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Comparison of bicarbonate and HEPES-buffered media on pregnancy rates after intrauterine insemination with cryopreserved donor sperm*

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Objective: We compared the pregnancy rates (PRs) after intrauterine insemination (IUI) with frozen donor sperm prepared in Ham's F-10 medium (Irvine Scientific, Santa Ana, CA) with bicarbonate buffer and synthetic human tubal fluid with HEPES buffer (Irvine Scientific).

Design: Women (n = 101) were randomized upon entry into the program, receiving sperm prepared in either Ham's F-10 or human tubal fluid medium their first treatment cycle. If pregnancy did not occur, the alternate medium was used to prepared sperm for the following cycle.

Setting: All patients were treated in our private care center.

Patients: Patients entering this study were normally ovulating women undergoing IUI with frozen donor sperm.

Main Outcome Measure: Pregnancy was used as our main outcome measure of success.

Results: After 324 cycles of treatment, the PR per cycle of IUI was 17.5% with sperm prepared in human tubal fluid which was significantly different ($P = 0.05$) from the PR (9.8%) after insemination with sperm prepared in Ham's F-10. There was no statistical difference in the number of motile cells inseminated in each of these groups.

Conclusions: Transitory exposure of the sperm in Ham's F-10 medium to the environment during preparation for insemination may result in an alkalinization of the medium that has a lasting influence on sperm fertility. Fertil Steril 56:540, 1991

The use of intrauterine insemination (IUI) with fresh and frozen sperm has gained widespread acceptance over the past several years for treatment of certain types of infertility. Because the use of fresh spermatozoa in a therapeutic donor insemination program is no longer acceptable,[1,2] programs must now use frozen sperm that has been quarantined. However, cryopreservation of sperm causes damage to sperm with a resultant loss of sperm viability and fertility.[3–5] Considering the lowered productivity of frozen-thawed sperm, it is essential to consider ways to improve the pregnancy rates (PRs) with frozen donor sperm. Strategies to improve PRs with therapeutic donor insemination may include different techniques for sperm preparation and cryopreservation, use of different media for sperm preparation, transcervical placement of spermatozoa into the reproductive tract, superovulation protocols, and better timing of insemination. We have previously demonstrated the superiority of IUI with frozen donor sperm over intracervical insemination in a prospective study.[6]

Although a variety of culture media have been described for sperm preparation for use with IUI,[7] the influence of these different media on PRs after

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therapeutic donor insemination has not been examined in a prospective fashion. The culture media used to prepare sperm for IUI are usually based on a modification of Krebs-Ringers bicarbonate solution. In these media, pH is regulated by incubation in a 5% CO_2 atmosphere. Alternate buffers such as N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES buffer) can be used to substitute for or replace sodium bicarbonate. The substitution of HEPES buffer allows incubation in atmospheric air. Although synthetic human tubal fluid medium with HEPES buffer has been successfully used in oocyte fertilization and culture,[8,9] there is some debate as to the ability of both media to equally support fertilization and embryonic development under in vitro conditions.[8,10] The short-term exposure of sperm prepared in Ham's F-10 medium (Irvine Scientific, Santa Ana, CA) to atmospheric air during insemination could cause a pH shift in the media and potentially lower sperm fertility.

In this prospective, randomized study, we have compared conception rates after IUI insemination with frozen-thawed sperm specimens prepared in bicarbonate or HEPES-buffered media in alternating trials of therapeutic donor insemination. We have quantitated sperm motility parameters after preparation and patient etiology to determine their influence on the success of therapeutic donor insemination. The success of the different media were judged on their influence on pregnancy outcome. We have also analyzed the timing of IUI and subsequent PRs in cycles with a single insemination.

MATERIALS AND METHODS

Patient Selection

Patients (n = 144) entering our therapeutic donor insemination program from March 1989 to July 1990 were considered for this study. All women undergoing therapeutic donor insemination had an initial fertility evaluation before acceptance into the program. Ovulatory status was documented by basal body temperature (BBT) charts and midluteal phase serum progesterone (P) levels or by endometrial biopsy. Tubal patency was evaluated by laparoscopy and/or by hystersalpingogram. Women with bilateral tubal obstruction or adhesions were eliminated from the program. Upon completion of the study, women were divided into two groups: women with no known female factor (n = 58) or women with one or more female factors (n = 43). Women with a female factor(s) were defined as having endometriosis, a cervical factor, chronic anovulation, a luteal phase defect, or sperm antibodies. Anovulatory women or women with midluteal phase P values < 5 ng/mL and/or menstrual cycles > 32 days in length were treated with 50 to 100 mg of clomiphene citrate. Patients were randomized upon entry into the study using a computer-generated random numbers table. The patients were inseminated by IUI with cryopreserved donor sperm washed and prepared in either Ham's F-10 or human tubal fluid.

The male partners in couples undergoing therapeutic donor insemination were either azoospermic or had a severe male factor. Male factor was defined as having one or more of the following sperm parameters: $<20 \times 10^6$ sperm/mL, <50% of all sperm showing motility, <50% normal forms, and/or an immunobead test (BioRad, Richmond, CA) > 60% positive for immunoglobulins (IgA or IgG) on the sperm or in their serum. All male factor couples (excluding azoospermic husbands or males with sperm antibodies) underwent at least four cycles of properly timed IUI with husband's sperm before entering the study.

Media Preparation

Medium was prepared from dry powder obtained from Irvine Scientific. Ham's F-10 (No. 9434, 25 mM sodium bicarbonate buffer final) and modified human tubal fluid, (No. 98270, 21 mM HEPES-4mM sodium bicarbonate buffer) were prepared in the laboratory and adjusted to 280 mOsm/kg with water. Ham's F-10 medium was equilibrated before use with humidified 5% CO_2:95% atmospheric air. Aliquots of media were checked periodically on a pH meter to ensure that the pH was correct. All media were assayed with a two-cell mouse embryo culture and stored at 4°C before use. All stock solutions were discarded 3 weeks after preparation.

Donor Selection

Donors were selected from healthy, medical student volunteers who were screened for heritable or sexually transmitted disease (STD) and who had good before and after thaw semen characteristics as described previously.[6] All donor samples were quarantined a minimum of 6 months before use. Donors were tested upon entry into the therapeutic donor insemination program and retested quarterly for STDs.

Sperm Cryopreservation, Thawing, and Preparation for Insemination

Fresh ejaculates were allowed to liquefy for 30 minutes before standard and computer-assisted se-

men analysis. Sperm were then suspended in a 7.5% glycerol-cryoprotectant medium[11] and frozen in a programmable rate freezer (Cryomed, Mt. Clemens, MI) as described previously.[6,12]

Sperm specimens were thawed at room temperature for 30 minutes before use. At this time, a small aliquot of each specimen was taken for standard and computer-assisted semen analysis before sperm washing. The thawed samples were resuspended in three volumes of wash medium and centrifuged at 200 × *g* for 6 minutes. After centrifugation, the sperm pellet was resuspended in 2 mL of fresh medium, mixed, and centrifuged again for 6 minutes at 200 × *g*. After the second wash, the sperm pellet was suspended in 0.5 mL of medium. There was no attempt to recover a swim-up fraction for insemination. Washed prepared samples were then placed in a 37°C incubator with either a 5% CO_2 atmosphere (Ham's F-10) or atmospheric air (human tubal fluid) until use. An aliquot of each washed sample was incubated for 30 minutes after washing and then subjected to standard and computer-assisted semen analysis.

Samples were prepared for insemination approximately 30 minutes after preparation. The samples were aspirated into a $3\frac{1}{2}$ French Tomcat catheter (Richmond Veterinarian Supply, Richmond, VA) that was connected to a 1-cc Becton-Dickinson syringe (Rutherford, NJ). The catheter was gently passed through the cervical canal into the uterine cavity 1 cm from the uterine fundus, and the contents of the syringe slowly injected. After 15 minutes of resting in the supine position, the patient resumed normal activities. A quantitative human chorionic gonadotropin-beta subunit was performed 2 weeks after insemination and repeated if positive. If both assays were positive, ultrasound confirmation of pregnancy was obtained after 6 weeks of gestation.

If pregnancy did not occur during the initial cycle, the patients were crossed over, and the alternate medium was used to prepare sperm for the following cycle. For each subsequent cycle the alternate sperm preparation medium was used. Patients received cryopreserved sperm from the same donor until they became pregnant or had undergone six unsuccessful cycles of IUI. After six cycles, they were assigned to a new donor.

Semen Analysis

Standard and computer-assisted semen analysis was performed on all samples. Computer-assisted semen analysis was performed using the CellTrak/S automated semen analysis system (Motion Analysis Corporation, Santa Rosa, CA). Aliquots (4 μL) of sperm were placed into 12-μm disposable counting chambers (Fertility Technologies, Inc., Natick, MA) on a 37°C microscope stage warmer.

The computer-assisted semen analysis system consisted of a Zeiss phase contrast microscope with a 20× objective, an RCA TC2812 video camera, Panasonic Monitor and videorecorder (Panasonic, Denver, CO), a VP110 microprocessor, a 80386 computer and software for automated semen analysis (Motion Analysis Corporation). Only fresh or thawed specimens (not recordings) were analyzed. The computer analyzed the field at 30 frames/s. Minimum velocity was set at 10 μm/s and maximum velocity at 400 μm/s. A minimum of 100 sperm was analyzed from each specimen.

Timing of Insemination

The timing of insemination for all patients entered into this study was determined by quantitative measurements of urinary luteinizing hormone (LH) levels beginning 2 to 3 days before the expected date of ovulation, as predicted from previous BBT charts. At least two to three urine specimens were analyzed each day for the level of urinary LH using a quantitative spectrofluorometric LH assay from Pharmacia (Pharmacia Diagnostics Inc., Columbia, MD). Specimens were collected in the morning (second voided urine) and early evening (8:00 P.M.). These samples were stored at 4°C by the patient and the laboratory until analysis. Urine samples were not indexed for creatinine. Samples were never stored >3 days. Most samples were stored a maximum of 24 hours. Additional urine samples were collected if the patient was able to store them properly. Inseminations were performed on the day of the LH peak and the following morning whenever possible. We defined a urine LH peak as value equivalent to 40 IU.

Statistical Analysis

Statistical analysis (Fisher's exact test) was performed using a statistics program (UTSTAT) on a Vax 8000 (Academic Computing Center, University of Texas Southwestern Medical School, Dallas, TX). Life table analysis was performed as described by Cramer et al.[13]

RESULTS

A total of 101 patients underwent 324 cycles of IUI in our therapeutic donor insemination program

Table 1 Influence of Media on PRs After IUI With Cryopreserved Donor Sperm

Media	No. of treatment cycles	Total no. of inseminations	Total no. of pregnancies	Pregnancy/treatment cycle
				%
Ham's F-10 medium	164	298	16	9.8[a]
Human tubal fluid	160	307	28	17.5

[a] The Ham's F-10 medium group is significantly different from the human tubal fluid group ($P = 0.05$).

during this study. The overall PR per cycle of IUI was 17.5% with sperm prepared in human tubal fluid which was significantly different ($P = 0.05$) than the 9.8% PR per treatment cycle observed when sperm prepared in Ham's F-10 medium (Table 1). The average number of inseminations per cycle was similar. There was an average of 1.8 inseminations/cycle using Ham's F-10 medium and 1.9 inseminations/cycle using human tubal fluid medium. Each patient was randomized into a different protocol for her initial treatment cycle. Although each patient served as her own control, it is possible that variables such as patient etiology, previous fertility and ovulatory status, the number of inseminations, and the total number of sperm inseminated may have influenced their outcome.

Patients were randomized into the two protocols without regard for their infertility status, years of infertility, or age. Because an unequal distribution of patients could have influenced our results, we examined patient demographics after the first cycle of treatment (Table 2). It is clear that patients were randomized equally between the two treatment groups. The major difference between these two treatments was the 23.1% PR observed in the human tubal fluid group during their first treatment cycle versus the 12.2% PR seen in the Ham's F-10 group after one cycle of treatment. The increased fecundity of sperm prepared in human tubal fluid continued to be superior to Ham's F-10 prepared sperm through the second and third cycles of inseminations. Because this study was a crossover design, we also examined the influence of known female and male factors on PRs for all insemination cycles (Table 3). Patients were divided into two groups depending on the absence or presence of sperm in the husband's ejaculate. After this, women were identified as having either no known fertility problem or were classified as having a female factor(s) as defined in Materials and Methods. The highest PRs were noted in husbands with azoospermia. Surprisingly, there was little difference between women with no known infertility problems and women with diagnosed female factors. No pregnancies were noted in women with moderate to severe endometriosis (14 treatment cycles). The presence of some sperm in the husband's ejaculate lowered the overall PRs when compared with azoospermic males.

Although sperm from the same donor was used in each arm of this study, it was possible that the different media used for preparation directly influenced sperm motility. An examination of sperm motility characteristics was made 30 minutes after preparation for IUI (Table 4). The average number of motile cells inseminated in this study (17 million) was sufficient to sustain the highest PR after IUI

Table 2 Patient Demographics

	Ham's F-10 medium	Human tubal fluid
No. of patients in first cycle of treatment	49	52
PR in the first treatment cycle (%)	12.2	23.1
Patient age (y)	33.5 ± 4.2[a]	32.5 ± 4.2
Length of infertility (y)	5.0 ± 2.5	4.8 ± 3.1
Patient etiology (no. of patients)		
Azoospermic—no female factor	20	23
Azoospermic—female factor(s)	12	12
Nonazoospermic—no female factor	8	7
Nonazoospermic—female factor(s)	9	10

[a] Values are means ± SD.

Table 3 Patient Etiology and PRs After Insemination With Frozen Donor Sperm

Etiology	No. of treatment cycles		No. pregnant[a]	
	Ham's F-10 medium	Human tubal fluid	Ham's F-10 medium	Human tubal fluid
Azoospermic males				
No female factor	44	48	7 (15.9)	11 (22.9)
Female factor(s)	52	45	3 (5.8)	9 (20.0)
Nonazoospermic males				
No female factor	47	45	5 (10.6)	5 (11.1)
Female factor(s)	21	22	1 (4.8)	3 (13.6)

[a] Values in parentheses are the percent of women becoming pregnant with each treatment cycle.

with cryopreserved sperm as shown in a previous study.[6] There was a statistically significant higher curvilinear velocity and the straight line velocity ($P < 0.001$) in the sperm prepared in Ham's F-10 medium compared with human tubal fluid. This would suggest a better vigor or motility for sperm prepared in Ham's F-10 medium after a short incubation period under proper gas and pH conditions.

Timing of IUI is important. Although we attempted to do two inseminations per LH-timed treatment cycle, it was not always possible. An examination of all single insemination cycles resulting in a pregnancy established that the average time of insemination in these women was 18.0 ± 12.6 hours after the LH peak. A retrospective analysis of all therapeutic donor insemination LH-timed IUI cycles with single inseminations, including other studies in our laboratory, with single inseminations is shown in Figure 1. These numbers suggest that the optimum time for IUI occurs approximately 11 to 20 hours after the peak LH is measured in urine.

DISCUSSION

Previously, women undergoing therapeutic donor insemination have not been considered candidates for IUI.[7] However, recent work has demonstrated a significant increase in PRs when IUI is contrasted with intracervical insemination using frozen donor sperm.[6,14] Cryopreserved sperm have a lowered fecundity when compared with fresh sperm, which is probably because of damage to sperm during processing and freezing.[3–5] To optimize PRs when using frozen donor sperm, we have examined several variables that may influence outcome and have designed prospective studies to improve sperm fertility potential. In this prospective randomized study, we have examined the influence of media used to wash and suspend cryopreserved sperm cells before IUI. The use of human tubal fluid medium significantly improved the PRs over Ham's F-10 medium. By the design of the study, the patient serves as her own control, and there was a good randomization of patients into the two treatments. The PR after insemination with Ham's F-10 prepared sperm (9.8%) observed in this study is similar to the 9.7% PR we reported in a previous study[6] using frozen donor sperm prepared in Ham's F-10 for IUI. Although both of these media perform well in a laboratory controlled trial in human in vitro fertilization,[8,10] there may be some environmental factor that can account for the difference in PRs. The answer may be the result of the exposure of sperm in Ham's F-10 medium to atmospheric air, with a resultant change

Table 4 Analysis of Washed Sperm Used in IUI[a]

Media	Total no. of inseminations	Postwash motility	Total motile sperm/IUI	Computer-assisted semen analysis[b]			
				VC	LIN	VSL	ALH
		%	$\times 10^{-6}$				
Ham's F-10 medium	298	36.2 ± 12.1	17.0 ± 14.5	41.7 ± 17.1[c]	50.4 ± 10.6	20.6 ± 10.0[c]	7.9 ± 6.9
Human tubal fluid	307	36.0 ± 13.5	17.5 ± 12.4	36.8 ± 14.3	48.7 ± 10.8	18.2 ± 8.3	7.6 ± 6.8

[a] Values are means ± SD of all samples used for insemination. These analyses were performed 30 minutes after the final sperm wash and suspension.

[b] VC, curvilinear velocity; LIN, linearity; VSL, straight line velocity; ALH, amplitude of lateral head displacement.

[c] Ham's F-10 medium values are significantly different from human tubal fluid values ($P < 0.001$).

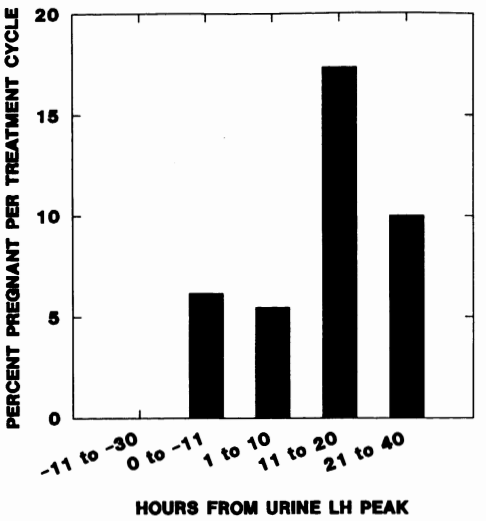

Figure 1 The PR per treatment cycle after LH-timed IUI. Only IUI treatment cycles in which a single insemination was performed are reported here. These data were gathered from several different studies that used LH-timed IUI with cryopreserved donor sperm. Urinary LH values were determined by a quantitative LH assay.

in pH after it leaves the incubator in the clinic and is prepared for IUI. However, we have no data on the viability or fertility of sperm after alkalinization of the media. Furthermore, these media differ significantly in the composition in their constituents (particularly potassium, calcium, phosphate, sodium, and glucose), which could influence sperm physiology and survival.[8] Further work will be required to establish which factor(s) are most important in maintaining sperm fertility under these conditions.

Sperm survival during long-term incubation is extremely dependent on the type of buffered media and the gas composition used for incubation. Ham's F-10 medium is a bicarbonate/carbon dioxide buffer system with 25 mM $NaHCO_3$, and it requires a gas atmosphere containing 5% CO_2 to maintain proper pH. Human tubal fluid buffered with HEPES (20 mM HEPES, 5 mM $NaHCO_3$) does not require a high CO_2 gas atmosphere to maintain pH. Previous work has demonstrated that sperm survival in Ham's F-10 medium with 0.5% human serum albumin is significantly decreased when incubated in atmospheric air over prolonged periods.[15] Decreased survival is probably the result of a rapid pH elevation when bicarbonate medium is exposed to air.[16] Although sperm motility may be unaffected by a brief exposure to an alkaline environment,[15] sperm may not be as fertile after exposure. Studies demonstrating the superiority of one sperm preparation technique over another need to be accompanied by parallel clinical studies to determine if fertility has been compromised by sperm preparation and manipulation.

The "gold standard" for comparing studies using artificial insemination by donor is by examination of results with the group of women with no known infertility problems who have husbands who are azoospermic.[6,17–24] Our previous study demonstrated a 16.4% PR in this group after IUI with cryopreserved donor sperm prepared in Ham's F-10 medium. In this study, the PR in this select group was 15.9% for patients after insemination with sperm prepared in Ham's F-10 medium and 22.2% after IUI with sperm prepared in human tubal fluid. Although all patients were encouraged to continue treatment until pregnancy occurs, some patients discontinued treatment after one or more treatment cycles, whereas others are still undergoing therapy. Using life table analysis, the theoretical probability of pregnancy[13] if each patient underwent six cycles of insemination with human tubal fluid prepared sperm would be 68.5% and 46.1% for sperm prepared with Ham's F-10 medium.

It would seem logical to perform IUI with washed sperm as close to the time of anticipated ovulation as possible for several reasons. It was not known in these women if there was cervical storage of sperm and a slow release of sperm from the cervical mucus, which would increase the chances of fertilization. The uterine or tubal environment might also be hostile to sperm longevity, particularly in patients with endometriosis or unexplained infertility. Previous work using urinary LH timing for both donor and husband IUI[6,7] suggest that an optimal window for insemination exists at or about 15 to 20 hours after the LH peak measured in urine. We found the optimal time of insemination in these studies to be approximately 18.0 ± 12.6 hours in cycles in which a single insemination was performed. This occurs about 30 to 33 hours after the initial surge in urinary LH. It should be understood that these times are based on a peak urine LH value that can be influenced by both the time interval between collections and urine volume.

Although further studies are needed, we conclude that IUI with human tubal fluid medium provides a superior PR with therapeutic donor insemination using frozen donor sperm. The success and outcome of therapeutic donor insemination can be influenced by several variables, and a refinement of techniques in the preparation of sperm for cryopreservation and for IUI should result in even higher PRs.

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