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ORIGINAL ARTICLE

Impact of semen-derived amyloid (SEVI) on sperm viability and motility: its implication in male reproductive fitness

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Abstract

Human semen contains a large number of macromolecules, including proteins/enzymes and carbohydrates, regulating and protecting sperm cells. Proteomic analysis of human seminal fluid led to the discovery of semen amyloids derived from short peptide fragments of the proteins prostatic acid phosphatase (PAP) and semenogelin (SG) which are known to play a crucial role in enhancing HIV infection. However, the relevance of their existence in human semen and role in maintaining sperm behavior remains unclear. Distinct physiological, biochemical, and biophysical attributes might cause these amyloids to influence sperm behavior positively or negatively, affecting fertilization or other reproductive processes. We assessed the direct effect of amyloids derived from a $PAP_{248–286}$ fragment, on sperm motility and viability, which are crucial parameters for assessment of sperm quality in semen. Co-incubation of human sperm with $PAP_{248–286}$ amyloids at normal physiological concentrations formed in buffer led to significant reduction in sperm viability, though approximately a 10× higher concentration was needed to show a similar effect with amyloid formed in seminal fluid. Both forms of $PAP_{248–286}$ amyloid also had a significant impact on sperm motility at physiological levels, in agreement with a previous report. Our study suggests that $PAP_{248–286}$ amyloids can directly influence sperm motility and viability in a concentration-dependent manner. We hypothesise that the direct toxic effect of $PAP_{248–286}$ amyloid is normally mitigated by other seminal fluid ingredients, but that in pathological conditions, where $PAP_{248–286}$ concentrations are elevated and it plays a role in determining sperm health and viability, with relevance for male fertility as well as sterility.

Keywords Semen-derived amyloids · $PAP_{248–286}$ · SEVI · Male sterility

Abbreviations

PAP	Prostatic acid phosphatase
SG	Semenogelin
SEVI	Semen-derived enhancer of viral infection
SP	Seminal plasma
DPBS	Dulbecco's phosphate buffer saline
RPM	Revolutions per minutes
CR	Congo red
TEM	Transmission electron microscope
SMI	Sperm motility index
PI	Propidium iodide
FACS	Fluorescence-activated cell sorting

Introduction

Human semen represents a highly complex biological fluid composed of sperm and seminal plasma (SP). Although these two components derive from different organs of the reproductive system, they are known to substantially influence each other's physiological and biochemical behavior (Eliasson 1982; Juyena and Stelletta 2012; Owen and Katz 2005). The biochemical composition of the seminal plasma is complex and differs remarkably from other body fluids (Eliasson 1982; Poiani 2006). The seminal fluid contains a variety of proteins and peptides, the majority of which are derived from seminal vesicles and the prostate gland (Owen and Katz 2005). These proteins are found to participate in the key events related to sperm function and fertilization, and hence contribute to a large extent to the

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maintenance of sperm quality (Juyena and Stelletta 2012). Due to the lack of knowledge regarding the physiological role of many of these proteins and peptides, our understanding of the molecular basis of the functioning of the male reproductive system remains limited.

Proteomic analysis of SP led to the discovery of amyloids in human semen (Munch et al. 2007; Roan and Greene 2007; Usmani et al. 2014). Amyloids are stable protein/peptide aggregates and consist of highly ordered cross-β-sheets in the core with a fibrillar morphology upon maturation. However, amyloidogenic proteins/peptides may exist in oligomeric and protofibrillar as well as fibrillar conformations (Glabe 2008). These amyloid structures, particularly the non-fibrillar ones, are usually pathogenic and are involved in the pathophysiology of several devastating disorders (Chiti and Dobson 2006; Eisenberg and Jucker 2012; Hammer et al. 2008; Johansson et al. 2016). Amyloid structures are toxic nature for different cells via various mechanisms including pore formation in cell membranes. The discovery of amyloids in human semen by Munch et al. (2007) led to an understanding of the involvement of amyloids in enhancing HIV infectivity. These amyloids are mainly derived from the proteolytic cleavage of prostatic acid phosphatase (PAP) and semenogelin, which are proteins found in human semen (Munch et al. 2007; Roan and Greene 2007; Roan et al. 2011).

One of the fragments released from PAP is a 39 amino acid residue peptide, commonly known as $PAP_{248–286}$ that possesses high aggregation propensity and forms well-defined amyloid-like fibrillar structures, termed Semen-derived Enhancer of Viral Infection (SEVI) (Munch et al. 2007). The terms 'SEVI' and '$PAP_{248–286}$ aggregates' correspond to the aggregated form of the peptide and have been used interchangeably in this manuscript. Subsequent studies revealed the mechanism of aggregation under different conditions and characterized the structural features of SEVI (French and Makhatadze 2012; Nanga et al. 2009; Ye et al. 2009). The positively charged SEVI fibrils efficiently bind to $CD4^+$ cells as well as HIV virions through electrostatic interactions, which serves as the basis for the underlying mechanism of SEVI-mediated enhancement of HIV infection (Roan et al. 2009, 2010). Further exploration established that SEVI exists naturally in human semen (Usmani et al. 2014) and that their relative concentration varies among individuals (Hartjen et al. 2012; Kim et al. 2010). The natural existence of amyloids in human semen might have physiological relevance in maintaining semen quality and sperm health. However, exactly what the physiological relevance of these amyloids is to the maintenance of sperm health and their subsequent implication for fertilization has not been fully elucidated yet (Castellano and Shorter 2012; Hammer et al. 2008; Malm et al. 2008; Southern 2013).

Several reports have suggested the possible role of SEVI at different stages of the reproductive process (Castellano and Shorter 2012). A recent study suggested that semen-derived amyloids help in eliminating physiologically weak sperms by enhancing their phagocytosis in the female reproductive tract (Roan et al. 2017). The fibrillar semen amyloids were shown to physically interact with the sperm, reducing overall motility and leading to their phagocytosis by vaginal macrophages (Roan et al. 2017). This exciting discovery suggests that a natural mechanism employed in the reproductive process for selecting the fittest sperm for fertilization. However, an amyloidogenic peptide can exist in a heterogeneous population of different conformations ranging from monomer, to oligomer, profibrils and mature fibrillar forms and their biological functions are conformation dependent. The aggregation of $PAP_{248–286}$ or SEVI formation in vitro is driven by concentration (usually ≥ 1 mg/ml) and other thermodynamic parameters (Martellini et al. 2011; Ye et al. 2009). The physiological concentration of semen-derived amyloidogenic peptides usually ranges from 28 to 267 µg/ml (Munch et al. 2007; Roan et al. 2014, 2011). Under in vitro conditions, the physiological concentration of $PAP_{248–286}$ is less than sufficient to form fibrillar amyloids. However, the expression level of PAP, the precursor protein for $PAP_{248–286}$ aggregates, is reported to be enhanced in azoospermia, as well as in prostate cancer (Gunia et al. 2009; Tasken et al. 2005; Vaubourdolle et al. 1985). These pathological conditions, among others, may, therefore, increase the amyloid load in human semen; apart from SEVI, there are several other amyloidogenic proteins and peptides such as CRES, SEM1, SEM2, and cystatin C and serum amyloid P component present in human semen that would increase the possibility of amyloids forming there (Castellano and Shorter 2012; Easterhoff 2013; Hewetson et al. 2017; Röcker et al. 2018; Whelly et al. 2014, 2016). We believe that the actual amyloid load in human semen is, therefore, underestimated and needs to be re-evaluated. In the current investigation, we, therefore, deal with the assessment of the direct effect of $PAP_{248–286}$ aggregates on human sperm. In addition to this, our description of the impact of amyloids on human sperm may be able to provide a new diagnostic strategy for the assessment of overall semen and sperm quality.

Materials and methods

Materials

Chemically synthesized $PAP_{248–286}$ peptide (Seq. GIHKQKEKSRLQGGVLVNEILNHMKRATQIPSYKKLIMY) was procured from GenScript, USA. Aggresome detection kit was purchased from ENZO Life Sciences, USA. Guanidium hydrochloride (GdHCl),

paraformaldehyde, and Congo red were procured from Sigma-Aldrich, St. Louis, MO. Live/dead sperm viability detection kit was procured from Invitrogen, USA.

Methods

Preparation of $PAP_{248–286}$ aggregates

To prepare amyloids, commercially procured synthetic peptide ($PAP_{248–286}$) was dissolved in 1× Dulbecco's phosphate buffer saline (DPBS). The concentration of peptide solution was estimated using Gill and Hippel method (Gill and Von Hippel 1989) and the final concentration of peptide was adjusted to 10 mg/ml. The solution was then subjected to incubation at 37 °C with agitation of 1200 RPM for 24 h on a thermomixer. Besides that $PAP_{248–286}$ aggregation was also performed in human seminal plasma in a similar manner to mimic its natural environment.

Characterization of $PAP_{248–286}$ aggregates

Congo-red (CR) binding Congo-red-binding assay is a gold standard method for amyloid detection. Congo red is an azo dye (benzidinediazo-bis-1-naphthylamine-4-sulfonic acid), which binds to the cross-β-sheet structure of amyloids and shows the bathochromic shift in its absorption maxima (Klunk et al. 1989). To assess Congo-red binding, an aliquot of the aggregated peptide was mixed with CR stock solution (2% w/v). The final concentrations of CR and peptide were adjusted to be 0.01% (w/v) and 50 µM, respectively. Thereafter, the absorption spectra were recorded on a UV–visible spectrophotometer (Halo DB 20, UK) between 400 and 600 nm.

Transmission electron microscopy (TEM) 5 µl of aggregated sample was loaded on a Formvar-coated copper grid and incubated for 30 s at room temperature and the extra sample was blotted out using filter paper strips. The grid was washed in three independent droplets of distilled water (~50 µl) each for 30 s to remove phosphate and other buffer components. Thereafter, the grid was negatively stained by submerging it in 3% (w/v) aqueous solution of ammonium molybdate for 30 s. This step was repeated three times, the grid was air dried, and the images were recorded at microscopy unit, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India on a transmission electron microscope (JEOL, Japan) accelerating at 80 kV.

Proteostat staining of amyloids The $PAP_{248–286}$ aggregates were further characterized by aggresome detection kit, which contains Proteostat dye; specifically meant for amyloid staining. To probe amyloid fibrils under a fluorescence microscope, 10 µl of aggregated $PAP_{248–286}$ was incubated with 90 µl of 2000 fold diluted Proteostat for 30 min. The sample was then centrifuged at 10,000*g* for 10 min. The pelleted aggregate was then re-suspended in 100 µl of 1× assay buffer provided with the kit. Thereafter, 10 µl of the sample was taken on a glass slide. The air-dried slides were mounted with 60% glycerol, covered with glass coverslips, and observed under a Nikon AIR confocal microscope equipped with Texas Red Filter set. The obtained images were analysed using NIS elements AR 4.00.04 software (Nikon Instruments, Shizuoka-ken, Japan).

Semen collection and isolation of sperms and seminal plasma

Fresh ejaculate (semen) was collected from the healthy volunteer working at molecular reproduction lab at Rajiv Gandhi Centre for Biotechnology (RGCB), Kerala, India. Sperm cells were isolated by centrifugation of liquefied semen at 500*g* for 10 min at 37 °C. The supernatant containing seminal plasma was collected in a sterile tube and stored at − 20 °C until use. The pellet containing sperms was re-suspended in Hank's balanced salt solution (HBSS) and incubated at 37 °C until use.

Binding of $PAP_{248–286}$ aggregates to human sperm

The physical interaction of amyloids with human sperms was probed by employing aggresome detection kit which consists of Hoechst 33342 and Proteostat dye and, therefore, facilitated co-staining of the sperm and amyloids, respectively. The sperm were incubated with $PAP_{248–286}$ aggregates prepared in the buffer and seminal fluid separately for 15 min at 37 °C. As a negative control, sperm was also incubated with the freshly dissolved peptide of identical concentration. Then, the incubated samples were centrifuged at 400*g* for 5 min at 37 °C to sediment the sperm. Thereafter, the samples were fixed for 30 min in 4% paraformaldehyde solution and subsequently stained with double detection reagent containing Proteostat and Hoechst 33342 both, to visualize the co-localization of sperm and $PAP_{248–286}$ aggregates. The samples were then centrifuged at 800*g* for 10 min and the pellet was re-suspended in 100 µl of the buffer supplied with the kit. 10 µl of this solution was then smeared on a glass slide. After 10 min, the smear was overlaid with 60% glycerol and covered with a coverslip. The images were recorded at 40× resolution using a standard Texas red filter set under a Nikon AIR confocal microscope and analysed using Elements AR 4.00.04 software (Nikon Instruments, Shizuoka-ken, Japan).

Effect of $PAP_{248–286}$ aggregates on sperm viability

The sperm viability was assessed using a Live/Dead sperm viability kit (Invitrogen, USA) following the manufacturer's protocol. This kit contains two differential dyes, i.e., SYBER-14 and propidium iodide (PI), which selectively stain live and dead sperm, respectively. To describe briefly, isolated sperm cells were incubated with different concentrations of $PAP_{248–286}$ aggregates (3.5–350 µM) for 30 min followed by addition of 2.5 µl of SYBR-14 and incubation for 5 min. Subsequently, 2.5 µl of PI was added to the solution and incubated for 2 min. The relative fluorescence intensities of both dyes were recorded on a flow cytometer (BDFACS Aria™, BD Bioscience, USA) by setting excitation and emission wavelengths 488/516 nm, 493/636 nm for SYBR-14 and PI, respectively. A total of 10,000 cells were quantified for each sample. The obtained data were finally analysed by BD FACSDiva™.

Effect of $PAP_{248–286}$ aggregates *on sperm motility*

The effect of $PAP_{248–286}$ aggregate amyloids on sperm motility was performed using sperm quality analyser (SQA-Vision, Medical Electronic Systems, USA) coupled with V-Sperm system ver.3.60. Alternatively, manual assessment of sperm motility was also carried out to see the effect of $PAP_{248–286}$ aggregates directly. For that, sperm cells were treated with different concentrations (3.5–350 µM) of $PAP_{248–286}$ aggregates and then incubated for 20 min at 37 °C. Sperm treated with different concentrations of the freshly dissolved peptide were considered as a negative control. Afterward, relative motility was assessed on a sperm analyser at KJK hospital in Thiruvananthapuram, India. For manual assessment of sperm motility, 5 µl of amyloid treated sperm suspension was taken on a glass slide and observed under a light microscope (Nikon, Japan). The mobility was recorded in the form of video files and later on, motile and non-motile sperms were manually counted by a group of unbiased volunteers.

Results

Binding of $PAP_{248–286}$ aggregates *to the sperm cells*

We began our study with the preparation of $PAP_{248–286}$ aggregates in 1X DPBS or in twofold diluted seminal fluid (to partially mimic its natural environment). The peptide formed a white milky aggregate upon incubation, and its amyloid-like characteristics were confirmed by the appearance of a red shift in its CR absorption maximum, ThT binding and fibrillar morphology under transmission electron microscopy, as shown in Fig. 1a, c, respectively. Alternatively, the amyloidogenic nature of these aggregates was further confirmed by staining with an aggresome detection kit (Enzo Life Sciences Inc., USA), which contains a fluorescent dye Proteostat, that gives red fluorescence upon binding with amyloids (Navarro and Ventura 2014; Shen et al. 2011). After staining with Proteostat the $PAP_{248–286}$ aggregates displayed red fluorescence (Fig. 1b) under a standard Texas red filter, thus exposing their amyloid nature.

To investigate the effect of semen-derived amyloids, the freshly isolated sperms were co-incubated with three different concentrations (3.5 µM, 35 µM, and 350 µM) of $PAP_{248–286}$ aggregates. Sperms were isolated from fresh ejaculate received from the registered volunteers at RGCB, Kerala, India, and washed thoroughly with the buffer to remove any intrinsic amyloids derived from semen. Subsequently, the quality of the isolated spermatozoa was further assessed under a light microscope. Semen samples containing motile and intact sperms were further processed for the assay. After incubation for 30 min, the sperms were treated and stained with Proteostat and Hoechst 33342 dye to probe the localization of the amyloids on sperm. The Proteostat and Hoechst 33342 dye selectively stain the $PAP_{248–286}$ aggregates and sperm nucleus, respectively. As shown in Fig. 2, the Proteostat intensity was found to be preferentially localized near the tail and mid-piece region of the sperms which suggests that amyloids are physically interacting with the sperm, irrespective of the aggregation medium (buffer and seminal fluid) used, which is also in agreement with the observation by Roan et al. (2017). Furthermore, as observed in Fig. 3, the Proteostat intensity was found to be considerably enhanced with increasing concentration of the aggregates. Parallel to that, the sperms were also incubated with the equivalent concentrations of freshly dissolved peptide; nevertheless, the un-aggregated peptides did not yield any observable signal. This signifies the fact that aggregated $PAP_{248–286}$ specifically interacts with sperms. The mid-piece region of sperm cells contains mitochondria that produce the energy required for motility and other metabolic activities and hence can determine their viability or otherwise (Amaral et al. 2013; Cardullo and Baltz 1991). Accumulation of amyloids on the cell surface is a hallmark phenomenon related to amyloid-induced cell toxicity in various amyloid-related human diseases, including Alzheimer's and Parkinson's diseases, and type II diabetes. Such interactions usually induce disruption of the membrane integrity and the cellular osmotic balance which ultimately results in cell death (Last et al. 2011; Ta et al. 2012; Walsh et al. 2014). Rationally, a similar outcome may emerge from the interaction of $PAP_{248–286}$ aggregates with sperm cells and might influence their physicochemical properties, although the amyloid induced toxicity is liable to vary for different amyloidogenic peptides. Therefore, we further examined the effect of such interactions on sperm

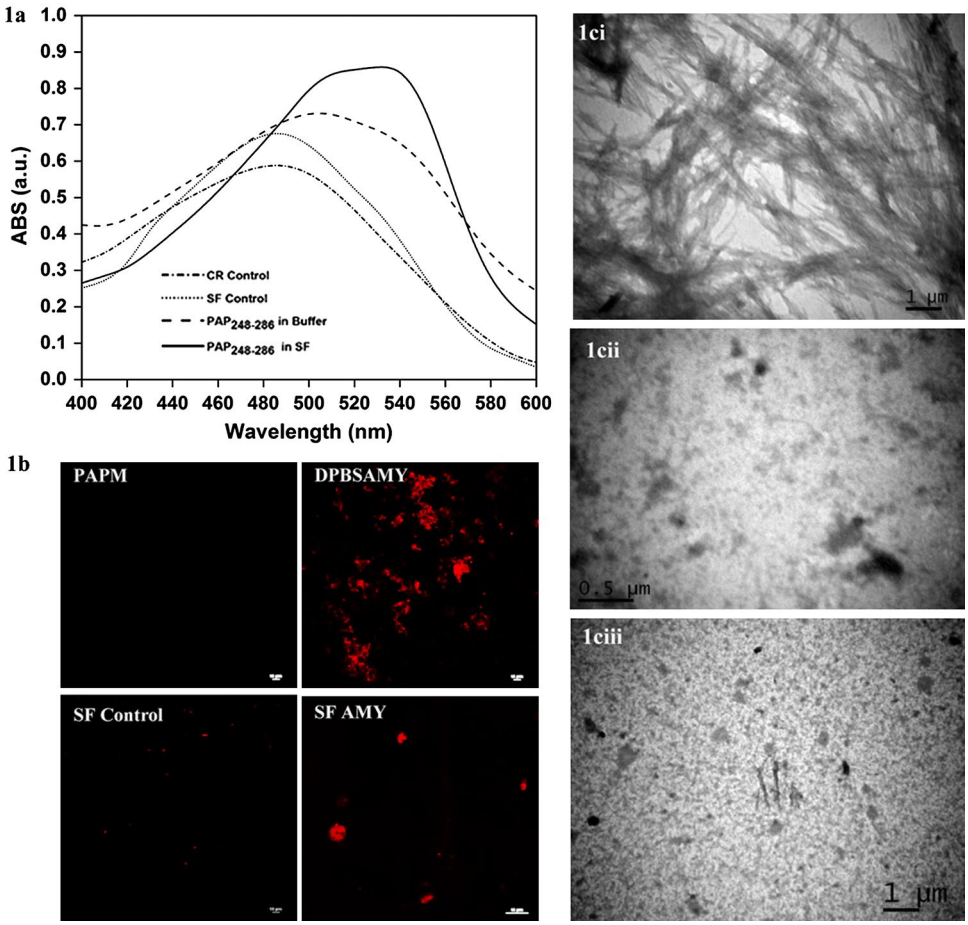


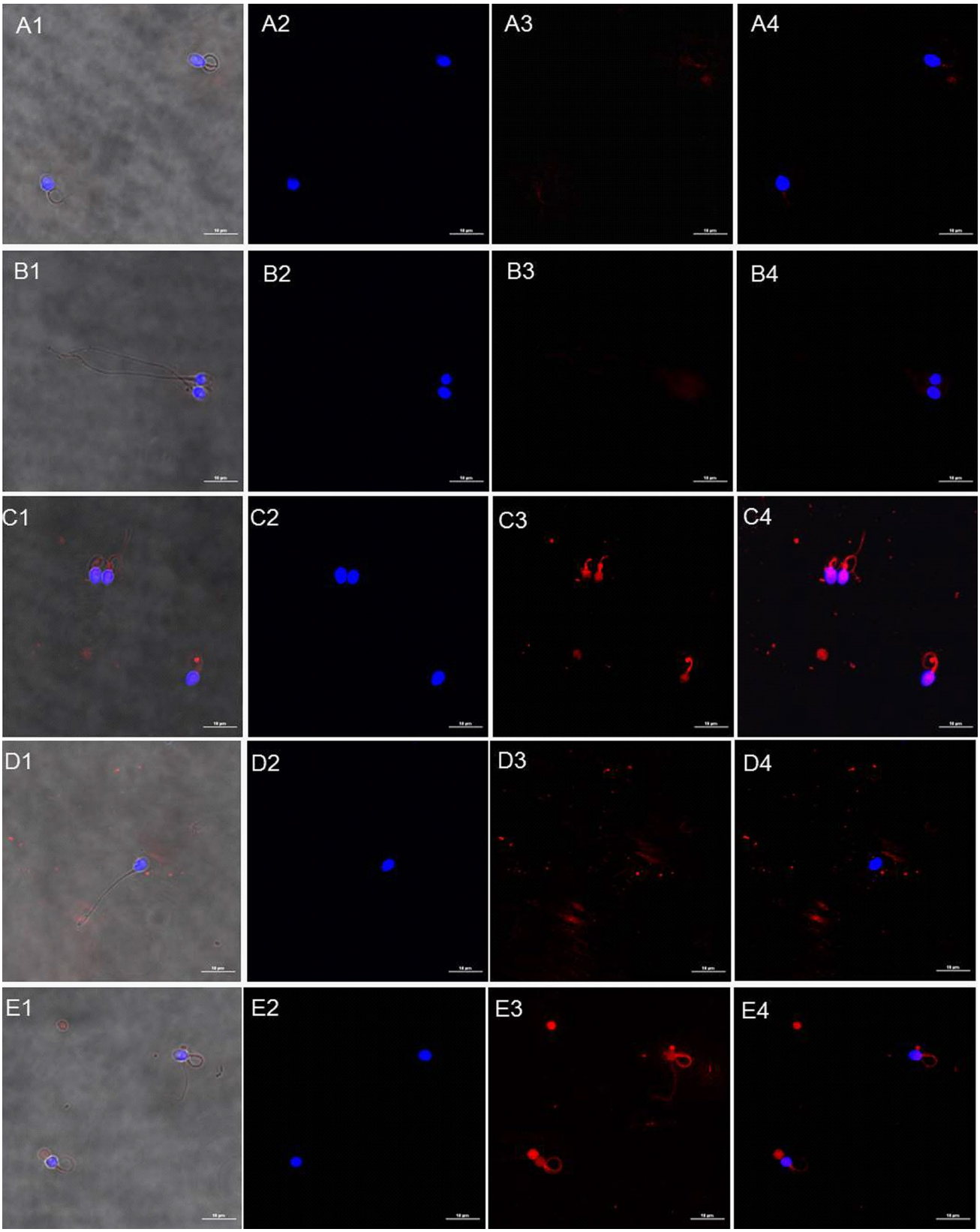
Fig. 1 Characterization of SEVI formed in buffer and seminal fluid. **a** Congo-red-binding assay of $PAP_{248–286}$ aggregates prepared in buffer and seminal fluid. The absorption maxima of Congo red shifted towards higher wavelength when CR binds with the aggregates that gave an indication of the presence of amyloid structures in the samples. **b** Proteostat staining of $PAP_{248–286}$ aggregates. DPBSAMY and SFAMY refer to amyloid formation in DPBS buffer and seminal fluid, respectively. PAPM and SF control correspond to monomeric peptide and seminal fluid taken as negative controls. The scale bar corresponds to 10 µm. **c** TEM analysis of $PAP_{248–286}$ aggregates formed in DPBS buffer (**ci**) and seminal fluid (**ciii**). Seminal fluid without $PAP_{248–286}$ (**cii**) was considered as a negative control

viability and motility that signify their pathophysiological status.

Effect of $PAP_{248–286}$ aggregates *on sperm viability and motility*

Sperm quality in a given semen sample can be influenced by various biochemical components of the seminal fluid (Juyena and Stelletta 2012) including semen-derived amyloids. Sperm viability and motility are the two vital characteristics that define the fertility fitness of a semen sample. After having confirmed the ability of $PAP_{248–286}$ aggregates to interact with human sperm, we further assessed the effect of $PAP_{248–286}$ aggregates on sperm cell viability and motility. To assess the effect, sperms were incubated with different concentrations of $PAP_{248–286}$ aggregates, and their viability and motility were measured using fluorescence-activated cell sorting (FACS) and sperm quality analyzer, respectively, as described in "Methods". FACS analysis enables a quantitative assessment of viability based on the uptake of live and dead cell marker dyes by the sperm cells in the presence and the absence of the amyloid. Under in vitro conditions, the functional characteristics of amyloids are largely defined by aggregation medium, and hence, the $PAP_{248–286}$ aggregates were grown in two distinct aggregation media (i.e., buffer and seminal plasma). The fibrillar structures of $PAP_{248–286}$ aggregates were commonly observed in the sample obtained from buffer; however, the $PAP_{248–286}$ aggregates grown in seminal plasma contained a comparatively lower number of fibrils along with many non-fibrillar aggregates. The rationale behind such an observation could be that molecular species such as heat shock proteins, clusterin, polyanions, and metal ions present in human semen could reduce the PAP248-286 fibrillation and downstream toxicity (Castellano et al. 2015; Elias 2015; Sheftic et al. 2012; Tan et al. 2013).

After incubation, the sperms were stained with SYBR-14 and PI. The relative proportions of PI^+ and $SYBR\text{-}14^+$ sperm cells were quantified in terms of their fluorescence intensity and dot scattergrams for each incubated sample. At the end, the data were normalized by considering the population of live sperm cells in control (i.e., without $PAP_{248–286}$ aggregates) samples as 100%. The effect of $PAP_{248–286}$ aggregates on sperm viability was found to be concentration dependent. It is evident from data presented in Fig. 4 that there is no recordable reduction in the signal for SYBR-14 positive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◂**Fig. 2** Confocal microscopic images showing binding of $PAP_{248–286}$ amyloids to sperm cells. Images **a1**–**a4** represents the buffer washed sperms (negative control). Images **b1**–**b4** showing sperms treated with the freshly dissolved peptide. **c1**–**c4**- and **e1**–**e4**-highlighting sperm bound amyloids (showed in red); prepared in buffer and seminal fluid, respectively. Images **d1**–**d4** represents respective control for amyloids grown in seminal fluid. Along with Proteostat staining, the samples were stained with a nuclear stain Hoechst 33342 (showed in blue). The poor Proteostat signal indicates endogenous semen-derived amyloids. Scale bar corresponds to 10 µm

events at the lowest concentration of $PAP_{248–286}$ aggregates (i.e., 3.5 µM). In the presence of 35 µM of $PAP_{248–286}$ aggregates, which is also the physiological concentration of the peptide in human semen (Roan et al. 2017), the viability was reduced to ≈ 85%. However, the same concentration of $PAP_{248–286}$ aggregates grown in diluted seminal fluid did not show a significant toxic effect (Fig. 4c). The sperm viability was further reduced to ≈ 50% in the presence of 350 µM of $PAP_{248–286}$ aggregates grown in buffer, and surprisingly, the un-aggregated $PAP_{248–286}$ was also found to be toxic at this concentration. This could be due to the fact that at this concentration, the peptide forms amyloid to some extent during co-incubation with sperms leading to sperm toxicity. On the other hand, the $PAP_{248–286}$ aggregates grown in diluted seminal fluid were found to be relatively less toxic and the viability was found to be ≈ 75%. 350 µM of $PAP_{248–286}$ peptide is non-physiological, but its overexpression has been observed in pathophysiological conditions (see above) and results in negative outcomes in terms of sperm function. Overall, the data establish the principle that a toxic effect can be shown for $PAP_{248–286}$ aggregates, and this sperm toxicity can be explained on the basis of amyloid theory which suggests that a given amyloid sample consists of structurally and functionally heterogeneous populations of aggregates including intermediate species, i.e., oligomers and protofibrils—along with the mature fibrillar structures (Tanaka and Komi 2015). At higher concentration, the probability of occurrence of toxic intermediate species would be higher and that could be the reason for the observed sperm toxicity. In addition, at higher concentrations, the number of both $SYBR^+$ and PI^+ spermatozoa decreased and there were more events giving fluorescence below the threshold signal and sorted as debris. In general, pore formation and membrane disruption are the most common mechanism of amyloid-induced cytotoxicity cited (Di Scala et al. 2016; Sciacca et al. 2012). However, the presence of toxic non-fibrillar structures in aggregated $PAP_{248–286}$ solution is yet to be revealed. Likewise, the physical interaction of $PAP_{248–286}$ aggregates with sperm cells could have resulted in membrane perturbation and leakage of macromolecules that further lead to fading of the PI-specific signal.

The toxicity of the $PAP_{248–286}$ aggregates prepared in buffer displayed higher toxicity than the $PAP_{248–286}$ aggregates formed in seminal fluid. The reduced amyloid toxicity could be due to structural variations in the $PAP_{248–286}$ aggregates formed in seminal plasma. Seminal fluid is highly crowded and likely to influence the aggregation of peptide—it is evident that the presence of macromolecular crowders significantly alters the aggregation pathway (Gaharwar et al. 2015). This observation indicates a possible natural strategy, employed to circumvent the toxic effect of $PAP_{248–286}$ aggregates in human semen either via remodelling of fibrils or by driving SEVI formation through off pathway non-toxic oligomer formation (Breydo and Uversky 2015). In addition, as already noted, the effect of other molecular species found in seminal fluid such as heat shock proteins, clusterin, polyanions, and metal ions could be another protective factor.

It was further anticipated that the toxic effect of the $PAP_{248–286}$ aggregates should be directly reflected in sperm motility. To do that, we employed a sperm quality analyser which assesses the sperm motility index (SMI) as a measure of variations in the optical density by moving sperms and estimates the percentage of progressive motility and non-motility of sperms in given samples. The progressive motility refers to sperms showing forward progression in a straight line or in a large circle. In contrast, non-progressive sperms do not make forward progression and swim in a tight circle. The sum of progressive and non-progressive sperm is referred to as the total motility (Elia et al. 2010). As shown in Fig. 5, the sperm motility was found to be considerably affected by the presence of $PAP_{248–286}$. In the presence of 35 µM, $PAP_{248–286}$ aggregates grown in buffer, and in seminal fluid, the motility was reduced to 80%, as 20% of the sperms became non-motile. Further loss of motility occurred with increasing concentration of $PAP_{248–286}$ and in the presence of 350 µM motility was reduced to ≈ 50%. Similarly, a partial reduction (≈ 15%) in sperm motility was observed in the presence of 350 µM freshly dissolved peptide. The pattern of reduction in sperm viability and motility in the presence of both kinds of $PAP_{248–286}$ aggregates was found to be very similar, but interestingly, the aggregates grown in seminal fluid were somewhat more effective in reducing the sperm motility than the aggregates formed in buffer. Again, this may reflect structural differences in the amyloids or other oligomeric species forming in the two separate media, with buffer-incubated peptide forming longer and denser fibrillar aggregates with a smaller diameter and peptide in seminal fluid-forming oligomeric and protofibrillar aggregates. Oligomers or protofibrils would have the capacity to interact with a greater surface area of the spermatozoa and this may be why they have more effect. Our sperm motility analysis data are found to be in agreement with the previous study, where SEM1 amyloids reduced the sperm motility significantly (Roan et al. 2017). It was evident that the semen-derived amyloids could entrap the spermatozoa and thereby decrease their motility. However, this effect was found to be

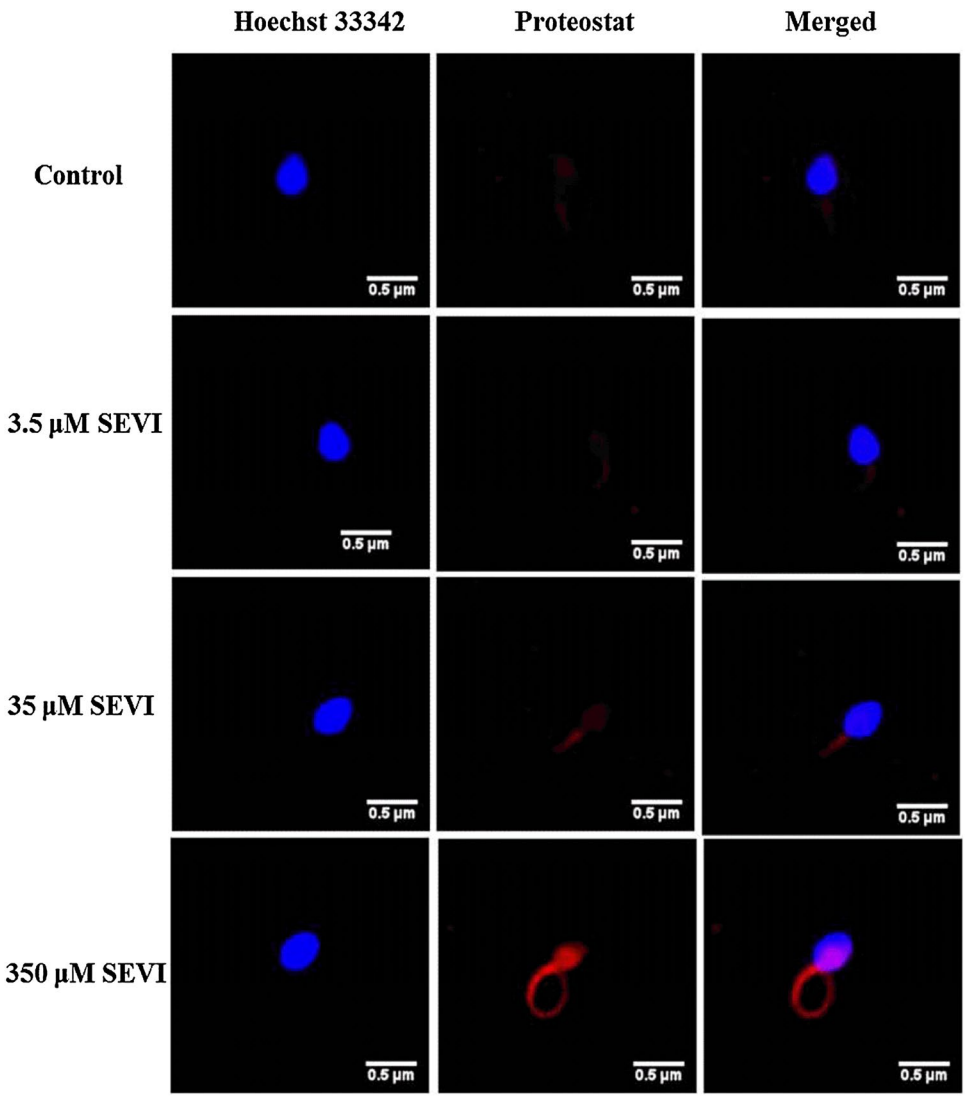

Fig. 3 Concentration-dependent variable sperm-binding affinity of $PAP_{248–286}$ aggregates. The binding affinity of SEVI was assessed at three different concentrations, as stated in the figure (panels 2, 3, and 4 from above). Increasing concentration improves the Proteostat signal and extends the binding region to sperm head. Panel 1 represents the control sample

reversible as amyloid gets degraded during the post ejaculation process, and hence, the motility gets restored (Roan et al. 2014). This observation indicates a physiological mechanism, where amyloids are employed to minimize the sperm motility temporarily by physical confinement to retain them inactive to minimize the energy loss until they are in the female reproductive tract for fertilization. This finding also suggests a pathological condition, wherein the absence of seminal proteases or high level of protease-resistant amyloids could be a potential cause of infertility by reducing the sperm viability or inhibiting sperm–egg fusion by permanent trapping of sperms.

Discussion

Although amyloids are generally considered to be insoluble toxic aggregates of misfolded proteins associated with several conformational disorders, recent evidence suggests that their non-toxic and functional attributes are vital for the survival of many organisms (Chiti and Dobson 2006; Fowler et al. 2007; Hammer et al. 2008). The fundamental basis for the difference between pathogenic and functional amyloids might lie in their conformational polymorphism. Due to the polydisperse nature of an amyloidogenic protein/peptide, it may exist in various conformations, i.e., oligomers, protofibrils, and fibrils with distinct structural and biological activities (Fändrich et al. 2009; Roberts 2016). Recent studies suggested the role of these functional amyloids in various biological activities of reproduction (Hewetson et al. 2017). For instance, sexual reproduction in eukaryotes requires the process of gametogenesis by which cells undergo meiosis to produce haploid germ cells. In yeast (*Saccharomyces cerevisiae*), Rim4 protein forms amyloids and mediates the translational control of cyclin B-type cyclin 3 (CLB3) mRNA during gametogenesis (Berchowitz et al. 2015; Ruggiu et al. 1997). Deleted in AZoospermia-like (DAZL) in mice and

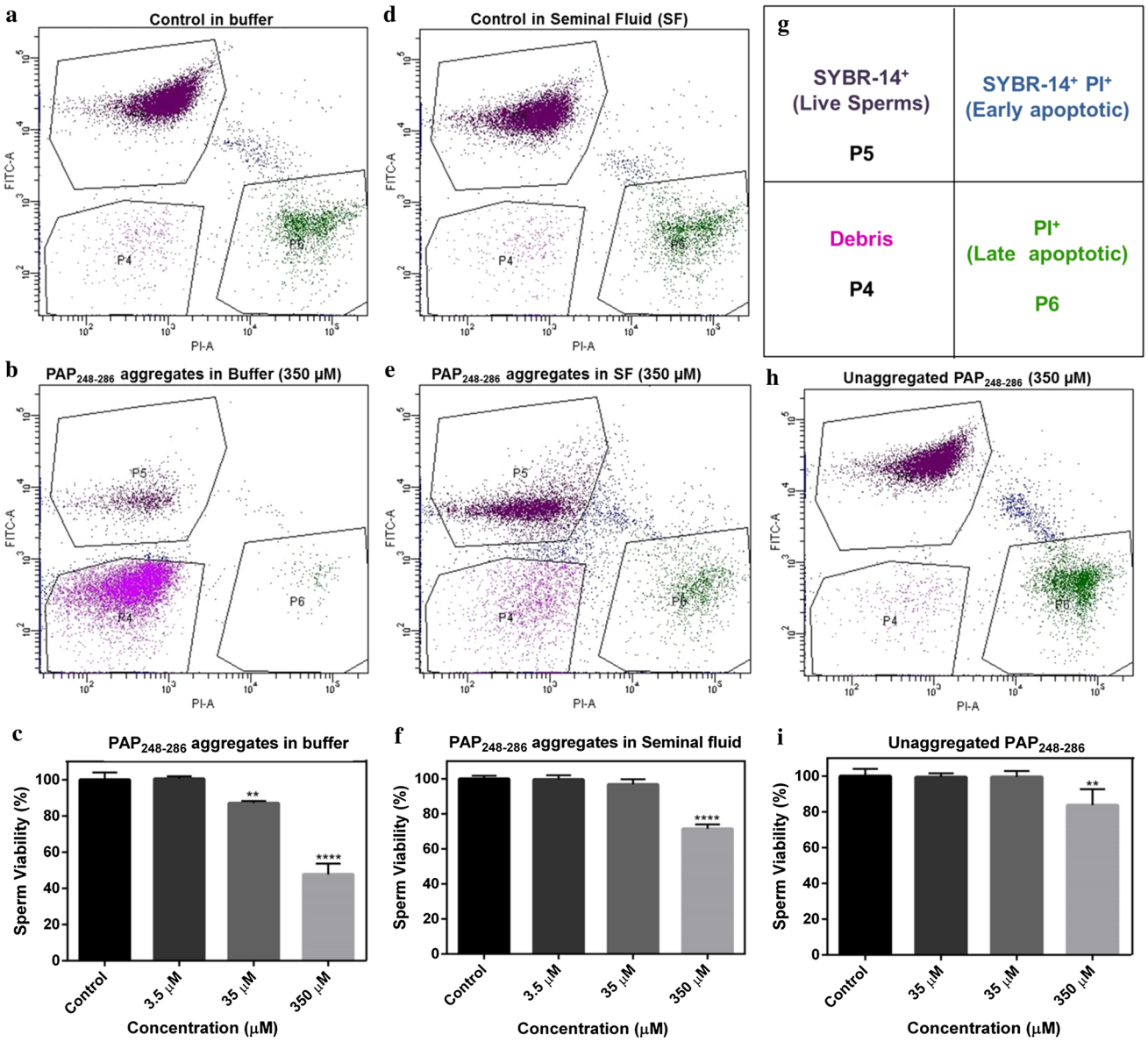

Fig. 4 Assessment of the effect of $PAP_{248–286}$ on sperm viability. FACS analysis of sperm viability in the presence of freshly dissolved $PAP_{248–286,}$ aggregated $PAP_{248–286}$ in buffer and aggregated $PAP_{248–286}$ in seminal fluid. The scatter plots (**a**, **b**, **d**, **e**, **h**) show the relative distribution of viable and dead sperm cells, wherein P4 denotes debris part, P5 corresponds to $SYBR^+$ sperms (live and intact sperms), P6 represents PI^+ cells (dead sperms), and the right unlabelled area includes double positives cells ($SYBR^+$ PI^+). **g** Demonstrates gating strategy and corresponding signal for differentially stained cell populations. The bar graphs in **c**, **f**, **i** show the percent viability of sperms after treatment with different concentrations of the freshly dissolved $PAP_{248–286}$ and $PAP_{248–286}$ aggregates. The data were plotted using Graph Pad prism 6.01 (Graph Pad software Inc., USA). The data were analysed using one-way ANOVA (Tukey's multiple comparisons test). *P* values of < 0.05 were considered statistically significant. The error bars represent ± SD

human meiosis-specific DAZ1–DAZ4 is functionally akin to Rim4 of yeast. Furthermore, amyloidogenic proteins are also studied in germ-line differentiation across several species from drosophila to mouse (Boke et al. 2016; Dosch et al. 2004). The acrosomal reaction, as found in humans and mice, constitutes the major event before the sperm fertilizes the egg, and basically involves release of hyaluronidase, digestion of the egg cumulous and zona pellucida, and fusion of sperm with egg. The proteins of the acrosome matrix (AM) of mice sperm were found to be amyloidogenic in nature. The physiological function of these AM amyloids is thought to relate to the sequential release of acrosome content during the acrosome reaction or to work as a receptor for gamete recognition (Chau and Cornwall 2011; Guyonnet et al. 2014). Similar amyloid-like structures are also found in the egg coat of fish and silk moth and protect those

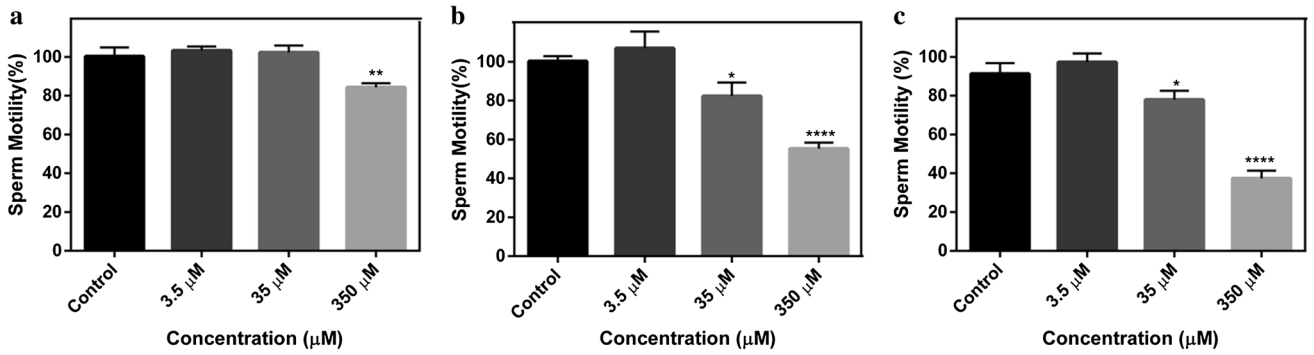

Fig. 5 Assessment of sperm motility in the presence of different concentrations of $PAP_{248–286}$ peptide and aggregates. **a** Data of sperm motility in the presence of monomeric $PAP_{248–286}$. Data **b** and **c** represent sperm motility in the presence of aggregated $PAP_{248–286}$ grown in 1× DPBS and seminal fluid, respectively. The data were plotted using Graph Pad prism 6.01 (Graph Pad software Inc., USA). The data were analyzed by performing one-way ANOVA using Tukey's multiple comparisons test. *P* values of < 0.05 were considered statistically significant. The error bars represent ± SD. The error bars represent ± deviation from the average value during the experiments

oocytes under stress conditions (Aagaard et al. 2006; Greve and Wassarman 1985; Iconomidou et al. 2000; Litscher et al. 2008; Louros et al. 2014; Podrabsky et al. 2001).

Sperm maturation is a process that occurs in the epididymis through which spermatozoa acquire motility and the ability to fertilize an oocyte. The mouse epididymis contains an extracellular amyloid matrix which has been shown to play a role in sperm maturation and protection (Whelly et al. 2012, 2016), suggesting a crucial role of amyloids at different stages of the reproductive process. The recent discovery of amyloids in human semen and its role in the enhancement of HIV infectivity led us to believe that amyloids may have additional functional attributes which may explain the relevance of their existence in seminal fluid. The co-existence of SEVI and sperm in semen asks the question of the functional role, if any, played by SEVI.

Sperm viability and sperm motility are two critical parameters that ensure the overall quality of sperm cells and their effectiveness during fertilization. Therefore, the analysis of sperm motility and viability is the central part of the evaluation of sperm quality and for the diagnosis of male sterility (Kordan et al. 2013; Macleod and Wang 1979; Wang and Swerdloff 2014). As a result, we investigated the effect of variable concentrations of peptide aggregates on sperm viability and motility. The current study suggests that SEVI has a direct role in defining the sperm fitness. We observed that sperm behavior, particularly in terms of motility and viability, was marginally affected at lower concentrations of the peptide aggregate. However, a considerable decrease in sperm motility and noticeable decrement in sperm viability were observed with increasing concentration of the peptide aggregates.

We could also find that the $PAP_{248–286}$ aggregates physically interact with sperm cells in a concentration-dependent manner. At low concentration, $PAP_{248–286}$ aggregate interacts in the mid-piece and tail region, whereas in the presence of higher concentrations, it binds all over the sperm surface. This interaction is likely to interfere with the sperm cell's membrane integrity and other biochemical processes. Since the concentration of the amyloids in human semen is known to vary among individuals (Hartjen et al. 2012; Kim et al. 2010) as well as in pathophysiological conditions mentioned previously, it is seems like that the higher concentration of the aggregates in physiological contexts leads to toxic effects on sperms. Furthermore, the kinds of tests of sperm viability preformed in vitro are relatively crude and may lack the sensitivity to detect some pathological effects of lower SEVI concentrations in vivo. Spermatogenesis is a continuous process and semen ejaculated at a given time might consist of a heterogeneous population of sperms with different ages and lifespan. We suggest that $PAP_{248–286}$ aggregates in semen influence the fitness of sperm in a concentration-dependent manner. Fertilization of an ovum with a sperm is a very well regulated process and it is the robust and active sperm that are expected to penetrate the egg shell for fertilization, in a process of natural selection. The mechanisms through which the physiologically active sperms are selected from a pool of millions of sperm cells include acidic vaginal pH mucosal immune response, and physical barriers such as cervical mucus (Barros et al. 1984; Suarez and Pacey 2006). The presence of amyloids in human semen may then act as another potential barrier that allows the selection of healthy sperms.

In the present study, we found that the presence of semen amyloids has a considerable effect on sperm viability and motility in the presence of a high concentration of $PAP_{248–286}$ aggregates. It may remain predominantly as a fibril and work non-specifically towards impeding sperm motility irrespective of their biological status. This observation, which we make for amyloid formed in seminal fluid and in buffer, is

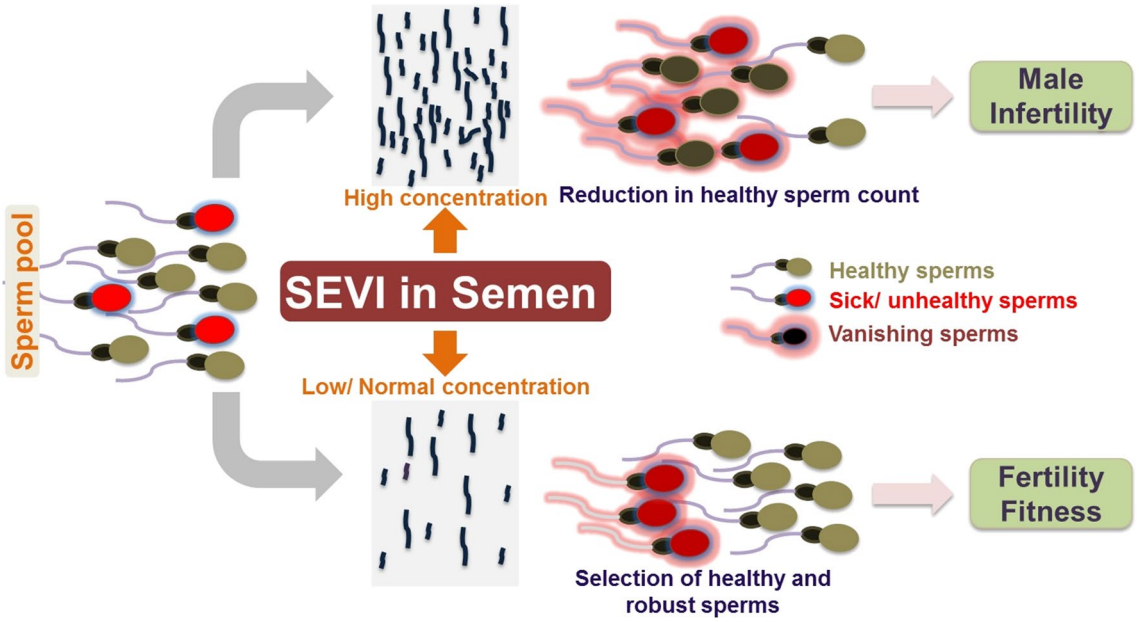

Fig. 6 Schematic representations of concentration-dependent differential activity of SEVI and regulation of sperm count in human semen. At low or normal concentration, SEVI selectively binds to unhealthy and physiologically weak sperms and drive them for clearance from the sperm pool and thereby works as natural barrier which provides most robust and fit sperms for fertilization. However, at high concentration, due to various physiological conditions, SEVI exhibits sperm toxicity irrespective of the health status of the sperm. Hence, the high concentration of SEVI may reduce the sperm count as well as quality and affect the incidence of fertilization

in agreement with the findings of Roan et al. (2017), where it was shown that semen amyloids help in the trapping and clearance of biologically unfit sperm cells (Roan et al. 2017). On the other hand, we find that for amyloid formed in buffer, including at physiological concentrations of 35 µM, there is a direct toxic effect on sperm cells.

Kim et al. (2010) reported that semen samples with elevated levels of amyloids enhance HIV infection by several folds. Such individuals are referred as super-spreaders of HIV (Kim et al. 2010). If as we observe that elevated levels of semen amyloids negatively influence the sperm quality/viability, then it will be interesting to investigate whether high amyloid load has any impact on their fertility. Considering the data altogether we propose a model (Fig. 6), to suggest that semen amyloids play an essential functional role during elimination of physiologically weak sperms and render the healthy sperms available in abundance to enable reproductive fitness. At the same time, we also suggest that high concentration of amyloids in semen might be detrimental for the sperm cell health in some individuals and be responsible for male sterility. However, differential levels of amyloids in fertile and infertile semen samples must be evaluated to validate the finding which is beyond the objectives of the current work. Hence, the current study provides a rationale which suggests that seminal amyloids may act as one of the potential biomarkers for the assessment of semen quality and diagnosing male infertility.

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Compliance with ethical standards

Conflict of interest The authors do not have any conflict of interest to declare.

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