Treatment of human sperm with serine protease during density gradient centrifugation

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Abstract

Purpose Seminal pathogens can bind specifically or non-specifically to spermatozoa, rendering semen decontamination procedures ineffective, whereby vertical or horizontal transmission of the infection could occur. Serine proteases have been demonstrated to effectively inactivate viruses and to break pathogen-sperm bonds. However, the addition of a protease to density gradient layers during semen processing could negatively impact on sperm parameters. This study investigated the effect of the addition of a recombinant, human-sequence protease (rhProtease) on sperm parameters during density gradient centrifugation.

Methods (i) Pooled semen samples (n=9) were split and processed by density gradient centrifugation, with the top density layers supplemented, or non-supplemented with rhProtease at three different concentrations (diluted 2, 10 and 20 times). Sperm parameters were then analysed by flow cytometry and computer-assisted semen analyses. (ii) Semen samples (n=5) were split and similarly processed using PureSperm® Pro, with rhProtease in the 40% density gradient layer, or standard PureSperm® not supplemented with rhProtease (Nidacon, International) respectively. The Hemizona assay was then utilized to compare sperm-zona binding post processing.

Results Evaluation of sperm parameters indicated that rhProtease did not, at any of the tested concentrations, have an impact on (i) mitochondrial membrane potential, vitality, motility, or (ii) zona binding potential.

Conclusion We report that the addition of rhProtease to density gradients is a non-detrimental approach that could improve the effectiveness of semen processing for the elimination of seminal pathogens, and benefit assisted reproduction outcome.

Key words

Density gradient centrifugation · Semen processing · Serine protease · Sperm parameters

Introduction

In an attempt to purify sperm and to eradicate pathogens, semen processing is performed by various assisted reproductive laboratories [1-3]. However, the binding of viruses such as human immunodeficiency virus-1 (HIV-1) [4,5] and other pathogenic micro-organisms, including: *Escherichia coli* [6,7], *Ureaplasma urealyticum* [8], *Mycoplasma hominis* [9], *Neiseria gonorrhoeae* [10] and *Chlamydia trachomatis* [11], to the spermatozoa, may result in the transport of these pathogens through the gradient layers during density gradient centrifugation (DGC). Processed sperm pellets, therefore, may remain positive for pathogens. The adherence of viruses to sperm membranes may contribute to the reported failure rate of 10% during semen processing for the elimination of HIV and hepatitis [12]. Consequently, techniques to inactivate pathogens and to break the virus-sperm bonds are, therefore, investigated.

Serine proteases such as chymotrypsin and trypsin are natural constituents of semen, that are secreted by the auxiliary seminal glands and play a role in semen liquefaction [13]. Tang *et al*. (1991) reported that HIV is susceptible to inactivation by trypsin at low concentrations [4]. Therefore, the effectiveness of DGC for the removal of viruses from semen could be improved by the addition of trypsin to density gradient layers [14]. In addition, serine protease has also been reported to remove bound proteins that may be responsible for sperm-antibody formation, thereby increasing fertilizing potential [15]. However, the effect of trypsin on human sperm parameters must first be considered. Binding of capacitated, acrosome-intact spermatozoa to the ZP3 and ZP4 glycoproteins of the zona-pellucida is a prerequisite for fertilization [16]. Trypsin could have a negative impact on sperm receptors, whereby sperm binding to the zona pellucida glycoproteins could potentially be compromised. Silva, Solana & Castro (1999), reported a decrease in the total and progressive motility, as well as vitality of bovine sperm treated for a period of 5 minutes with trypsin at a concentration 0.3% [17].

Any potential negative effects of reduced motility or vitality on sperm fertilizability [18] will cause any semen processing method to be undesirable. The current study, therefore, investigated the effect of semen processing by DGC, supplemented with a recombinant, human-sequence serine protease (rhProtease), on human sperm parameters.

Materials and methods

Institutional approval for the study was received from Steve Biko Academic Hospital and the Medical Research Council's Ethics Committee, University of Pretoria (protocol number 37/08). Informed written consent was received from all participants.

Processing of semen using density gradients supplemented with rhProtease.

Prior to experimentation, the proteolytic activity of rhProtease (Nidacon International, Mölndal, Sweden) was tested and confirmed by the effective trypsinization of HeLa cells [19] Density gradients (40 & 80%) were prepared by diluting PureSperm®-100% with PureSperm® Buffer (Nidacon, International). rhProtease was added to the 40% gradient at dilutions of 0, 2, 10 and 20 times. Semen samples with parameters in the normal ranges, as

described by the World Health Organization (WHO) [20], were pooled (n=2 samples/pool) and divided into test (n=3 samples) and control (n=1) samples. The samples were processed by DGC according to the product manufacturer's guidelines using the supplemented, or non-supplemented, density layers, respectively. Sperm parameters were evaluated 16 to 18 hours after processing and the experiment was repeated nine times.

Flow cytometry controls

Sperm vitality (apoptosis & necrosis) and mitochondrial membrane potential were determined by means of flow cytometry (FC500, Beckman Coulter, Brea, CA, USA). Protocols were set up and validated by the inclusion of the following controls: 1) sperm apoptosis was induced by incubation with 1μM staurosporine (Sigma-Aldrich, St Louis, MO, USA) for 18 hours at 37°C [21]; 2) Annexin V-FITC binding to sperm was blocked by incubating sperm, according to the manufacturer's guidelines, with purified recombinant Annexin V (BD Biosciences, San Diego, CA, USA); 3) necrosis was induced by incubating 1 x 10^6 sperm (1ml) with 2μl of Triton X (Sigma-Aldrich); and 4) mitochondrial membrane potential ($\Delta\psi_m^{high}$) was abolished by incubating sperm with 50μmol/l carbamoylcyanide m-chlorophenylhydrazone (mCLCCP, Sigma-Aldrich) for 15 minutes at 37°C [22].

Vitality

Sperm vitality post-processing was determined using a BD Pharmingen™ FITC Annexin V apoptosis detection kit (BD Biosciences). Aliquots (5 x 10^6 sperm) from the processed samples were washed twice; first in 2ml of cold phosphate-buffered saline (Sigma-Aldrich) and, secondly, in 2ml of cold Annexin V binding buffer. Sperm pellets (100μl) were obtained and 5μl of Annexin V-FITC and propidium iodide (PI) were added. The samples were vortexed and incubated (24°C) in the dark for 15 minutes, binding buffer (400μl) was then added and flow cytometry evaluations were performed within 20 minutes after staining. Annexin V-FITC and PI stained sperm were detected in the FL1 and FL3 channels, respectively.

Mitochondrial membrane potential

Mitotracker Red CMX Ros (Molecular Probes, Eugene, USA) (50µg) was diluted in 100µl DMSO (Sigma-Aldrich) to prepare a stock solution that was stored at -20°C. Processed sperm samples (5 x 10^6 cells) were re-suspended in 1ml PureSperm® Wash (Nidacon, International). Mitotracker (2.6µl from the stock solution) was added to the sperm, and the suspensions were incubated at 37°C for 15 minutes. The cells were washed, re-suspended in 1ml PureSperm® Wash, and flow cytometry was performed to determine the percentages of cells with high mitochondrial membrane potential ($\Delta\psi_m^{high}$) using the FL3 channel.

Computer assisted semen analyses (CASA)

Two-chamber, 20 µm deep, Leja® counting chambers (Leja Products, The Netherlands) were pre-warmed to 37°C. Duplicate sperm aliquots (5µl) were loaded into both chambers, then at least 200 sperm and 10 microscope fields were evaluated per chamber [20] by means of CASA (MediaLAB, version 5.4 Altdorf, Germany) at 200 times magnification (Axioskop 40; Zeiss, Göttingen, Germany).

Sperm-zona interaction

The hemizona assay was utilized to evaluate sperm-zona binding potential [23]. Semen samples (n=5) with parameters in the normal ranges, as described by the WHO [20], were received from donors (n=5). Samples were split and processed by means of DGC, using PureSperm® Pro (Nidacon, International), with rhProtease in the 40% density gradient layer, or standard PureSperm® not supplemented with rhProtease (Nidacon, International). Prepared sperm samples were kept at room temperature (37°C) until insemination of the hemizonae.

Non-viable, unfertilized oocytes remaining from the Unit's *in vitro* fertilization (IVF) program were bisected by means of micromanipulation (Transferman; Eppendorf, Hamburg Germany) at 200 times magnification (Axiovert 200; Zeiss) using a 3.5 mm, 30 degree blade (BD Micro-Sharp™, Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). Droplets of 50µl PureSperm® Wash (Nidacon, International) were prepared in culture dishes (Falcon

353004; Becton Dickenson Co.) and covered using FertiCult mineral oil (FertiPro, Beernem, Belgium). Hemizonae were placed in the micro-droplets and any attached sperm remaining from IVF were stripped by vigorous pipetting using 80µm pipette (Flexipet®; Cook, Limerick, Ireland). Procedures were performed at room temperature (24°C) and oocytes were bisected on the day of use.

Hemizonae (n=7 pairs per semen donor) were individually inseminated with 1 x 10^4 progressively motile test or control sperm. After an incubation period of 16 hours at 37°C, the hemizonae were transferred to freshly prepared micro droplets. Loosely bound sperm were removed by pipetting 4 times using a 130µm pipette tip (Flexipet®; Cook). The numbers of tightly bound sperm were double-blind counted by two evaluators at 400 times magnification (Axiovert 200; Zeiss).

Statistical analysis

Sperm parameters were examined after semen processing, using top density gradient layers containing rhProtease diluted 20, 10, and 2 times, and were compared to similar processing without rhProtease. Stata Statistical Software: Release 10 [24] was used to perform a mixed model analysis, and to preserve the 0.05 level of significance, each test was done at the Bonferroni adjusted level of significance of 0.017, i.e. 0.05/3 [25].

Stata Statistical Software was also used to compare the number of sperm bound to hemizonae post processing using PureSperm® and PureSperm® Pro. This comparison was performed using a mixed-model approach under the maximum likelihood option. This method controls for the dependence of data associated with specific semen donors.

Results

Activity of rhProtease

The rhProtease used to supplement the PureSperm® density gradient layers, was confirmed to be active by trypsinization. Incubation of cultured HeLa cells with the rhProtease resulted in the cells becoming round and dislodged from the bottom of the cell culture dish after 2 minute incubation at 37°C.

Flow cytometric controls

The incubation of sperm with staurosporine and recombinant Annexin was effective for the induction of apoptosis and blocking of Annexin V-FITC binding, respectively. Fluorescence intensities of Annexin V-FITC stained sperm, after treatment with staurosporine and recombinant Annexin, are illustrated in Figure 1A. Figure 1B illustrates the necrotic status of sperm after treatment with Triton X, and Figure 1C illustrates the abolishment of $\Delta\psi_m^{high}$ by treatment of sperm with mCLCCP.

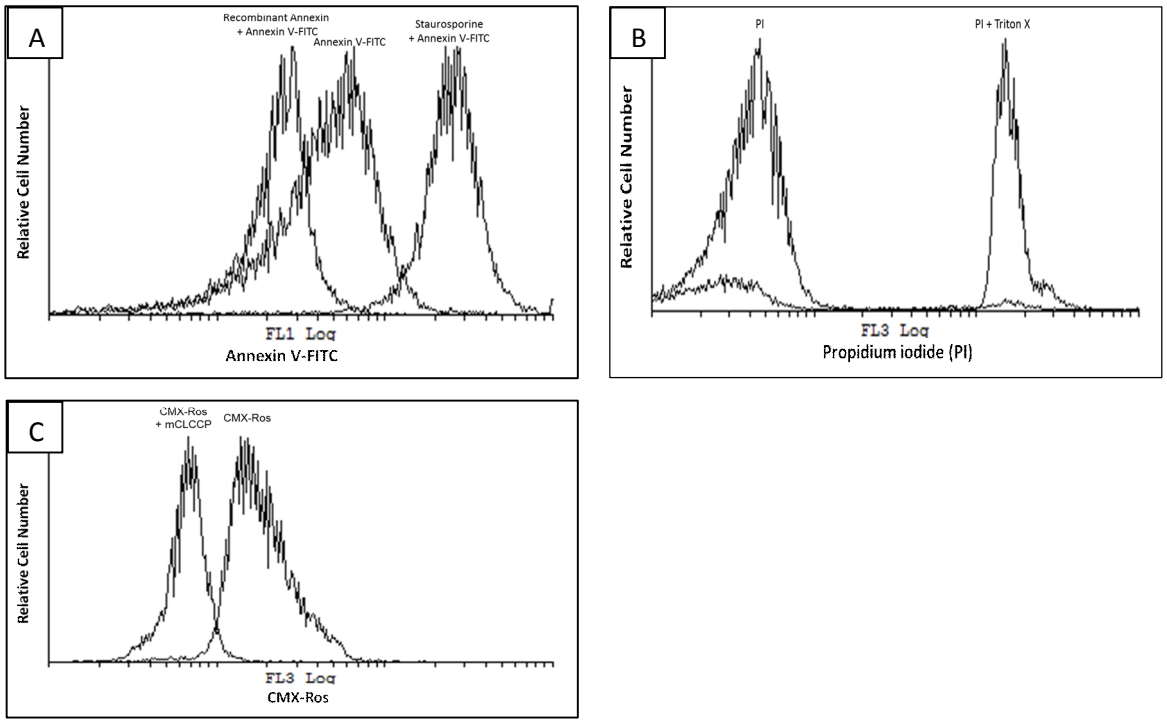

Fig 1: Fluorescence intensity of treated sperm, included as controls, during set up of flow cytometry protocols; (A) Staurosporine to induce apoptosis, and purified recombinant annexin to block sperm annexin V-FITC binding; (B) Triton X induced necrosis; (C) mitochondrial membrane potential was abolished by treatment with mCLCCP.

Vitality, mitochondrial membrane potential and motility parameters

Processing of semen samples by means of DGC with the top density layer containing rhProtease, diluted 2, 10 and 20 times, did not have a negative impact on apoptotic and necrotic status, mitochondrial membrane potential (Table 1), or any of the motility parameters of sperm including: progressive motility, average path velocity, curvilinear velocity and straight line velocity (Table 2).

Table 1: Apoptotic/necrotic status (AV-PI-) of sperm treated with rhProtease compared to untreated sperm

rhProtease concentration	(AV-PI-)			MMP		
	Mean	SD	P-Value	Mean	SD	P-Value
0 x Trypsin	91.772	8.782		90.469	3.943	
20 x Diluted	93.641	5.231	0.079	87.549	6.391	0.090
10 x Diluted	93.748	4.936	0.064	86.517	6.076	0.022
2 x Diluted	93.369	3.801	0.134	88.647	5.073	0.290

Table 2: Progressive motility of sperm treated with rhProtease compared to untreated sperm

rhProtease concentration	Progressive motility			Average path velocity			Straight line velocity			Curvilinear velocity		
	Mean	SD	P-Value	Mean	SD	P-Value	Mean	SD	P-Value	Mean	SD	P-Value
0 x Trypsin	83.889	5.476		42.202	11.520		38.474	11.261		59.731	13.882	
20 x Diluted	82.222	7.567	0.281	41.657	12.598	0.703	37.793	12.224	0.666	59.225	13.982	0.713
10 x Diluted	84.000	5.220	0.943	42.938	12.465	0.607	39.252	12.438	0.621	61.212	13.748	0.281
2 x Diluted	84.222	6.305	0.829	43.408	10.419	0.399	39.459	10.367	0.532	60.904	12.010	0.281

Sperm-oocyte interaction in the hemizona assay

Processing of semen using PureSperm® Pro with rhProtease did not cause a reduction in the number of sperm bound to the hemizonae when compared to processing with standard PureSperm® (P=0.732), not supplemented with rhProtease. The results are summarized in Table 3.

Table 3: Numbers of sperm bound to the hemizonae post-processing with standard Puresperm® compared to PureSperm® Pro

Treatment	Mean number bound	SE	95% CI
PureSperm (-rhProtease)	26.26	6.60	13.33; 39.19
PureSperm Pro (+rhProtease)	28.51	6.60	15.58; 41.44

Standard error (SE) & 95% confidence interval (CI)

Discussion

Treatments of sperm for 5 minutes at concentrations of 25-100µg trypsin/ml has been found previously to effectively inactivate HIV-1, depending on the number of viral copies present [4]. Loskutoff *et al*. (2005) reported a significant reduction in the infectivity of HIV-1 RNA after a brief (1 minute) exposure to 0.25% trypsin and the effective removal of HIV-1 and HCV RNA from spiked human semen by processing semen with density gradients supplemented with trypsin at the same concentration [14]. Furthermore, trypsin treatment at a concentration of 0.3% and 0.25% has successfully been utilized to inactivate bovine herpesvirus-1 during the washing of bovine semen [26].

Another application may be in cases of semen hyper-viscosity, where the progressive motility of sperm is decreased [27], potentially resulting in decreased sperm yield during semen processing. Hyper-viscous semen samples could therefore be treated to reduce viscosity [28]. This is of importance especially in patient populations with high incidences of sexually transmitted infections such as human immunodeficiency virus [29] and *Ureaplasma urealyticum* [30,31], with increased prevalence of seminal hyper-viscosity. The addition of trypsin to semen has been proven to effectively reduce seminal viscosity [32,33], whereby sperm yield during processing could also be improved.

The ability of serine proteases to inactivate and remove viruses attached to sperm [14] and the potential to prevent sperm-antibody formation [15], together with improved sperm yield during processing, demonstrates the advantages for use as a supplement during semen processing. The addition of rhProtease to density layers in combination with the usage of a ProInsert™ (Nidacon, International), could therefore be beneficial, specifically when assisting patients with potentially infected semen samples [34]. However, the effect rhProtease has on sperm parameters should be considered prior to its inclusion into DGC protocols. Therefore, we evaluated sperm parameters post-processing to determine whether rhProtease could have any detrimental effects.

This study indicated that semen processing with the top density gradient layer supplemented with rhProtease, diluted 0, 2, 10 and 20 times, did not impact negatively on: sperm apoptotic status, necrotic status, mitochondrial membrane potential, or motility. Sperm-zona binding potential was similar for sperm processed with the rhProtease contained in PureSperm® Pro, when compared to the standard PureSperm® (Nidacon, International) without rhProtease. The low concentrations of rhProtease added to the density gradient layers, together with the washing step after exposure to the enzyme and silane-coated silica particles, could contribute to the lack of a negative impact of rhProtease on sperm parameters. These results are in agreement with that of other researchers. Mattson (2008) reported that the treatment of bovine sperm with trypsin had no negative impact on sperm parameters or *in vitro* embryo production [35]. In another study, fertilization rates and the number of *in vivo* produced transferrable bovine embryos were improved by the processing of semen with density gradients supplemented with trypsin [36]. Loskutoff *et al*. (2005) reported that DGC of semen with trypsin added to density layers had no detrimental effects on sperm motility or viability [14]. Furthermore, trypsin treatment of human sperm, prior to swim up, resulted in increased motility and adenosine triphosphate (ATP) concentration [37]. Westhoff and Kamp (1997) suggested that the increased ATP concentration could be attributed to the activation of the glycolytic enzyme glyceraldehyde triphosphate dehydrogenase (GADPH) by trypsin [38], explaining the improved motility.

In conclusion, the addition of rhProtease to the top layer during density gradient centrifugation is non-detrimental to human sperm. The usage of rhProtease improves (i)

sperm yield of hyper-viscous semen samples, (ii) the effectiveness of semen processing for the elimination of seminal pathogens and, potentially, (iii) the prevention of sperm-antibody formation, all of which could enhance the outcome assisted reproduction.

Acknowledgements

The authors are grateful to Prof. P. Becker, Biostatistics Unit Medical Research Council (MRC) for the statistical evaluations; to Nidacon International for providing the PureSperm® products – The authors do not have a commercial or other association with the products that might cause a conflict of interest; to Mr. A. Stander, Department Physiology, University of Pretoria for the confirmation of rhProtease activity and the validation of flow cytometry protocols, and to the MRC for funding the research. The views expressed by the authors do not necessarily reflect the views of the MRC.

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