Silane-Coated Silica Particle Colloid Processing of Human Sperm

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Purpose: *The purpose of this study was to determine differences in the quality of human sperm processed through different lots of silane-coated silica particle colloid solutions. The objectives were to compare (a) sperm kinematic parameters, (b) the sperm acrosome status, (c) the membrane integrity of the head and tail regions, (d) the DNA normality, and (e) the heat-inducible hyperactivation motility after processing sperm through either a Silane-coated silica particle colloid solution, a Percoll solution, or a simple centrifuge sperm wash (control).*

Methods: *Sperm cells were derived from pooled cryopreserved–thawed specimens of several donors (n = 10). The pooled sperm were divided and processed through either the centrifuge wash, the 90:47% two-layer Percoll, or one of three lots of silane-coated silica particle colloidal solutions from three vendors. Aliquots of sperm cells were analyzed using the Hamilton–Thorn HTM-C motility analyzer for differences in kinematics and hyperactivation. Sperm were also analyzed for membrane integrity at both head and tail regions, normal morphology, acrosome status, and viability. Sperm undergoing apoptosis were determined using the acridine orange stain. Processed sperm were also incubated at 40°C for 4 hr and the quality of the sperm was assessed using the heat-induced hyperactivation and motility parameter.*

Results: *The data showed that after sperm processing, the number of sperm recovered was higher for the three lots of colloids (silane-coated silica particle colloid solutions) compared with Percoll processing. Total sperm motility was higher in the colloidal washes compared with the control. There were no differences in motility between Percoll- and colloid-processed sperm. In contrast, the percentages of sperm exhibiting progressive motility or hyperactivation varied among the different lots of colloid solutions. The Percoll wash solution yielded the highest percentage of sperm with intact tail membranes, whereas some lots of colloid solutions disrupted sperm head membranes. The percentages of sperm undergoing apoptosis varied for the different lots of colloid solutions. There was a marked increase in hyperactivation associated with one colloid solution after heat induction.*

Conclusions: *The results demonstrated variability in the different lots of silane-coated silica particle colloid solutions for processing sperm. Each lot of colloid solution excelled at improving different sperm parameters. The silane-coated silica particle colloid solutions were shown to be effective in recovering motile sperm compared with Percoll but the types of motility and sperm quality varied for the different lots of colloid solutions. Due to the variability in lots of silane-coated silica colloid solutions, reported studies based on only one lot or one source of colloid solution may be difficult to interpret. Furthermore, it may be advantageous to select the best lot of silane-coated silica particle colloid solution to produce the highest number of sperm exhibiting the ideal parameters for use in assisted reproduction technologies.*

KEY WORDS: spermatozoa; Percoll; isolate; Silane; polyvinylpyrrolidone; sperm wash.

INTRODUCTION

The polyvinylpyrrolidone-coated silica particle colloidal solution commonly known as Percoll is no longer available for the processing of human sperm for assisted reproductive technology. The substitute material is the silane-coated silica particle colloidal solution, touted as being equivalent to or better than Percoll for discontinuous gradient centrifugation to separate motile sperm from nonmotile sperm (1,2). However, other than product literature with a few poorly detailed graphs of this material in sperm, there is no other information available concerning the new material. In the past, Percoll gradient centrifugation had been used to process cryopreserved sperm after thawing to

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remove seminal plasma and cryoprotectant materials. Information on the use of silane colloid processing of cryopreserved–thawed sperm or the existence of lot-to-lot variation in silane material is lacking. The hypothesis was that there were no differences in the quality of cryopreserved–thawed sperm processed through different lots of silane-coated silica particle colloid solutions. The objectives were to compare (a) sperm kinematic parameters, (b) the sperm acrosome status, (c) the membrane integrity of the head and tail regions, (d) the DNA normality, and (e) the heat-inducible hyperactivation motility after processing sperm through either one of three lots of silane-coated silica particle colloid solutions from three vendors, Percoll solution, or simple centrifuge sperm wash (control). The information obtained will assist clinicians in the development of quality control procedures for selecting the appropriate lot of silane-coated silica particle colloid solutions for assisted reproductive technologies.

MATERIALS AND METHODS

Sperm cells were derived from pooled cryopreserved–thawed specimens of several donors (n = 10). Cryopreserved-thawed sperm were used in this study because (a) it would generate new information about the effect of silane colloid processing of cryopreserved–thawed sperm because it is standard practice to wash cryopreserved–thawed sperm frozen directly from raw semen to remove seminal plasma and cryoprotectant material, and (2) the cryopreserved donor sperm had been tested and would not add confounding factors such as sexually transmitted diseases and seasonal effects to the study. Different combinations of pooled sperm specimens were taken randomly from 4 donors of the available 10 donors and combined for each run in the study. The pooled sperm were divided and processed through either the centrifuge wash, 90:47% two-layer Percoll (Perception, Fertility Technologies, Natick, MA), or one of three lots of silane-coated silica particle colloidal solutions (termed colloidal solutions for short) from different commercial vendors (in random order not related to the designated lot number in this study: ISolate, Irvine Scientific, Santa Ana, CA; PureSperm, GenX International, Inc., or NidaCon Laboratories, Madison, CT; Enhance-S plus, Conception Technologies, San Diego, CA). The experiment was repeated 12 times, and the means were obtained for each of the tested parameters.

In this study, the simple spin-down or centrifuge wash was designated the control. The centrifuge wash procedure involved mixing a portion of the pooled thawed semen with an equal volume of HEPES-buffered human tubal fluid (HTF; Irvine Scientific, Irvine, CA), pH 7.2, 282 mosmol/L, culture medium supplemented with 3.5% human serum albumin fraction V (Irvine Scientific, Irvine, CA), and the mixtures were centrifuged for 10 min at 300g at room temperature (23°C). Each resultant sperm pellet was resuspended in 0.4 ml of modified HTF medium. An aliquot (0.1 ml) from each control suspension was placed in an Eppendorf microfuge tube and incubated at 40°C in room air for exactly 4 hr (3) to heat-induce sperm hyperactive motility (4). The remaining portions of sperm suspensions were incubated at 23°C next to the Percoll and silane-coated silica particle colloid processed sperm as described below.

The wash procedures for the Percoll and the three silane-coated silica particle colloid solutions were similar (1). Briefly, centrifuge tubes were prepared by pipetting 1.5 ml of the "lower" solution (usually the 90% solution) to the bottom of the tube, followed by pipetting 1.5 ml of the second "upper" solution (usually a 47% solution) over the lower layer. The pooled semen (1 ml) were then carefully layered as the topmost layer in the tube. The tubes were centrifuged at 300g for 20 min. The supernatants were discarded, and each sperm pellet was mixed with 1.5 ml of modified HTF medium. The tubes were centrifuged at 300g for 10 min. The resultant pellet from each tube was resuspended with 0.4 ml modified HTF medium. An aliquot (0.1 ml) from each sperm suspension was placed in an Eppendorf microfuge tube and incubated at 40°C in room air for exactly 4 hr to heat-induce sperm hyperactive motility (3,4). The remaining portions of sperm suspensions were incubated at 23°C next to the tubes of centrifuge-processed sperm.

Small aliquots (10μl each) were removed from the tubes of incubated sperm (23 and 40°C) for analyses of kinematic motility using the computerized Hamilton Thorn HTM-C computer-aided sperm analyzer (CASA). Analyses were done at hr 0 and at hr 4. The settings for the HTM-C analyzer were as described previously (5). Sperm (10 μl each) were analyzed in 20-μm deep glass slides with coverslips prewarmed at 37°C on a slide warmer. A minimum of 100 sperm cells was analyzed for each tube.

A drop from each sperm suspension was also removed at the start of incubation and an air-dried smear was made at this time for the Spermac acrosome procedure as reported previously (6). Briefly, each

dried smear was fixed (5 min, 23°C) in a formalin solution (Fixative I) provided in the spermac kit (Stain Enterprises, Onderstepoort, South Africa, distributed by Fertility Technologies, Natick, MA). Each slide was processed through stain solutions A, B, and C (1 min, 23°C). The stained slides were air-dried and analyzed under oil immersion (×1000) for the percentage of sperm with intact acrosome. Sperm with intact acrosome have normal oval-shaped heads, with the anterior acrosomal region staining dark green (6) and the postacrosomal region staining red–pink. Sperm lacking this red–pink color were not counted. Sperm lacking acrosomes have white or red anterior acrosomal heads. Sperm heads that showed peeled acrosomal membranes or partial green coloration were considered acrosome defective. The percentage of sperm with intact acrosomes was calculated by dividing the number of sperm with dark green acrosomes over the total number of sperm multiplied by 100.

Supravital staining of sperm to determine viability was performed on a third aliquot of sperm as described previously using 0.5% Eosin Y stain (7). The term viability used in this study was defined as "the ability to exclude dyes which have low permeability in the membrane lipid bilayer."

A fourth aliquot from each sperm suspension was also removed at the start of incubation and tested for membrane integrity at both the head and the tail regions as reportedly (8). Briefly, sperm (0.1 ml) were placed into 1.0 ml of a modified hypoosmotic solution (8,9) and incubated upright at 37°C for 30 min. After the incubation period, an aliquot (5 μl) was pipetted from the bottom of the tube on to a glass slide and a coverslip placed on the top of the droplet. The percentage of sperm with intact head membranes was calculated from the number of sperm with clear, non-red-colored heads (unstained) divided by the total number of sperm (sperm with non-red-colored heads plus stained red-colored heads) and multiplied by 100. A total of 100 sperm cells (irrespective of whether or not the cells were swollen) in several random fields was analyzed for each sample (light microscope at 400× magnification). The percentage of sperm with an intact tail membrane was calculated from the number of sperm displaying types B to G tail coiling over the total number of sperm multiplied by 100. The results were then recorded and analyzed.

Acridine Orange Test Procedure for Sperm Apoptosis

After 4 hr of incubation at 23°C, an aliquot (0.1 ml) of each sperm suspension was added to 6 μg/ml acridine orange (AO) dye dissolved in phosphate-buffered saline (PBS) in a dark-brown Eppendorf tube and the contents were mixed by gently tapping the tube. The entire procedure was done with the room lights off and in diffuse room lighting only. The tubes were centrifuged at 300*g* for 5 min and the supernatant was discarded. The pellets were washed in 1.0 ml PBS by centrifugation at 300*g* for 5 min. The washed pellets were resuspended in 0.1 ml PBS, and a drop was removed from each tube, placed on a glass slide, and covered with a coverslip. The AO-stained sperm were examined in an ultraviolet (UV) fluorescent microscope at 400× magnification. A total of 100 cells was analyzed for each type of processed sperm. Sperm undergoing apoptosis where the DNA became fragmented or denatured to single strands would stain orange–red at the head, while healthy sperm with double-stranded DNA would stain the head green (10). The percentage of sperm with apoptosis was calculated by dividing the number of orange–red sperm by the total sperm and multiplying by 100. To discriminate between nonviable sperm and sperm just starting the process of apoptosis, the percentage of viable sperm was determined during fluorescent microscopy using supravital Eosin-Y stain as described earlier.

Statistical Analyses

The results (Table I) are expressed as mean±standard deviation. The means were analyzed using the Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

The results (Table I) showed that after sperm processing, the numbers of sperm recovered were higher for the three lots of colloids (silane-coated silica particle colloid solutions) compared with Percoll processing. In terms of total motility, up to four times greater sperm motility was obtained after processing through the colloidal washes (Percoll as well as silane-coated silica particle colloid solutions) compared with the centrifuge wash (control). Colloid 2 yielded a higher percentage of sperm motility compared with colloid 1. However, there were no differences in motility between Percoll-processed sperm and sperm processed through the three lots of colloid solutions. In contrast, the percentages of sperm exhibiting progressive motility varied among the different lots of colloid solutions. Sperm processed through the colloid 3 solution had the highest percentage total progression,

Table I. A Comparison of Mean Sperm Kinematic Parameters at hr 0 After Processing Pooled Cryopreserved–Thawed Human Sperm Through Different Lots of Silane-Coated Silica Particle Colloid Solutions (n = Number of Sperm Analyzed)

Parameter	Control (n = 2046)	Percoll (n = 1145)	Colloid 1 (n = 1186)	Colloid 2 (n = 753)	Colloid 3 (n = 359)
% sperm recovery	76.3 ± 12.5	11.5 ± 2.9[a,b,c,d,*]	20.3 ± 3.6[a,b,c]	19.2 ± 5.3[a,c,f]	33.3 ± 4.7[a,d,e,f]
% motility at hr 0	12.0 ± 9.8	± 20.9[a]	41.1 ± 20.1[a,b]	51.8 ± 10.9[a,b]	48.0 ± 7.9[a]
% total progression	3.6 ± 4.6	12.8 ± 8.4[a]	9.1 ± 5.0[a,b]	11.8 ± 6.9[a]	17.3 ± 8.8[a,b]
Average path velocity (μm/sec)	43.3 ± 7.5	36.8 ± 7.1	39.2 ± 5.4	40.3 ± 7.3	48.0 ± 3.6
Cuvilinear velocity (μm/sec)	54.0 ± 11.2	47.8 ± 9.5	48.0 ± 8.1	46.8 ± 9.6	53.0 ± 3.6
Straight line velocity (μm/sec)	22.7 ± 7.4	23.4 ± 7.7	24.3 ± 3.9	25.0 ± 5.5	34.0 ± 9.3[a]
Amplitude of lateral head (μm)	1.2 ± 1.1	2.1 ± 1.0[a]	2.0 ± 1.1	1.3 ± 0.5	0.9 ± 0.3
Beat cross-frequency (Hz)	6.5 ± 3.0	6.8 ± 2.4[b,d]	6.5 ± 2.4[c,e]	4.6 ± 2.4[a,b,c]	4.6 ± 2.7[a,d,e]
Hyperactivation (%)	1.0 ± 1.7	1.3 ± 3.0	1.0 ± 2.0	1.5 ± 2.9[b]	0.7 ± 0.9[b]
Minor axes (μm)	3.1 ± 0.4	3.5 ± 0.2	3.6 ± 0.3	3.3 ± 0.2	3.1 ± 0.2
Major axes (μm)	5.6 ± 0.6	5.4 ± 0.8	5.6 ± 0.8	6.2 ± 1.3	5.2 ± 0
Intact tail membrane (%)	61.6 ± 13.8	83.5 ± 5.4[a,b,c,d]	66.5 ± 16.8[c]	60.2 ± 19.2[b]	66.7 ± 7.4[d]
Intact head membrane(%)	10.4 ± 9.6	7.7 ± 6.2[b,d]	7.2 ± 6.3[c,e]	4.9 ± 3.5[a,b,c]	1.0 ± 1.4[a,d,e]
Acrosome intact (%)	11.9 ± 4.5	14.9 ± 5.3[b,c]	14.5 ± 5.4[d,e]	17.5 ± 4.2[a,b,c]	16.9 ± 5.4[a,d,e]
Normal morphology (%)	13.9 ± 4.3	22.9 ± 8.9[a]	21.6 ± 9.5[a]	25.2 ± 6.7[a]	21.9 ± 5.0[a]
Apoptotic sperm at hr 4 (%)	26.7 ± 21.9	19.0 ± 19.9[a,b]	15.6 ± 14.6[a,c]	21.0 ± 19.0[a]	28.5 ± 18.5[b,c]
Viability at hr 4 (%)	32.2 ± 15.4	30.9 ± 32.7[b]	31.8 ± 23.8[c]	52.5 ± 7.6[a,b,c,d]	40.0 ± 20.0[d]

[a] Different from control mean ($P < 0.05$).
* Mean values with similar superscripts (b–e) in rows are different ($P < 0.05$).

although this was not different from that of Percoll-processed sperm. The beat cross-frequency parameter was lower for colloid lots 2 and 3 compared with the other wash solutions. Hyperactivation was higher in sperm processed through colloid 2 compared with colloid 3. However, this was not significantly different from Percoll-processed sperm. There were no differences in the remaining sperm kinematic parameters.

In terms of membrane integrity, the Percoll wash solution yielded the highest percentage of sperm with intact tail membranes. There were no differences among the three lots of silane-coated silica particle colloid solutions. However, processing through two of the three lots resulted in disrupted membranes at the sperm head in comparison with the other wash solutions. Colloid 3-processed sperm had the lowest, while Percoll-processed sperm had the highest, percentage of undisrupted sperm head membranes among the colloidal solutions.

The percentages of sperm with intact acrosomes varied across the different colloid solutions. The highest percentage of sperm with intact acrosomes was found in colloid 2-processed sperm. All colloid-processed sperm showed improved sperm morphology over the control. However, the percentages of sperm undergoing apoptosis varied for the different lots of colloid solutions. Colloid 1 was best in reducing the percentage of apoptotic sperm but had only a low percentage of viable cells. Colloid from lot 3 produced the highest percentage of sperm with apoptosis.

When the incubated sperm were analyzed after 4 hr, the colloid-processed sperm had significantly higher motility compared with the control (Table II). Colloid from lot 2 had the highest motility, followed by Percoll-processed sperm. In addition, colloid 2- and Percoll-processed sperm exhibited sustained motility, whereas the other colloid solutions resulted in decreased sperm motility. A higher percentage decrease in sperm motility after 4 hr of incubation suggested the presence of sperm cell damage. There was decreased hyperactivation in all the sperm groups, with the largest decrease observed in colloid 3-processed sperm. A decrease in sperm hyperactivation suggested that fewer sperm have the capacity to fertilize oocytes since hyperactivation has been linked to sperm capacitation. In this

Table II. Longevity of Sperm Motility After Processing Cryopreserved–Thawed Human Sperm Through Different Lots of Silane-Coated Silica Particle Colloid Solutions

	% Motility at hr 4	% Change in motility from hr 0	% Decrease in hyperactivation from hr 0
Control	18.8 ± 13.8	+56.0	−60.0
Percoll	48.2 ± 24.8[a,c,*]	+4.6[a]	−69.2[a,c]
Colloid 1	30.5 ± 15.7[a,b,c]	−25.8[a]	−60.0[c]
Colloid 2	50.7 ± 8.7[a,b]	−2.1[a]	−86.7[a,c]
Colloid 3	37.3 ± 18.5[a]	−22.3[a]	−100[a,c]

[a] Different from control mean ($P < 0.05$).
* Mean values with similar superscripts (b and c) in columns are different ($P < 0.05$).

study, colloid 1 fared better than Percoll in terms of causing the smallest loss in percentage hyperactivation.

When the processed sperm were incubated at 40°C for 4 hr, there was a marked increase in hyperactivation in colloid 2-processed sperm, with smaller yet appreciable increases in both colloid 1 and colloid 3 (Table III). In terms of percentage hyperactivation after heat induction, the Percoll and all three lots of colloid solutions were comparable. However, in terms of motility, sperm processed through colloid 1 had the lowest motility among sperm processed through colloidal solutions which included Percoll.

DISCUSSION

The present study demonstrated significant variability in different lots of silane-coated silica particle colloid solutions in affecting sperm recovery, kinematic parameters, membrane integrity, viability, heat-induced hyperactivation, and apoptosis. The results illustrate the advantages of an optimization test of lots of colloidal solutions before use in assisted reproductive technology. The study also showed that the use of silane-coated silica particle colloid solutions resulted in a higher recovery of sperm compared with Percoll. This is especially important when it comes to processing the semen of oligozoospermic patients (2), where the number of sperm cells is the limiting factor. Sperm processed through the colloid solutions were significantly more motile than centrifuge-processed sperm. This difference, however, was not related to sperm velocities, which were similar for all groups tested. Differences in the pH and osmolarity of the solutions were also ruled out, as these were similar.

Table III. Heat-Induced Hyperactivation (HIH) Motility in Human Cryopreserved–Thawed Sperm After Processing Through Different Lots of Silane-Coated Silica Particle Colloid Solutions

	% Motility after heat induction	% Hyperactivation after heat induction	% Increase in hyperactivation after heat induction
Control	25.3 ± 13.7	0.8 ± 1.3	+50.0
Percoll	56.3 ± 22.7[a,b,*]	1.8 ± 2.7[a]	+350.0[a]
Colloid 1	41.3 ± 16.1[a,b,c]	1.3 ± 2.1[a,b]	+225.0[a]
Colloid 2	56.7 ± 25.1[a,c]	2.2 ± 1.7[a]	+1000.0[a]
Colloid 3	52.0 ± 21.2[a]	2.5 ± 2.3[a,b]	+250.0[a]

[a] Different from control mean ($P < 0.05$).
* Mean values with similar superscripts (b and c) in columns are different ($P < 0.05$).

The property of selecting for motile sperm has been shown for Percoll solutions and the results were consistent with previous findings (1,2). A difference noted in using the colloid solutions was the observation that the sperm formed a pellet at the bottom of the tube in the lower (90%) colloid layer, whereas in Percoll the sperm were distributed unevenly in the entire lower layer, making it difficult to observe a distinct sperm pellet.

Some of the lots of silane-coated silica particle colloid solutions yielded more apoptotic sperm or sperm with denatured DNA. Such types of apoptotic or denatured sperm have been programmed for cell death (11). It is not known if the silane-coated silica particles can cause sperm denaturation or if the packaging process or a factor(s) present in certain lots of colloid solutions affected the sperm DNA, and more studies are needed to clarify the effect of silane on sperm DNA. It is interesting that Percoll was effective in reducing the number of apoptotic sperm by almost 30%. Unlike previous studies of acridine orange (10), acid treatment of the sperm was not necessary here because we wanted to determine the actual number of apoptotic sperm. Acid pretreatment would be used only if an artificial stressor effect was required to break the weaker protamine disulfide bridges and DNA linkages, thus revealing immature apoptotic sperm (11). Also, acid pretreatment would result in a higher number of apoptotic sperm, making it difficult to separate effects due to sperm washing from effects due to incomplete sperm maturation. It was noteworthy that the results also showed that an increase in apoptotic sperm was not evident from an assessment of sperm morphology, nor was it evident from the sperm viability data.

The applicability of silane-coated silica particle colloid processing of cryopreserved–thawed sperm was demonstrated in this study. The processed sperm maintained their motility over the course of 4 hr, depending on the lot of colloid solution used. However, hyperactivation decreased during this period. Hyperactive motility of sperm (4) is an important marker of capacitation, an important step in the preparation of sperm for fertilization (12,13). In contrast to the decreased hyperactivation at 23°C, when heat (40°C) was applied to the sperm over the same period, all groups of sperm showed corresponding increases in motility and hyperactivation. Although the percentages of hyperactivation were low, the differences are interesting. Note that the percentages of hyperactivation observed in cryopreserved–thawed sperm are generally lower than the percentages seen for fresh sperm.

A recent report linked the increase in sperm motility after incubation at 40°C to a successful in vitro fertilization outcome (3). The results of the present study showed that the percentage increase in heat-induced motility was dependent on the lot of colloid solution. Hence, the selection of the colloid solution that produced the greatest increases in heat-induced motility would be an advantage for assisted reproduction technologies such as artificial insemination. More studies are needed to improve the quality of the colloids for sperm separation (14).

In summary, the data demonstrated variability in the different lots of silane-coated silica particle colloid solutions in processing sperm. Each lot of colloid solution excelled at improving different sperm parameters. The silane-coated silica particle colloid solutions were shown to be effective in recovering motile sperm compared with Percoll. Due to the variability in lots of silane-coated silica colloid solutions, reported studies based on only one lot or one source of colloid solution may be difficult to interpret Furthermore, it may be advantageous to analyze different lots of colloid solutions to determine the best lot that will yield the highest number of sperm exhibiting the ideal parameters for use in assisted reproduction technologies.

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