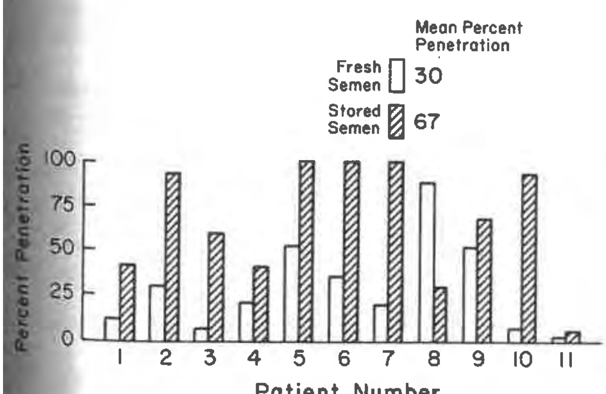

Figure 3
Penetration of zona-free hamster eggs by spermatozoa from infertility patients with apparently normal semen evaluations. Results with fresh semen are compared with semen stored for 48 hours at 2° C to 5° C.

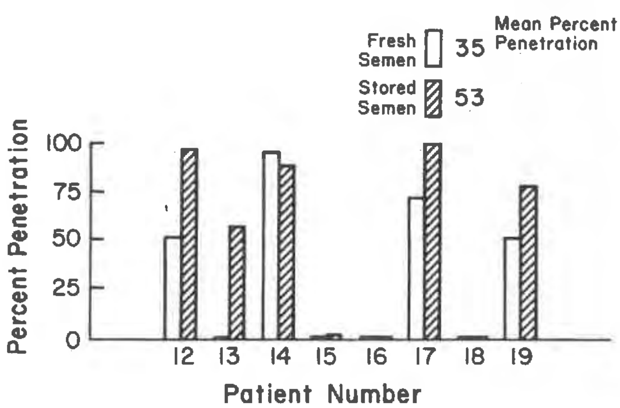

Figure 4
Penetration of zona-free hamster eggs by spermatozoa from infertility patients with abnormal semen evaluations. Results with fresh semen are compared with semen stored for 48 hours at 2° C to 5° C.

Table 2. *Sperm Penetration of Zona-Free Hamster Eggs After Storage of the Semen in TEST Yolk Buffer for 48 Hours at 2° C to 5° C*[a]

Group	% of Eggs penetrated		No. of sperm per penetrated egg	
	Fresh semen	Stored semen	Fresh semen	Stored semen
Fertile donors	61 ± 8	83 ± 8	1.04 ± 0.25	3.19 ± 1.12
Patients: normal semen	29 ± 8	67 ± 10	0.42 ± 0.14	1.99 ± 0.52
Patients: abnormal semen	35 ± 10	47 ± 12	0.80 ± 0.33	1.47 ± 0.62

[a]Values are mean ± standard error of the mean.

can change a "negative" test into a "positive" one. The results of the assay on fresh semen from patients 1, 3, 10, and 13 could have been interpreted as abnormal by contemporary criteria. Yet in each of these cases, the penetration rate reached the normal range after the semen was stored for 48 hours. These results are consistent with the findings of Binor et al.,[21] who were able to improve the hamster egg penetration rates in 31% of 41 cases by incubating spermatozoa in a TEST yolk buffer for 24 hours or more.

The clinical application of our knowledge of sperm physiology requires appreciation of the limitations of current technology. In spite of intensive research with human gametes and those of laboratory animals, we still do not understand the precise relationship between in vitro fertilization tests and sperm function in vivo. In vitro tests with zona-free hamster eggs have revealed significant differences in "fertilizing capacity" among fertile men.[19] The results of the present study demonstrate that the percentage of penetrated hamster eggs can be altered in vitro by modifying the environment of the sperm cell. This biologic complexity makes it almost impossible to compare the results of egg penetration assays carried out in different laboratories and limits the confidence with which a fertility diagnosis can be made at the present time on the basis of egg penetration tests alone. With further study, we may be able to define standard, optimum conditions for this assay. In the future, we could manipulate these systems to increase the in vitro fertilizing capacity of spermatozoa from subfertile men and make possible the treatment of these cases by in vitro fertilization and embryo transfer.

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