

Effect of treatment of seminal viscosity difficulties with α -chymotrypsin on the recovery of spermatozoa for assisted reproductive technologies: comparison between the SpermPrep™ filtration and Percoll gradient centrifugation methods

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ABSTRACT

Objectives: The study was designed to assess the impact of limited proteolysis, using α -chymotrypsin in high viscosity semen specimens, on the qualitative and quantitative characteristics of spermatozoa recovered via the SpermPrep filtration and Percoll gradient centrifugation methods for possible use in assisted reproductive technologies (ART).

Design: Controlled clinical study.

Setting: Andrology Institute of Lexington.

Patients: Thirty patients producing high viscosity semen specimens, which otherwise were within normospermic limits, participated in this study.

Main Outcome Measures: Treatment of high viscosity semen specimens via limited proteolysis using α -chymotrypsin and preparation via the SpermPrep™ filtration or Percoll gradient centrifugation methods.

Results: The method of semen liquefaction as applied in this study aided in the recovery of greater numbers and higher quality spermatozoa ($P < 0.05$) as compared to the conventional method. Improvements in all qualitative measurements assessed were noted between the conventional and the α -chymotrypsin liquefied specimens regardless of the sperm selection method employed.

Conclusions: The limited proteolysis of high viscosity semen specimens with α -chymotrypsin was shown to be a treatment which can assist significantly in the handling and preparation of human seminal specimens with viscosity difficulties. Furthermore, limited proteolysis of high viscosity semen specimens with α -chymotrypsin assisted in the recovery of greater numbers and higher quality spermatozoa which can be used further in the various assisted reproductive techniques.

Key words: Semen viscosity, proteolysis, coagulation, liquefaction, α -chymotrypsin

INTRODUCTION

Fresh ejaculated human semen is normally ejaculated in the liquid state followed by immediate coagulation and the coagulum does not liquefy for at least 20 to 30 min (1-6).

Commonly, semen samples are rather viscous even after 30 to 45 min (3,4). However, if liquefaction is not complete and the semen remains viscous, the indications are that some abnormality of the normal semen producing mechanisms exists and more specifically lack of an enzymatic process catalyzed by a proteolytic enzyme present in the prostatic secretions (3,4). The coagulation and subsequent liquefaction of semen and its physiological significance is not clear. Delayed or failure of liquefaction, and high viscosity (hyperviscosity) are conditions equated with

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infertility, since it is claimed that the spermatozoa are tangled in the fibrous or mucoid mass in the semen and are prevented from migrating properly from the seminal plasma into the cervical track fluids and ascending to the site of fertilization (2, 7-9). With the development of the various forms of assisted reproductive technologies (ART) and the need to obtain the maximum numbers and quality of spermatozoa from the ejaculated semen has brought the semen viscosity into focus again. Viscous specimens are extremely difficult to manipulate in vitro and may not allow the proper separation and isolation of spermatozoa for assessment, sperm preparation or performance of ART procedures such as intrauterine insemination (IUI) and IVF (6, 10,11). Semen liquefaction can be induced in vitro via mucolytic agents, dilution pretreatment or mechanical disruption of the mucous material (1, 2, 6, 7, 10, 12-17). Mucolytic agents such as α -chymotrypsin, α -amylase and dithiothreitol have been employed to improve semen liquefaction and viscosity difficulties (6, 10, 16). In general, it has been shown that treatment of semen with α -chymotrypsin improves the in vitro fertilizing ability of high viscosity semen specimens (10, 18).

The objective of this study was to assess the impact of limited proteolysis, using α -chymotrypsin in high viscosity semen specimens, on the qualitative and quantitative characteristics of spermatozoa recovered via the SpermPrep™ filtration and Percoll density gradient centrifugation methods for possible use in ART.

MATERIALS AND METHODS

Semen Collection and Assessment

Thirty ejaculates of high viscosity which otherwise were within normospermic limits were used in this study. All patients that participated in this study were instructed to collect a semen specimen within 4 days of abstinence at intercourse via the use of a non-spermicidal condom-shaped semen collection device (Male Factor Pak ; ZDL, Inc., Lexington, KY, USA) as described by Zavos et al. (19). Patients delivered the specimens to our andrological facilities within

30 min following collection and the semen specimens were assessed for concentration ($\times 10^6$ sperm/ml), motility (%), grade of motility (0 to 4), morphology (% normal) and for the sperm membrane functional integrity as measured by the hypoosmotic swelling (HOS) test (20). The sperm motility index (SMI) was also assessed via the sperm quality analyzer (SQA; IntroTech, San Diego, CA) as previously described (3, 4, 21-23). The SQA is a device that combines optical and computing technologies to measure the relative quantity of motile spermatozoa (3, 4, 21-23). The SQA uses light passed through a small sample of the semen to detect variations in optical density that result from the movement of spermatozoa. The SMI measurement was performed over a 40 sec period, which was comprised of 4 (10 sec) measurement intervals, and the mean SMI value was then displayed and recorded (21-23). The viscosity of seminal specimens was determined by measuring the length of the thread that is produced when aspirating the semen via a Pasteur pipette and pulling when in contact with the semen specimen. The length of the thread was assessed to the nearest approximate cm, in a similar fashion as when measuring the 'Spinbarkeit' of cervical mucus (24, 25). Semen specimens were considered hyperviscous when the length of the thread was equal or greater than 1 cm, and were further used in this study.

Semen Preparation and Hyperviscosity Treatment

Following seminal evaluation, the specimens were divided into four aliquots (Aliquot 1 to 4). Aliquot 1 and 2 were liquefied by diluting 1:1 (v/v) with modified Ham's F-10 medium containing 3% (w/v) bovine serum albumin (SpermPrep™ medium; ZDL, Inc.), mixed with a Pasteur pipette 10 to 20 times and used for SpermPrep™ or Percoll selection. Aliquot 3 and 4 were liquefied via the Viscolytic System (VLS) following the manufacturer's specifications (ZDL, Inc.). The VLS is a α -chymotrypsin-based (40 to 60 units per mg protein from bovine pancreas) method that allows limited proteolysis of the seminal plasma. The α -chymotrypsin was added to the Ham's F-10 (5.0 mg/ml), and the solution was mixed 1:1 (v/v) with the semen. The semen

specimens were incubated for 20 to 30 min (35 C) and evaluated periodically (5 min intervals) for liquefaction. The specimens were further diluted with 4.0 ml of Ham's F-10 without α -chymotrypsin immediately after liquefaction (14.5 \pm 2.3 min), centrifuged at 400 x g for 7 min (sperm wash) and reconstituted in 0.5 ml of Ham's F-10. The sperm wash procedure was performed twice and the washed spermatozoa were further processed via the SpermPrepTM filtration and the Percoll density gradient centrifugation methods.

SpermPrepTM Filtration Method

The SpermPrepTM I filtration method (ZDL, Inc.) was used as previously described (26-30). The Sephadex beads used in the SpermPrepTM filters are made out of a physiologically inert polysaccharide derivative which at hydration and subsequent swelling and enlargement (3 times the original size) develop rough ridges on their surface which further aid in the entrapment and removal of dead or immotile spermatozoa. Before use, the SpermPrepTM I was hydrated by placing 4.0 ml of the corresponding media in the barrel of the column. The filter matrix was gently mixed with the media to form a suspension ensuring that air bubbles were removed from the bottom of the filter. The beads were allowed to settle for 10 min to the bottom (sedimentation) and to undergo

complete hydration. When the sedimentation was completed, the bottom closure was removed and 2.0 to 3.0 ml of the medium was allowed to run through. This step normally enables the removal of any small bubbles and debris from the filter line. The filter was capped again with the bottom closure and the filtrate was discarded. The capped filter was placed into a 15.0 ml conical-centrifuge tube and held (37°C) until the sperm specimen was ready for filtration. The sperm specimen was placed in the filter and was gently mixed with the Ham's F-10 medium to form a uniform suspension and to prevent all the sperm from setting directly onto the filter and possibly clotting the filter. As the sperm specimen was added and mixed, the bottom closure was removed and filtration began. Filtration was continued for 10 min, and more medium was periodically added to the filter to maintain the level of medium at its original level. This step maintained a uniform hydrostatic pressure on the filter during the total filtration time. At the end of filtration, the filter was closed with the bottom closure and removed from the centrifuge tube. The filtrate was collected in the centrifuge tube, centrifuged, resuspended in 0.5 ml of Ham's F-10 medium and processed further for final evaluation as previously described.

Table 1 Sperm characteristics of high viscosity semen specimens liquefied with or without α -chymotrypsin (means \pm SD).

Liquefaction method	Semen characteristics assessed					
	Count ($\times 10^6$)	Motility (%)	Grade (0 to 4)	HOS* (%)	Morphology (% normal)	TFSF ($\times 10^6$)
Fresh	86.7 \pm 14.3	44.6 \pm 9.1	2.4 \pm 0.4	82.6 \pm 7.0	57.1 \pm 3.8	18.2 \pm 4.2
Conventional †	84.6 \pm 10.8	53.1 \pm 7.2	2.9 \pm 0.3	83.2 \pm 6.8	56.7 \pm 4.1	21.2 \pm 5.0
α -chymotrypsin‡	85.0 \pm 7.1	57.2 \pm 5.0	3.3 \pm 0.2	82.9 \pm 6.7	58.3 \pm 4.0	23.5 \pm 4.5

*Hypoosmotic swelling (HOS) test (% swollen spermatozoa).

† Total functional sperm fraction (TFSF; $\times 10^6$).

‡ Semen specimens were liquefied via dilution with Ham's F-10 followed by pipetting 10 to 20 times.

§ Semen specimens were liquefied via dilution with Ham's F-10 medium containing α -chymotrypsin (5.0 mg/ml).

Table 2 Recovery of spermatozoa via the SpermPrep™ and Percoll gradient centrifugation methods following liquefaction with or without α -chymotrypsin (means \pm SD).

Treatments	Sperm characteristics assessed					
	Count ($\times 10^6$)	Motility (%)	Grade (0 to 4)	HOS* (%)	Morphology (% normal)	TFSF† ($\times 10^6$)
Percoll‡	23.4 \pm 5.1	68.0 \pm 8.6	3.3 \pm 0.3	80.1 \pm 9.1	80.6 \pm 9.1	10.3 \pm 3.2
Percoll-VLS§	35.3 \pm 4.7 [¶]	88.4 \pm 6.1 [¶]	3.6 \pm 0.3 [¶]	87.3 \pm 8.7 [¶]	83.1 \pm 8.7 [¶]	15.1 \pm 3.0
SpermPrep™‡	28.6 \pm 4.3 [#]	75.6 \pm 6.3 [#]	3.5 \pm 0.2 [#]	83.2 \pm 7.3 [#]	84.3 \pm 7.3 [#]	22.6 \pm 3.6
SpermPrep™-VLS§	41.7 \pm 4.1 ^{¶¶}	89.7 \pm 5.7 ^{¶¶}	3.7 \pm 0.2 ^{¶¶}	91.4 \pm 6.7 ^{¶¶}	88.9 \pm 6.7 ^{¶¶}	30.3 \pm 3.0

*Hypoosmotic swelling (HOS) test (% swollen spermatozoa).

† Total functional sperm fraction (TFSF; $\times 10^6$).

‡ High viscosity semen specimens were prepared by diluting 1:1 (v/v) with modified Ham's F-10 medium, mixed with a Pasteur pipette 10 to 20 times and used for SpermPrep™ or Percoll selection.

§ High viscosity semen specimens were prepared by diluting 1:1 (v/v) with Ham's F-10 medium containing α -chymotrypsin (VLS; 40 to 60 units per mg protein) and used for SpermPrep™ filtration or Percoll gradient centrifugation selection.

¶ Significant differences noticed in specimens treated with or without α -chymotrypsin and prepared via the same selection method ($P < 0.05$).

¶¶ Significant differences noticed between specimens treated with α -chymotrypsin and prepared via the SpermPrep™ filtration or Percoll gradient centrifugation methods ($P < 0.05$).

Significant differences noticed between specimens treated without α -chymotrypsin and prepared via the SpermPrep™ filtration or Percoll gradient centrifugation methods ($P < 0.05$).

Percoll Density Gradient Centrifugation

A discontinuous two layer technique was used for the Percoll separation method (30). An isotonic 90% (v/v) Percoll stock solution was prepared by adding the corresponding Ham's F-10 media (10X BSA-free media) to 100% Percoll (9 Percoll:1 Ham's F-10). The Ham's F-10 media had 10 times (10X) the solute concentration, and therefore the osmolarity, of the original Ham's F-10 (280 mOsm/l) in order to obtain an isotonic medium upon dilution with 100% Percoll. The Percoll stock solution was diluted further to obtain a 45% (v/v) Percoll solution. Percoll solutions (1.0 ml aliquots) were layered starting with the 90% solution at the bottom. The sperm specimen (0.5 ml) was layered onto the top of the upper Percoll layer (45%), and was centrifuged for 20 min at 300 x g. The 90% Percoll fraction containing the sperm pellet was collected (after removing the upper layer) with a disposable Pasteur pipette and washed once more. The recovered spermatozoa were resuspended in 0.5 ml of Ham's F-10

medium and processed for final evaluation as previously described.

Statistical Analysis

The results obtained were reported as means \pm SD. The variables studied were analyzed via analysis of variance. The Least Significance Difference method was employed to detect statistical significance among the variables studied (31).

RESULTS

The results obtained in this study are summarized in Tables 1 and 2. The average time interval for semen liquefaction in hyperviscous specimens processed with α -chymotrypsin was 14.5 \pm 2.3 min. The treatment of semen specimens with high viscosity difficulties via the VLS method (α -chymotrypsin), followed by sperm preparation and selection using the SpermPrep™ or Percoll gradient centrifugation methods,

improved the recovery of greater numbers and higher quality spermatozoa than treatment without α -chymotrypsin ($P < 0.05$). Improvements in all qualitative measurements assessed were noted between semen specimens treated with or without α -chymotrypsin regardless of the method employed for sperm preparation and selection. The most efficient method of preparing spermatozoa was via α -chymotrypsin treatment in conjunction with SpermPrep™ filtration ($P < 0.05$). The total functional sperm fraction (TFSF; $\times 10^6$ spermatozoa), an inclusive term that incorporates the quantitative and qualitative sperm characteristics was calculated for all specimens recovered. The TFSF was calculated as the product of sperm count by % motility by % normal morphology by % swollen spermatozoa (HOS test). The TFSF for specimens treated with or without α -chymotrypsin and prepared via Percoll centrifugation was 12.8 and 18.2, respectively. The TFSF for specimens treated with or without α -chymotrypsin and prepared via SpermPrep™ filtration was 25.9 and 33.4, respectively. The TFSF was improved by a factor of approximately 1.4 in specimens treated with α -chymotrypsin, regardless of preparation method. The SMI values were 47.6 ± 6.3 and 53.5 ± 6.3 ($P > 0.05$) in specimens prepared without α -chymotrypsin and selected via Percoll centrifugation and SpermPrep™ filtration methods, respectively. The SMI values were 68.7 ± 6.1 and 81.1 ± 6.3 ($P < 0.05$) in specimens prepared with α -chymotrypsin and selected via Percoll centrifugation and SpermPrep™ filtration methods, respectively. The SMI values for specimens prepared with α -chymotrypsin, and selected via Percoll centrifugation and SpermPrep™ filtration methods, were significantly different than those prepared without α -chymotrypsin, regardless of the selection method applied ($P < 0.05$).

DISCUSSION

Delayed or failure of liquefaction, and high viscosity (hyperviscosity) are conditions equated with infertility, since it has been suggested that the spermatozoa are tangled in the fibrous or

mucoid mass in the semen and are prevented from migrating properly from the seminal plasma into the cervical track fluids and ascending to the site of fertilization (2, 7-9). Viscous specimens are extremely difficult to manipulate in vitro and may not allow the proper separation and isolation of spermatozoa for assessment, sperm preparation or performance of ART procedures such as IUI and IVF (6,10,11). Treatment of high viscosity specimens with α -chymotrypsin does not appear to decrease pregnancy rates when employed for IUI and various ART procedures (10, 16). Honea et al. (10) reported that limited proteolysis of high viscosity semen specimens with α -chymotrypsin improved the sperm capacitation and penetration index as measured by the zona-free hamster oocyte test. The authors indicated that approximately 63% of the patients, which had a previous history of SPA abnormal results, were able to improve their semen parameters via limited proteolysis treatment using α -chymotrypsin. When the same patients underwent IVF therapy, the fertilization rates improved from 12% to 88% after preparing the semen via α -chymotrypsin treatment. It has also been suggested that preparation of semen specimens via various selection techniques could improve the fertilization ability of the spermatozoa by isolating the highly motile and morphologically normal sperm population (26-30, 32, 33). Treatment of high viscosity semen specimens via limited proteolysis with α -chymotrypsin in conjunction with the performance of sperm selection for ART could improve those pregnancy rates even further.

The results obtained in this study showed that the treatment of semen specimens with high viscosity difficulties via limited proteolysis with α -chymotrypsin, followed by sperm preparation and selection using the SpermPrep™ or Percoll gradient centrifugation methods, improved the recovery of greater numbers of high quality spermatozoa as compared to specimens processed without α -chymotrypsin. The limited proteolysis of high viscosity semen specimens with α -chymotrypsin was shown to be a treatment which can assist significantly in the handling and preparation of human seminal specimens with viscosity difficulties. Furthermore, limited

proteolysis of high viscosity semen specimens with α -chymotrypsin assisted in the recovery of greater numbers and higher quality spermatozoa which can be used further in the various assisted reproductive techniques including IUI. Further studies are currently being carried out at our facilities to evaluate the effects of limited proteolysis via α -chymotrypsin on hyperviscous seminal specimens with various spermatogenic deficiencies.

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