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GAMETE BIOLOGY

Identification and characterization of RSIY-11, a novel seminal peptide derived from semenogelin-1, which acts as a neutral endopeptidase inhibitor modulating sperm motility

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Abstract

Purpose Based on prior reports demonstrating that neutral endopeptidase (NEP) inhibitors increase sperm motility, the goal of our studies was to identify endogenous seminal peptides that inhibit NEP and investigate their potential effect on sperm motility.

Methods Peptidomic analysis was performed on human seminal fluid, identifying 22 novel peptides. One peptide, named RSIY-11, derived from semenogelin-1, was predicted through sequence analysis to be a substrate and/or potential inhibitor of NEP. Enzymatic analysis was conducted to determine the inhibitory constant (Ki) of RSIY-11 as an inhibitor of NEP. Total and progressive sperm motility was determined at baseline and 30 and 60 min following addition of RSIY-11 to seminal fluid in 59 patients undergoing an infertility workup at an urban medical center. Additionally, the effects of RSIY-11 on sperm motility were evaluated in 15 of the 59 patients that met criteria for asthenospermia.

Results RSIY-11 was shown to act as a competitive inhibitor of NEP with a Ki of 18.4 ± 1.6 μM. Addition of RSIY-11 at concentrations of 0.75 μM, 7.5 μM, and 75 μM significantly increased sperm motility at all time points investigated, with increases of 6.1%, 6.9%, and 9.2% at 60 min, respectively. Additionally, within the subgroup of patients with asthenospermia, RSIY-11 at concentrations of 0.75 μM, 7.5 μM, and 75 μM significantly increased sperm motility at all time points investigated, with increases of 7.6%, 8.8%, and 10.6% at 60 min, respectively.

Conclusions RSIY-11 is a newly identified semenogelin-1-derived peptide present in seminal fluid. RSIY-11 acts as a potent competitive inhibitor of NEP, which when added to seminal fluid significantly increases sperm motility. RSIY-11 could play a potential role in the treatment for male factor infertility related to asthenospermia and improve intrauterine insemination outcomes.

Keywords Semenogelin · Asthenospermia · Sperm motility · Neutral endopeptidase inhibitor · RSIY-11

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Introduction

Infertility affects approximately 1 in 8 couples, with male factor infertility increasingly recognized as a significant contributing factor to the overall incidence rate of infertility. In 2016, the Centers for Disease Control reported that male factor infertility was the leading single diagnosis for patients undergoing in vitro fertilization or intracytoplasmic sperm injection, accounting for 32% of cases [1]. Although male factor infertility is multi-factorial, sperm motility is one of the most significant factors in predicting male fertility [2–4]. After ejaculation, semen forms a semisolid coagulum that entraps immotile spermatozoa [5]. Sperm motility is initiated upon the proteolytic cleavage of the coagulum and the release of sperm into the female reproductive tract where it develops progressive motility and hyperactive motility upon reaching the oviduct and oocyte [6]. Asthenospermia, reduced sperm motility, is defined by the World Health Organization as < 40% motile sperm or < 32% of sperm with progressive motility [7].

Numerous proteins and peptides are present in seminal fluid [8, 9], and several peptides such as enkephalins and tachykinins [10–12] are implicated in maintaining sperm motility. The major seminal fluid protein is semenogelin-1, which forms the coagulum. A well-characterized breakdown product of semenogelin-1 is seminal plasma motility inhibitor (SPMI), which, as its name suggests, functions as an inhibitor of sperm motility [5, 13, 14]. Cleavage of semenogelin-1 into peptides is primarily mediated by prostate-specific antigen (PSA, also known as kallikrein 3); this enzyme is also able to degrade SPMI [15]. Although the prevailing view is that the primary biological function of semenogelin-1 and semenogelin-derived peptides is to inhibit sperm motility, it was proposed ~ 30 years ago that semenogelin-1 is processed into a "motility-activating peptide" [16]. However, this peptide has not previously been identified.

The activity of peptides in mediating sperm motility is likely regulated through the action of peptidases, in particularly neutral endopeptidase (NEP), which determines the stability and duration by which peptide signaling molecules act on receptors located on the sperm membrane surface. NEP is detectable in the seminal plasma, where its activity is primarily localized to prostasomes and sperm cells [11]. Previous reports have demonstrated inhibition of NEP by synthetic inhibitors, such as thiorphan or phosphoramidon, results in improved sperm motility [10, 12]. Opiorphin, an endogenous peptide which acts as a potent NEP inhibitor, is found in human seminal fluid, and we have previously reported that supplementation of opiorphin to seminal fluid has a positive effect on sperm motility [17–19].

Recognizing the potential role that endogenous peptides present in seminal fluid may have on sperm motility, we evaluated the peptidome of human semen. Unlike proteomic studies that have previously been performed on seminal fluid [8, 9], peptidomics studies focus exclusively on the endogenous peptides present in a biological sample [20, 21]. Peptidomic screening identified 22 peptides in seminal fluid that were derived from semenogelin-1 and semenogelin-2. We performed a detailed evaluation of a novel peptide that we named RSIY-11. Amino acid sequence analysis predicted RSIY-11 acts as a substrate and/or competitive inhibitor of NEP. In the current report, we demonstrated that RSIY-11 is an NEP inhibitor and a positive regulator of sperm motility.

Materials and methods

All studies involving human subjects were approved by the Institutional Review Board of Albert Einstein College of Medicine.

Peptidomic analysis of seminal plasma

Semen from two donors, both with normal sperm motility parameters, was analyzed separately by peptidomics analysis. Freshly collected semen was immediately centrifuged at 13 krpm for 5 min at 4 °C to remove sperm from the seminal plasma. Approximately 0.6 ml of the supernatant (seminal plasma), from 1 ml of semen, was added to 2.4 ml of cold acidified acetone (HCl/H_20/acetone; 1:5:40, v/v). The samples were incubated for 10 min at − 70 °C to precipitate proteins and centrifuged at 13 krpm for 10 min at 4 °C. The supernatant was removed and dried under vacuum for approximately 4 h at ambient temperature.

To separate peptides from proteins, samples were resuspended in 1 ml of H_2O and applied to centrifugal filter units with a 10-kDa molecular weight cut-off cellulose membrane (Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane; Millipore, Sigma, USA). Samples were centrifuged at 2300×*g* for 30 min, and the flow-through containing the peptides was recovered. To remove salts, the samples were loaded onto a C18 reverse phase spin column (Pierce, Thermo Fisher, USA), washed with dilute trifluoroacetic acid in water, and the peptides eluted with 70% acetonitrile. The C18 eluate was dried in a vacuum centrifuge and stored frozen until analysis on liquid chromatography/mass spectrometry (LC/MS).

LC-MS/MS analyses were performed on a Synapt G2 mass spectrometer coupled to a nanoAcquity capillary liquid nanochromatography (LC) system (Waters, Milford, MA, USA). The peptide mixture (5 μl) was desalted online for 5 min at a flow rate of 8 μl/min of phase A (0.1% formic acid) using a Symmetry C18 trapping column (5-μm particles, 180-μm inner diameters, 20-mm length; Waters). The mixture of trapped peptides was subsequently separated by elution with a gradient of 7–65% of phase B (0.1% formic acid in acetonitrile) through a BEH 130 C18 column (1.7-μm particles, 75-μm inner diameter, 200-mm length; Waters) in 60 min. The data

were acquired in the data-dependent mode, and the MS spectra of multiple-charged protonated peptides generated by electrospray ionization were acquired for 0.3 s from *m*/*z* 300–1600. The three most intense ions exceeding base peak intensity threshold of 2500 counts were automatically selected and dissociated in MS/MS by 15- to 60-eV collisions with argon for 0.3 s. The typical LC and electrospray ionization conditions consisted of a flow rate of 250 nl/min, a capillary voltage of 3.0 kV, a block temperature of 70 °C, and a cone voltage of 50 V. The dynamic peak exclusion window was set to 90 s.

MS spectra were analyzed using the MassLynx 4.1 software (Waters). To identify peptides, MS/MS data were analyzed using the Mascot search engine (Matrix Science Ltd., UK) and the NCBI human database (310106 sequences). No cleavage site was specified. Modifications included N-terminal protein acetylation, methionine oxidation, and deamination of Asn/Gln (which was initially detected in the present study from de novo sequencing and then included in the Mascot search parameters). All search results were manually interpreted to eliminate false positives, as described [22, 23].

Prediction of peptides with NEP inhibitor function and synthesis of RSIY-11

Peptides identified by LC-MS were screened as potential substrates/inhibitors of NEP through use of the MEROPS database (https://www.ebi.ac.uk/merops/ [24]). This screen identified a peptide VQKDVSQRSIY (referred to as RSIY-11, a peptide of 11 amino acids with a C-terminal amino acid sequence of RSIY). RSIY-11 was synthesized by GenScript (Piscataway, NJ, USA).

Enzymatic characterization

Michaelis-Menten Constant A fluorometric assay was performed using recombinant human NEP enzyme at 0.1 μg/ml (R&D Systems, Minneapolis, USA) and fluorogenic NEP peptide substrate ((7-methoxycoumarin-4-yl)-acetyl-R-P-P-G-F-S-A-F-K-(2, 4-dinitrophenyl)-OH, R&D Systems). In a 96-well format plate, 50 μl of NEP enzyme was added to 50 μl of 50 mM of Tris 0.05%, Brij-35 buffer (R&D Systems) and 50 μl of fluorogenic NEP peptide substrate at concentrations ranging from 0.03 to 33 μM for a total volume of 150 μl. Fluorescence was recorded at 1-min intervals for 15 min using a 96-well plate reader (BMG Labtech, Ortenberg, Germany) with excitation and emission wavelengths of 320 and 405 nm, respectively. The assay was run in triplicate and the Michaelis-Menten equation was used to calculate the Michaelis-Menten constant (Km) of the substrate.

Inhibitory constant and inhibitory nature of RSIY-11 and opiorphin The inhibitory nature and inhibitory constant (Ki) of RSIY-11 were determined using the fluorometric assay described above. Fifty microliters of substrate (10 μM, 3.3 μM, 1.1 μM, or 0.37 μM) was added to 50 μl of NEP enzyme (0.1 μg/ml) followed by 50 μl of synthetic RSIY-11 or opiorphin at a range of concentrations from 2 mM to 0.31 μM. A Dixon plot was generated to obtain the Ki of RSIY-11 and opiorphin, and a Lineweaver-Burk plot was generated to evaluate the inhibitory nature of RSIY-11 (using GraphPad Prism, La Jolla, CA, USA).

Pre-incubation of RSIY-11 The level of NEP inhibition by RSIY-11 was compared between assays that included or did not include pre-incubation of NEP with RSIY-11 (8.2 μM). The fluorometric assay (as described above) was prepared with 50 μl of recombinant human NEP enzyme (0.1 μg/ml) and 50 μl of synthetic RSIY-11 followed by pre-incubation for 3 or 24 h at 37 °C prior to the addition of fluorogenic NEP peptide substrate to determine enzymatic activity. A negative control was also performed with addition of 50 μl of phosphate-buffered saline (PBS) in place of RSIY-11. Following the addition of substrate, the fluorescence was recorded in 1-min intervals over a 15-min period, as described above. The assays were run in triplicate.

Semen and motility analysis

Following IRB approval, semen samples were collected from men who were seen at the Montefiore's Institute of Reproductive Medicine and Health. Inclusion criteria were men presenting for a routine semen analysis for an infertility workup > 18 years old with at least 2 days of sexual abstinence. Exclusion criteria were men < 18 years old, azoospermia, or any known history of HIV, prostate surgery, benign prostatic hyperplasia, exogenous androgen use, untreated thyroid disease, hyperprolactinemia, marijuana or opioid use, clomiphene or human chorionic gonadotropin use within the previous 90 days, kidney failure, cirrhosis, history of gonadotoxic chemotherapy, history of testicular or prostate cancer, cystic fibrosis, abnormal karyotype, and history of cryptorchidism.

Specimens from 59 patients were collected and allowed to liquefy at 37 °C for 30 min. Ninety-nine microliters of raw semen was divided into 6 aliquots and subsequently analyzed prior to and after addition of one of the following five treatments: PBS, opiorphin (200 μM), or RSIY-11 (0.75 μM, 7.5 μM, or 75 μM). The sixth aliquot was not treated. The two peptides (opiorphin and RSIY-11) were synthetic to avoid the possibility that biological effects were due to a contaminating peptide. An aliquot (5 μl) of each of the samples was then counted in a 4-chamber Microcell disposable counting chamber slide (Vitrolife, Goteborg, Sweden). Total motility (TM) including progressive motility (PM) and non-progressive motility (NP), as well as immotile sperm (IM), was evaluated in duplicate immediately (zero time point)

and at 30 and 60 min following the addition of peptides. TM, PM, NP, IM, and sperm concentration were evaluated under a phase-contrast microscope at × 200 magnification. An eyepiece with a 10 × 10 grid was used to count the sperm in all 10 chambers. Concentration was reported as million/milliliter. PM was defined as spermatozoa moving linearly or in a large circle, whereas NP was defined as movement not meeting the criteria for progressive movement. TM was calculated by PM + NP/PM + NP + IM. PM was calculated by PM/PM + NP + IM. All readings were performed in duplicate by one or two trained examiners. If there was a > 20% discrepancy between the first and second reading, a third evaluation was performed and all values were averaged.

The last 14 semen samples of the 59 evaluated were tested with a broader range of concentrations of RSIY-11 and opiorphin using the following additional concentrations: RSIY-11 diluted to 0.075 μM, 0.0075 μM, or 0.00075 μM and opiorphin diluted to 20 μM, 2 μM, or 0.2 μM. Additionally, a separate analysis on the effects of RSIY-11 and opiorphin was performed on patients that met the criteria for asthenospermia (< 40% motility or < 32% progressive motility).

Statistical analysis

We used a repeated measures analysis of variance (ANOVA) model to compare TM and PM at baseline with all other experimental conditions: i.e., PBS (negative control), opiorphin at 200 μM (positive control), and RSIY-11 at 0.75 μM, 7.5 μM, and 75 μM concentrations (a similar comparison was performed for the lower test doses, data shown in Supplementary Fig. 2A–C). We also compared TM and PM for three time points 0, 30, and 60 min within each condition. A $p < 0.05$ (2-sided) was considered to represent a statistically significant difference compared with baseline with lower p-values of < 0.01 and < 0.001 denoting more statistically significant results (represented in figures with ** and ***, respectively). We also carried out a sub-analysis to determine if RSIY-11 and opiorphin increased sperm motility in patients with asthenospermia. These data were also analyzed using a repeated measures ANOVA model, with a p value < 0.05 considered to represent a statistically significant difference compared with baseline. All data analysis was carried out in Stata version 14.

Results

Identification of semen peptides and prediction of RSIY-11 as a potential NEP inhibitor

Peptidomic analysis of human seminal fluid from two healthy male donors detected over 100 peptides of which 22 were identified by MS/MS sequence analysis (Table 1). The remainder of the peptides could not be conclusively identified because there was no high-quality MS/MS data, a common occurrence with peptidomics [25]. These 22 peptides represent 18 distinct peptides, with 4 of the peptides identical in sequence to other identified peptides but with post-translational modifications that altered their masses. These modifications include deamidation of Asn, which was converted into Asp, and loss of water (18 Da) from the C-terminal region of the peptide (Table 1). All of the identified peptides were derived from two proteins, semenogelin-1 and semenogelin-2, which are known to be secreted into seminal fluid [26]. Six of the 14 distinct peptides identified from semenogelin-1 were related to each other, with three ending in –RSIY and another three ending in –SSIY (Table 1). In addition, three peptides were found that were similar to these peptides, but which lacked two C-terminal amino acids, ending in –RS (two peptides) or –SS (one peptide).

Based on the sequence of semenogelin-1, the cleavage site required to generate the –RSIY and –SSIY peptides is Y-S, which is a favored cleavage site for the enzyme kallikrein 3, also known as prostate-specific antigen (PSA). The peptides ending in –RS and –SS are not likely to be products of direct PSA cleavage of semenogelin-1, but these shorter peptides could be formed by the action of NEP on the –RSIY and –SSIY peptides; NEP typically cleaves 2 residues from the C-terminus of peptides. Based on the data for NEP compiled in the MEROPS database, this enzyme was predicted to cleave the C-terminal –IY from either –RSIY or –SSIY peptides, generating the observed shorter peptides (Table 1). However, the residues within the –RSIY and –SSIY sequence are not the optimal residues for NEP cleavage, as indicated in the MEROPS database, suggesting that these would be weak substrates for NEP. With other peptidases, weak substrates can be good inhibitors, and therefore, it was predicted that these peptides would be competitive NEP inhibitors. Therefore, the 11-residue –RSIY peptide was chosen for further studies and named RSIY-11 based on its length (Table 1).

RSIY-11 is a competitive inhibitor of NEP with a lower Ki than opiorphin

A fluorogenic substrate was used to assay NEP; this substrate was found to have a Km of 1.1 μM in our assays (Supplementary Fig. 1). The Lineweaver-Burk plot demonstrated that RSIY-11 acts as a competitive inhibitor of NEP, and a Dixon plot revealed the Ki of RSIY-11 to be 18.4 ± 1.6 μM (Fig. 1a, b). In comparison, the Ki of opiorphin (a well-document endogenous NEP inhibitor) was found to be 265 ± 62 μM (data not shown).

Table 1 Human seminal peptides identified by MS/MS sequence analysis

Peptide sequence	*z*	Observed Mass	Theoretical Mass	Diff (ppm)
Semenogelin-1 (SEMG1)				
VQKDVSQRSIY	3	1321.694	1321.699	− 4
ENGVQKDVSQRS	2	1345.650	1345.659	− 6
SSSTEERRLHY	2	1363.644	1363.648	− 3
GENGVQKDVSQRS	3	1402.687	1402.680	5
GENGVQKDVSQSSIY	2	1609.728	1609.758	− 19
GEDGVQKDVSQSSIY deamidation of Asn3	2	1610.752	1610.742	6
YGENGVQKDVSQSSIY	2	1772.820	1772.822	− 1
YGEDGVQKDVSQSSIY deamidation of Asn4	2	1773.815	1773.806	5
SQTEKLVAGKSQIQAPN	2	1797.963	1797.958	2
PSQDQGNSPSGKGISSQY	2	1835.838	1835.829	5
YGENGVQKDVSQRSIY	3	1841.897	1841.891	3
YGEDGVQKDVSQRSIY deamidation of Asn4	3	1842.868	1842.875	− 4
ITIPSQEQEHSQKANKISYQ	4	2328.181	2328.171	4
SSSTEERRLHYGENGVQKDVSQSS -18 Da from Cterm region	4	2661.277	2661.248	11
SSSTEERRLHYGENGVQKDVSQSS	3	2679.249	2679.248	0
SQKQITIPSQEQEHSQKANKISYQ	3, 4	2799.416	2799.415	0
SSSTEERRLHYGENGVQKDVSQSSIY	3	2955.404	2955.396	3
SSSTEERRLHYGENGVQKDVSQRSIY	5	3024.449	3024.465	− 5
Semenogelin-2 (SEMG2)				
STEERRLNY	2	1166.581	1166.568	11
SSSTEERRLNY	2	1340.645	1340.632	10
YQSSSTEERRLNY	3	1631.753	1631.754	− 1
NVVDVREEHSSKLQ	2	1638.832	1638.833	− 1

z, charge; *mass*, monoisotopic mass of uncharged peptide; *diff*, difference between observed and theoretical mass in parts per million (ppm). Deamination of Asn (N) converts this residue into Asp (D)

Pre-incubation of RSIY-11 with NEP reduces the level by which RSIY-11 inhibits NEP

To test if incubation of RSIY-11 with NEP influenced the degree of NEP inhibition, the peptide was incubated with enzyme for either 0, 3, or 24 h before adding substrate and measuring enzyme activity. After pre-incubation of RSIY-11 with NEP for 3 h, inhibition of NEP was 26% ($p < 0.05$), compared with NEP incubated and assayed in the absence of RSIY-11 (Fig. 2). Pre-incubation of RSIY-11 with NEP for 24 h led to a comparable inhibition of NEP of 29% ($p < 0.05$), relative to NEP incubated and assayed in the absence of RSIY-11 (Fig. 2). Under identical conditions but without the pre-incubation step, the same concentration of RSIY-11 resulted in a 47% reduction in NEP activity ($p < 0.01$) (Fig. 2). This result strongly suggests that RSIY-11 is both an inhibitor and a substrate of NEP. Because NEP was still active after 24 h incubation and the degree of inhibition did not change between 3 and 24 h incubation; this result implies that RSIY-11 is cleaved to a product that is also an inhibitor of NEP, although less potent than the 11-residue peptide.

Addition of RSIY-11 or opiorphin to semen samples increases sperm motility

Patient and baseline semen parameters are shown in Supplementary Table 1. The average age of the patients ($n = 59$) included in this study was 38.5 ± 1.1 years old. The average BMI was 29.3 ± 5.9 kg/m^2, which is in the overweight range. The average time from semen sample collection to evaluation was 3 h and 28 min. The average number of days since the last ejaculation was 4.3 ± 2.0 days. The average volume, concentration, pH, and morphology (Kruger scale) were 3.1 ± 1.6 cc, 47.3 ± 32.9 million/ml, 7.8 ± 0.27 pH, and 8.6 ± 3.1, respectively.

Following addition of opiorphin (200 μM) or RSIY-11 (0.75 μM, 7.5 μM, or 75 μM) to semen samples, there was a significant increase in sperm motility (both TM and PM), compared with baseline ($p < 0.001$) (Fig. 3). An increase in motility was observed immediately following addition of opiorphin or RSIY-11 and was sustained when motility was determined after 30 and 60 min ($p < 0.001$) (Fig. 3a–c). The average increase in total motility with opiorphin (200 μM) immediately and at 30 and 60 min following addition was

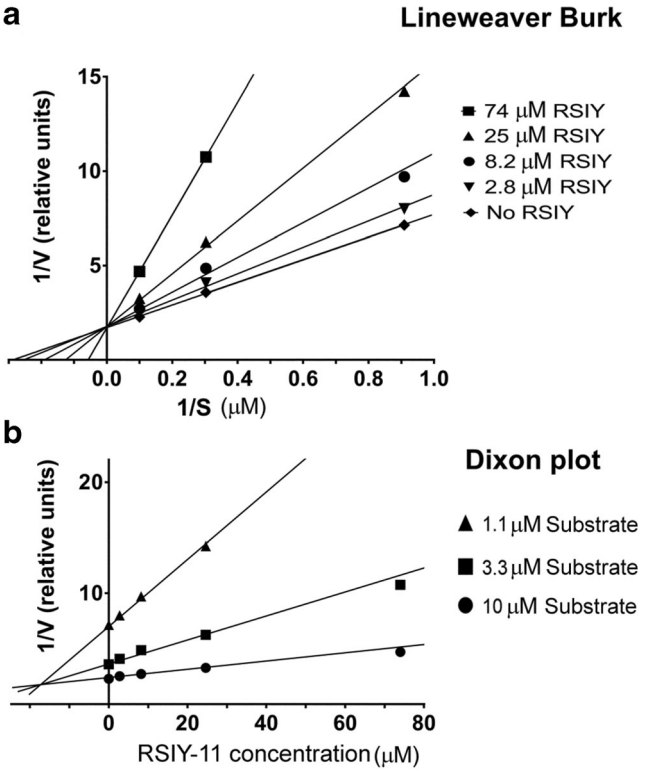

Fig. 1 Determination of the inhibitory nature and Ki of RSIY-11. NEP activity was determined fluorometrically for a range of concentrations of RSIY-11 (2.8 to 74 μM) and fluorogenic NEP substrate (1.1, 3.3, or 10 μM). Data was used to generate **a** Lineweaver-Burk plot and **b** Dixon plot

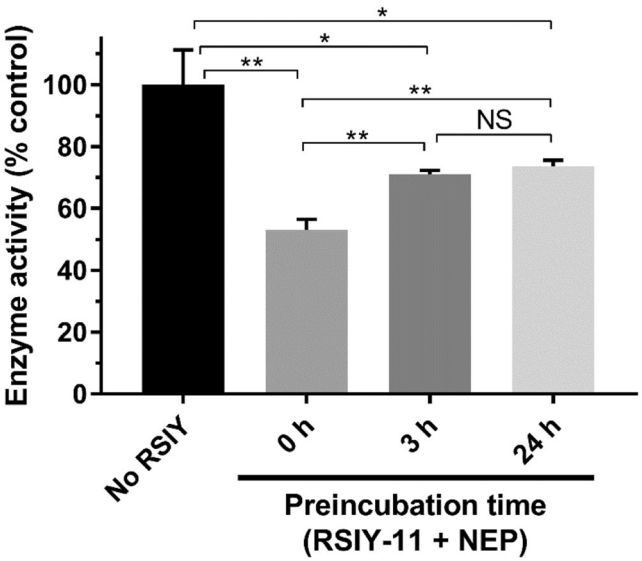

Fig. 2 Determination of the effect of pre-incubation of RSIY-11 with NEP on inhibition. Fifty microliters of recombinant human NEP enzyme (0.1 μg/ml) and 50 μl of RSIY-11 (8.2 μM) were incubated for 0, 3, or 24 h at 37 °C prior to the addition of 50 μl of fluorogenic NEP substrate to determine enzymatic activity, and then, activity was measured every minute over 15 min. A control was performed by incubating NEP for the indicated time without RSIY-11, which was used as the 100% for each time point. Assays were performed in triplicate and averaged. Enzymatic activity was expressed as a percentage of the activity of the control. Error bars represent standard deviation. Asterisks (*) = statistically different from the control, *p value < 0.05; **p value < 0.01. NS no significant difference

9.1%, 6.9%, and 8.6%, respectively ($p < 0.001$). The average increase in total motility with RSIY-11 at 0.75 μM immediately and at 30 and 60 following addition was 9.8%, 7.2%, and 6.1%, respectively. The average increase in total motility with RSIY-11 at 7.5 μM immediately and at 30 and 60 following addition was 8.6%, 7.6%, and 6.9%, respectively. The average increase in total motility with RSIY-11 at 75 μM immediately and at 30 and 60 following addition was 8.2%, 6.6%, and 9.2%, respectively. A significant difference in TM and PM was not seen between baseline and addition of PBS at any time point (Fig. 3a–c).

In the case of patients meeting the definition of asthenospermia ($n = 15$), a significant increase in TM and PM was seen following addition of opiorphin and RSIY-11 at all concentrations and time points tested (Fig. 4a–c).

The effect of RSIY-11 and opiorphin on sperm motility is dose dependent

In 14 patient samples, the effect of adding lower concentrations of opiorphin and RSIY-11 on sperm motility was investigated. Highly significant increases ($p < 0.001$) in PM were seen at 0, 30, and 60 min after addition of 20 μM opiorphin to semen samples with an average increase of 11.3 ± 2.5%, 7.7% ± 2.4%, and 7.8% ± 2.3%, respectively, and a highly significant increase ($p < 0.001$) was seen in TM at the 0-min time point (average increase of 11.5% ± 2.3%, Supplementary Fig. 2). The 2 μM or 0.2 μM concentration of opiorphin did not result in highly significant improvements of TM or PM at any of the time points tested, although the 0.2 μM concentration did show some changes with significance of $p < 0.05$ or < 0.01 (Supplementary Fig. 2). After addition of RSIY-11 at lower concentrations (0.075 μM, 0.0075 μM, or 0.00075 μM) no significant increase in TM or PM was observed at any of these test concentrations or at any time point (Supplementary Fig. 2).

Discussion

NEP is a ubiquitous enzyme present in human sperm cells and prostasomes, where it degrades peptides thereby reducing their biological activity. Endogenous opioids and bradykinins are amongst the substrates of NEP and have been shown to play a positive role in sperm motility [27–29]. Several previous studies have demonstrated that both synthetic NEP inhibitors and opiorphin (an endogenous NEP inhibitor) can increase sperm motility [10, 12, 18]. To gain insight into the

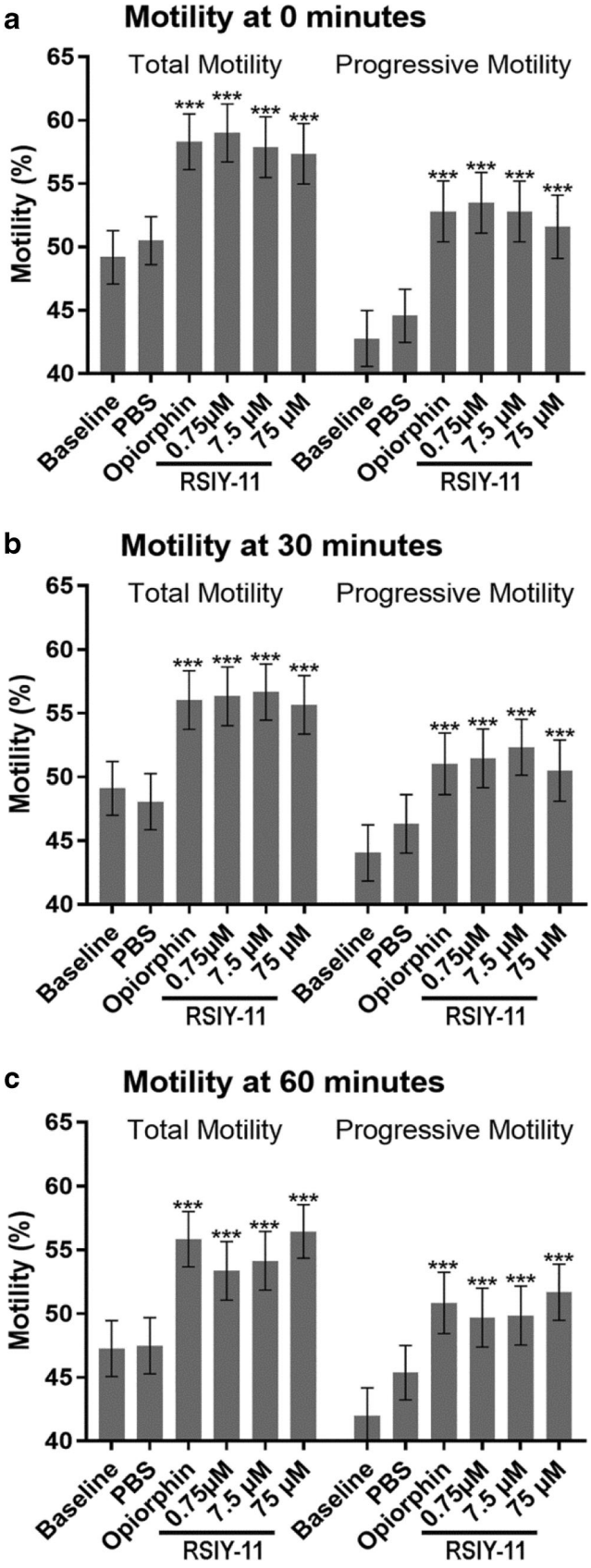

Fig. 3 Sperm motility following addition of RSIY-11 or opiorphin to semen samples. Semen samples were collected from 59 patients, allowed to liquefy at 37 °C for 30 min, and then 99 μl of collected semen was divided into 5 aliquots and subsequently analyzed prior to, and after the addition of, the following final concentration of peptides: PBS, opiorphin (200 μM), or RSIY-11 (0.75 μM, 7.5 μM, or 75 μM). In addition, one aliquot was set aside without addition of any peptide or PBS, which served as our baseline. Five microliters of semen was then placed into a 4-chamber microcell disposable counting chamber slide, and the percent of total motile sperm (TM) and percent of sperm exhibiting progressive motility (PM) were evaluated in duplicate for each patient sample. The graphs show the average for the entire patient population at **a** time zero, **b** at 30 min, and **c** 60 min following the addition of peptides. Error bars represent the standard error of the mean. Asterisks (*) = statistically different from the control; *p value < 0.05, **p value < 0.01, and ***p value < 0.001

peptides present in semen and if they might play a role in sperm motility, we performed a peptidomic analysis. Whereas proteomic analysis has been extensively applied to studies on semen [8, 9, 30–34], our use of peptidomic analysis is unique to the field. Proteomic analysis is focused on identifying proteins, usually after digestion with trypsin, and therefore cannot distinguish between those peptides that are present in semen from those that are generated artificially during sample preparation. In contrast, peptidomics determines the native forms of peptides in a sample, thereby providing information about the biologically active forms.

Our peptidomic analysis of seminal fluid identified 18 distinct peptides, all of which were derived from semenogelin-1 and semenogelin-2, which are major proteins present in seminal fluid. Several of these novel peptides terminated in RSIY, and some were further truncated by 2 residues from the C-terminus, which is a hallmark of NEP cleavage. Because the RSIY sequence was predicted to be a weak substrate of NEP, based on the cleavage site preferences of NEP, it was a logical candidate to test as an NEP inhibitor. We confirmed that RSIY-11 acts in vitro as a competitive inhibitor of NEP. As with other NEP inhibitors which act as positive modulators of sperm motility, RSIY-11 increased both total and progressive sperm motility [10, 12, 18].

Determining the Ki demonstrated that RSIY-11 inhibits NEP with approximately 15-fold greater potency than opiorphin. Although the ability of opiorphin and RSIY-11 to improve sperm motility was comparable, this is likely due to the differences in peptide concentrations used in the sperm motility assays. Opiorphin was tested at 200 μM, whereas RSIY-11 was effective at much lower concentrations, consistent with its greater potency for NEP. The 200 μM opiorphin concentration was based on a previous study from our laboratory where this concentration demonstrated significant improvement in sperm motility, albeit with a smaller patient sample size [18]. It is also possible that the peptides are inhibitors of other enzymes; for example, opiorphin is known to inhibit aminopeptidase N (APN) which is present on sperm

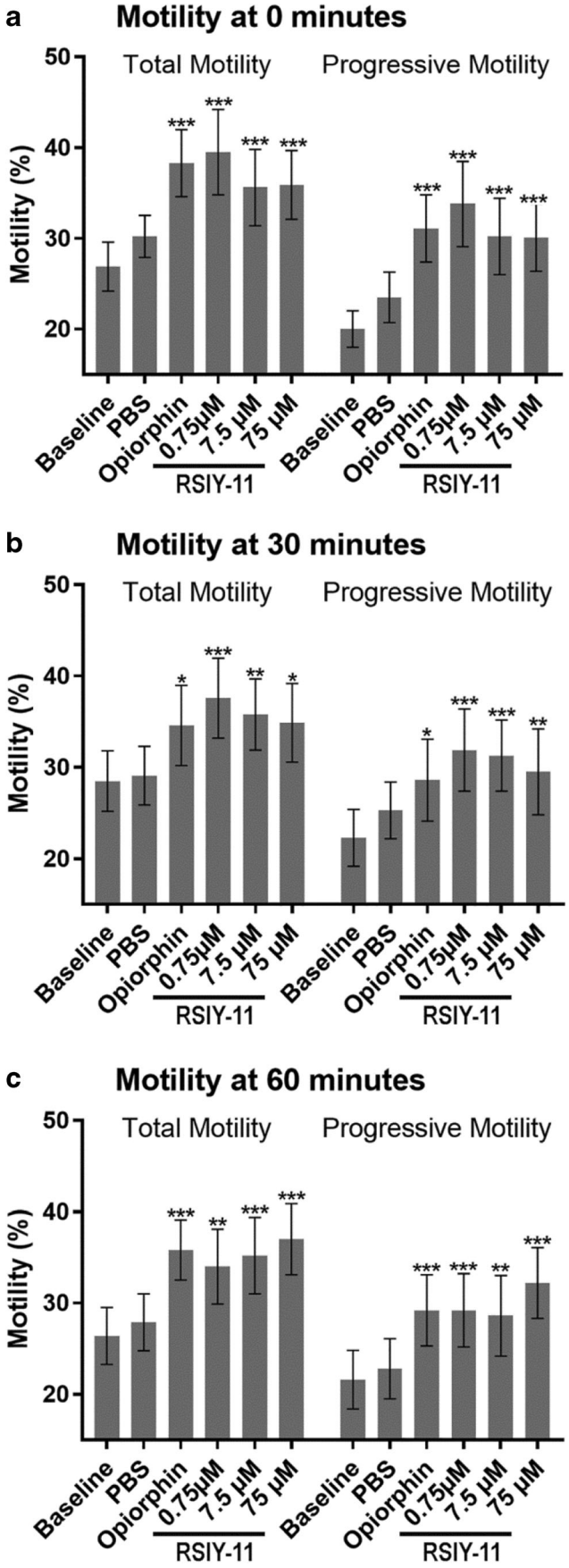

Fig. 4 Sperm motility following addition of RSIY-11 or opiorphin to semen samples from patients with asthenospermia. Similar experiments to those shown in Fig. 3 were performed on semen samples from 15 patients with asthenospermia. The graphs show the average for the entire patient population at **a** time zero, **b** at 30 min, and **c** 60 min following the addition of peptides. Error bars represent the standard error of the mean. Asterisks (*) = statistically different from the control; *p value < 0.05, **p value < 0.01, and ***p value < 0.001

cells and prostasomes [11, 29, 35]. Serial dilution of RSIY-11 to concentrations of 0.75 μM, 7.5 μM, and 75 μM did not generate a graded decrease in sperm motility, but instead showed the same improvement in sperm motility as the higher concentration, although further dilution to 0.075 μM was without effect. It is possible that the effect of RSIY-11 on sperm motility is binary; a certain level of NEP inhibition improves sperm motility beyond which there is no further improvement.

Pre-incubation of RSIY-11 with NEP enzyme for 3 and 24 h resulted in significantly less inhibition compared with no pre-incubation; however, compared with our negative control (no addition of RSIY-11 or opiorphin), it still resulted in a significant level of inhibition. These results are consistent with the competitive inhibitory nature of RSIY-11 to NEP, suggesting that RSIY-11 is a substrate for NEP and undergoes proteolysis. However, it remains to be determined if the remaining inhibitory action of RSIY-11 is due to its incomplete breakdown or if products of its breakdown are also NEP inhibitors, albeit with reduced efficacy.

It is interesting to note that although addition of RSIY-11 and opiorphin to semen on average resulted in improved sperm motility, this was not universal and varied greatly between patients. A possible explanation is that semen from different patients expresses different levels of proteolytic activity. For example, it has been reported that patients with asthenospermia have different levels of APN activity in semen compared with control patients [36]. Further studies are necessary to determine if improvement in sperm motility with addition of RSIY-11 is correlated to levels of RSIY-11 and/or opiorphin and activity levels of NEP and/or APN enzymes.

Our results reveal that sperm motility in patients with asthenospermia improved following the addition of RSIY-11 and opiorphin to semen. Therefore, potential clinical applications of RSIY-11 may be in treatment strategies for male factor infertility related to asthenospermia. For example, addition of RSIY-11 to sperm prior to intrauterine insemination (IUI) may be of benefit to patients with asthenospermia as positive effects on sperm motility persist up to 60 min following addition. In contrast to the sperm samples used for IUIs which undergo washing procedures, the sperm used in the present studies were in unprocessed seminal fluid. Although present on sperm, NEP is found at higher concentrations in prostasomes [11, 29] which are known to play a key role in

modulating sperm motility [37, 38]. Washing sperm samples may therefore remove a significant proportion of NEP activity, which may mute the benefit of supplementing sperm with RSIY-11 for IUI.

NEP is widely expressed throughout the body, being found in the brain, intestinal and kidney epithelial cells, neutrophils, thymocytes, and lung, and plays a role in several physiological processes [39–43]. Systemic application of RSIY-11 to treat male factor infertility in its present form is therefore unlikely.

Based on its amino acid sequence, RSIY-11 is clearly a semenogelin-1-derived peptide. Semenogelin-1 is the most abundant protein in human semen, is involved in keeping human semen in a coagulative gelatinous form upon ejaculation, and functions as an anti-motility factor [5]. Liquefaction is associated with sperm motility and occurs upon degradation of semenogelin-1 by PSA [15]. PSA is known to cleave the Tyr-Ser bond within semenogelin-1 that is located on the C-terminus of RSIY-11 [15]. The enzyme responsible for the N-terminal cleavage necessary to produce RSIY-11 is not yet known. A number of peptidases have been identified from proteomic analysis of seminal fluid [8, 9], and further studies are necessary to identify the one responsible for the N-terminal cleavage needed to produce RSIY-11.

The prevailing view is that the primary biological function of semenogelin-1 and semenogelin-derived peptides is to inhibit sperm motility. However, 30 years ago, there was speculation that semenogelin-1 could be processed into a "motility-activating peptide" [16]. Our studies on RSIY-11 have validated the existence of such a peptide; future studies may identify additional peptides that are also able to activate sperm motility.

The generation of RSIY-11 and SPMI likely occurs through the action of proteases responsible for the initial liquefaction and breakdown of semenogelin-1. The presence in semen of both positive and negative peptide regulators of sperm motility suggests an environment of checks and balances to motility determined by the ratios of different peptides. Given that the variation in activity and rates by which different proteases generate peptides, the ratios of positive and negative peptide regulators of motility would likely change over time, providing a mechanism for the progressive changes observed in sperm motility that follows ejaculation.

In conclusion, RSIY-11 is a peptide present in seminal fluid generated from the breakdown of semenogelin-1. It functions as a relatively potent competitive inhibitor of NEP and when added to human semen, increases sperm motility. Further studies are needed to evaluate the concentration of RSIY-11 in human semen, the relation of asthenospermia to levels of RSIY-11, and its evaluation as a therapeutic option for couples suffering from infertility due to male factor infertility.

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Compliance with ethical standards

All studies involving human subjects were approved by the Institutional Review Board of Albert Einstein College of Medicine.

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