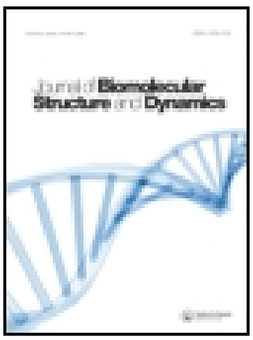

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Characterization of human prostasomes protein Clusterin (macromolecule) – a novel biomarker for male infertility diagnosis and prognosis

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ABSTRACT

Prostasomes, a secretory particle from prostate gland in human seminal fluid plays a role in enhancing the fertility and its absence or less presence will lead to male infertility. Very few fertility associated proteins were detected in prostasomes. In order to isolate the prostasomes from ejaculated semen, the characterization was done using biochemical, molecular and *in silico* methods. The objective of current work on prostasomes is to identify a novel protein biomarker for the diagnosis and prognosis of male infertility. Semen samples were collected, primary semen analysis was done and prostasomes were isolated from ejaculated semen of fertile and infertile categories. Biochemical parameters like protein content, total antioxidant capacity, cholesterol content were evaluated in prostasomes. The critical expressed protein was identified by using SDS page and MALDI TOF techniques. Isolated particle from the semen samples was confirmed as prostasomes from SEM analysis and particle size analyzer. In MALDI results, the maximum hit was obtained against Clusterin that been reported to be involved in capacitation and motility of sperm. Structural modeling and molecular dynamics were carried out on Clusterin and elucidate the structural insights in the dynamic system. Overall, our study reported the novel biomarker Clusterin in prostasomes for diagnosis and prognosis of male infertility.

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KEYWORDS
Male infertility; prostasomes; Clusterin; threading; molecular dynamics simulation

1. Introduction

The release of extracellular vesicles by various accessory sex glands into the surrounding environment has been the subject of increasing interest in the past few years, which in turn made a path to create the scientific committee in 2012 called international society for extracellular vesicles. Realistic evidence exists that vesicles allow the exchange of complex information. Prostasomes are one sub-type of secreted vesicles. Most of the research in the field of prostasomes and human reproduction is currently going in Department of Medical Sciences, Uppsala University, Sweden. Sperm motility was the major parameter that plays a major role in the fertility of human (Kudavidanage et al., 2020). The decrease in the number of motile sperms always tends to reduce the fertility rate. Also, it has been proved that prostasomes play a major role in inducing the forward motility to the sperm (Ronquist, 2015). Sperm forward motility and curvilinear motility was very important key factor because sperm has to travel in the cervical mucus and has to penetrate zona pellucida (Ronquist, 2015). Inclusion of prostasomes in the cryoprotective media as well as in the swim up and swim down media, it tends to increase the number of motile and forward progression sperms after frozen and thaw. Immunosuppressive mechanism in the female genital tract is the important mechanism where lot of work has to be carried out. Prostasomes found to have the capacity to adhere with spermatozoa soon after ejaculation. This mechanism was found to be important in playing the immunosuppressive role in the female genital tract. They were found to be inhibitors of lymphocyte proliferation and phagocytic activity (Tarazona et al., 2011).

The key finding from University of Extremadura, Immunology unit, Spain concluded that prostasomes does not pass the cervix barrier and does not enter the uterus; response in the lower reproductive tract (vaginal mucosa, cervix and ectocervix) (Tarazona et al., 2011). Future studies should aim to determine the role of specific epididymal or accessory gland components in the maturation and activation of sperm and to clarify the role of secreted vesicles such as epididymosomes and prostasomes on sperm maturation and function (Hernandez-Silva & Chirinos, 2019). Prostasomes protein peptides were found to have antibacterial activity. Many researchers proved that in the presence of the prostasomes the growth of the bacterium was inhibiting in a dose dependent manner. These show clearly the role of prostasomes in the antibacterial activity in the female reproductive tract (Kumar et al., 2018).

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It has been reported that more than 60 nm of prostasomes were recruited by the live sperm cells under neutral or slightly alkaline pH not more than 7.5; these processes were done only when the sperm cells were in capacitation induction conditions. Authors further states that the prostasomes are recruited by live sperm cells in response to early capacitation events in the uterus and stay attached to those sperm cells until the final approach to the oocyte–cumulus complex at which point they may fuse to transfer molecular components that facilitate hyper motility and the acrosome reaction (Aalberts et al., 2013; Holt & Fazeli, 2010; Mannowetz et al., 2011; Park et al., 2011).

Lipopolysaccharide binding protein (LBP) which is produced in the epididymis and the functions were associated with spermatozoa and prostasomes (Malm et al., 2005). The fusion of prostasomes with spermatozoa is also a way for LBP and other prostasomes associated antimicrobial proteins, like secretolytin to reach the uterus. At the outside of cervix, where the spermatozoa are deposited, the environment is very rich in a number of bacteria, both Gram-positive and Gram-negative.

Extracellular vesicles were in general found to be the novel mediators of cell-cell communication (Vickram et al., 2020). Being prostasomes is an extracellular vesicle is also an emerging target for the cancer therapy (Prostate cancer). Certainly, much is still to be established regarding the prostasomes and its relationship to prostate cancer development and progression. Clusterin protein presence in prostasomes helps in proper capacitation and motility. As the literature shows many lagging research points in the prostasomes, this research focuses on the identification and characterization of fertility associated protein in prostasomes that helps in the diagnosis of human male infertility. This research focuses proper isolation of prostasomes from the ejaculated semen of different infertile and normospermia categories. Also, the biochemical and molecular characterization of prostasomes will be the focus of this article. After identification of the fertility associated protein, this research also focuses on to characterize the protein by *in silico* methods.

2. Materials and methods

2.1. Semen sample collection

All the semen samples for this research were collected from the Milan Fertility center, Bangalore, Karnataka. The semen samples were collected from the patients who came for Andrology department at Milann fertility center, males were asked for the abstinence time (it should be minimum 4 days and maximum 7 days) and then noted. The patients were asked to collect the semen samples through masturbation in a neat, sterilized, toxic free wide mounted plastic container at the sample collection room, andrology department. Immediately, after collection the secret code, age and place of the patient was noted on the container. The samples were allowed for liquefaction and the time was noted.

2.1.1. Semen parameter evaluation

The semen samples were then processed for semen parameter evaluation including sperm count, sperm motility and morphology by using computer assisted semen analysis (CASA) instrument (Peiris & Moore, 2001). The values for each parameter was noted down and compared with standard semen parameter values set by World Health Organization values. Based on these values, semen samples were categorized into various infertile category and normospermia.

2.1.2. Institutional review board approval

All the semen samples have been collected with patients consent. Also, we explained to each of participants about the purpose of collection in layman language and proceeded further. The human ethical clearance for handling semen samples has also been approved by IRB (institutional review board for ethics).

2.1.3. Semen samples inclusion criteria

Males leading very normal life with regular unprotected sex after marriage were included in the study. Samples were collected and immediately processed for the semen parameter analysis. The patient's history also recorded.

2.1.4. Semen samples exclusion criteria

Male's semen sample with any sexually transmitted infections, particularly human immune deficiency virus, and other sort of major infection that could cause hurdle to others were excluded from the study. Males with obstructive azoospermia also excluded from this study as we could not access any interpretations with those samples. The patients with already diagnosed as prostate cancer also excluded from this study, as we correlates only protein level and no way involved with oncology. A separate study is going on with prostasomes and oncology.

2.1.5. Statistical software

Mean and standard error of mean and other statistics needed for this research were evaluated with the help of graphpad prism version 5.01. Image J software was used to analyze the intensity of bands in SDS PAGE.

2.2. Prostasomes isolation

All the procedures hereafter from seminal plasma isolation to prostasomes isolation were carried out between the temperature ranges from 0 to 4 °C. Semen samples were centrifuged for 10 min at 1000*g* (at 4 °C) to recover spermatozoa from seminal plasma. The cell debris and others were also removed by centrifuging at 10,000*g* for 20 min at 4 °C. The remaining seminal plasma was ultracentrifuged at 1,05,000*g* for 2 h in order to pellet the prostasomes. The isolated amorphous prostasomes has to be purified for further research (Eswaran et al., 2018).

2.2.1. Prostasomes purification

For this, a resuspension isotonic TRIS-HCL at pH 7.6 was prepared and then the prostasomes were resuspended. For further purification of prostasomes, Sephadex column G-200 was used with equilibration buffer as TRIS-HCL in order to separate the prostasomes from other particles present in it. The eluant in the column chromatography was the equilibration buffer TRIS-HCL and the fractions were collected with a regular interval of 1 min as time and fractions were collected for 50 min. Each fraction was monitored with the UV spectrophotometer at 280 nm. Fractions with highest peaks were selected and checked for the presence of purified prostasomes by aminopeptidase N/CD13 activity. This is believed to be perfect marker for exhibiting the presence of prostasomes (Eswaran et al., 2018).

2.3. Instrumentation

2.3.1. Size (dynamic light scattering [DLS]) and stability analysis (zeta analysis)

The isolated prostasomes was analyzed through nanoparticles analyzer–DLS which works obeying the principles of light scattering (HORIBA SZ-100) to determine the average size. The operating conditions for DLS were set as follows; temperature at 25 °C and scattering angle of 90°. In order to estimate the stability and integrity of the prostasomes zeta analysis was carried out. The experiments were repeated twice to check the reproducibility and consistency in the obtained results.

2.3.2. Morphological studies: scanning electron microscopy

Scanning electron microscope (SEM) analysis results in the morphological characterization of the prostasomes. The sample preparation is done by dropping the prostasomes on a glass coverslip, allowing it to dry overnight at 37 °C. Moisture free sample were then gold coated under closed conditions and were analyzed through SEM (Carl Zeiss microscopy Ltd, UK).

2.3.3. Energy dispersive X-ray analysis

The elemental composition making the sample and their pattern were studied by performing EDX i.e. energy dispersive X-ray analysis (OXFORD). The EDX values were obtained for the same sample which was used for SEM analysis.

2.4. Biochemical assays

Total protein estimation by Lowry's method in purified prostasomes: Once the prostasomes were purified by the above said protocol, then the total protein present in the prostasomes were evaluated by using standard Lowry's protocol 1951. For the better understanding also, the protein present in seminal plasma, debris, spermatozoa were also evaluated for each sample separately and average was calculated.

2.4.1. Total antioxidant capacity

Total Antioxidant Capacity for each semen samples in their seminal plasma was evaluated by using Assay Kit (ab65329) purchased for ABCAM Pvt. Ltd. The standard protocol was followed. Antioxidant evaluated according to the uric acid equivalent as per the kit protocol.

2.4.2. Total cholesterol estimation in purified prostasomes

Total cholesterol content in the purified prostasomes was evaluated by using Cholesterol Assay Kit (ab133116) which was purchased from Abcam Pvt. Ltd. The standard protocol mentioned with the kit was followed for the calculations.

2.5. Molecular Assays

2.5.1. SDS PAGE analysis

All the samples used for biochemical assays were also used to identify the fertility-associated protein in prostasomes; prostasomes in its purified form isolated from different semen samples' categories was subjected to SDS PAGE analysis. The CBB stating protocol was used to stain the gel. The protein band which was differentially or less expressed in different infertile categories was subjected to MALDI TOF MS analysis and then Mascot search for identification of protein. The intensity of the band was accessed through image J software.

2.5.2. MALDI TOF analysis

Based on intensity level seen with protein bands from SDS PAGE gel was excised and dehydrated with 40% 50 mM ammonium bicarbonate and 50% acetonitrile. Trpsin digestion standard protocol was used. Voyager-DE STR instrument in linear or reflectron mode was used to acquire MALDI TOF MS spectra. 2,5-Dihydroxybenzoic acid matrix was used throughout the procedure.

2.5.3. Mascot search

The *m/z* ion values were searched in mascot MALDI TOF MS ions search, database as SwissProt, taxonomy as humans and enzyme as trypsin.

2.5.4. Identification of protein

Based on the maximum hits in mascot search, the protein was identified and its function was retrieved from UNIPROT database.

2.6. In silico analysis

The protein sequence of human Clusterin (449 amino acids) was retrieved from UniProt database (Uniprot id: P10909). *In silico* modeling of human Clusterin was carried out using I-Tasser server based on the threading or folds recognition method. The quality of the Clusterin 3D structural model was verified with quality index in ProSA server (Wiederstein & Sippl, 2007). Structural refinement was done by construction of Ramachandran plot in Rampage.

Table 1. Comparison of semen parameters between different categories.

Semen category	Volume (ml)	pH	Sperm concentration (millions/ml)	Total motility (%)	Rapid progressive motility (%)	Normal morphology (%)
Oligoasthenospermia ($N = 35$)	2.8 ± 0.7	7.8 ± 0.1	4.7 ± 0.5	5.2 ± 1.5	2.01 ± 0.5	17.3 ± 2.2
Asthenospermia ($N = 35$)	2.2 ± 0.3	7.8 ± 0.0	28.7 ± 4.1	08.2 ± 1.9	4.5 ± 1.5	12.5 ± 1.2
Azoospermia ($N = 10$)	2.1 ± 0.2	7.7 ± 0.1	Nil	Nil	Nil	Nil
Normospermia ($N = 35$)	3.2 ± 0.6	7.7 ± 0.2	85.6 ± 7.2	42.32 ± 10.3	28.5 ± 5.5	22.2 ± 3.8
Oligospermia ($N = 35$)	2.8 ± 0.4	7.7 ± 0.1	7.2 ± 0.8	19.8 ± 2.5	20.4 ± 5.6	22.5 ± 3.1
Control ($N = 10$)	3.5 ± 0.8	7.7 ± 0.0	89.5 ± 15.2	48.6 ± 6.7	31.0 ± 4.8	40.4 ± 4.5

All the values expressed as mean ± standard error of mean

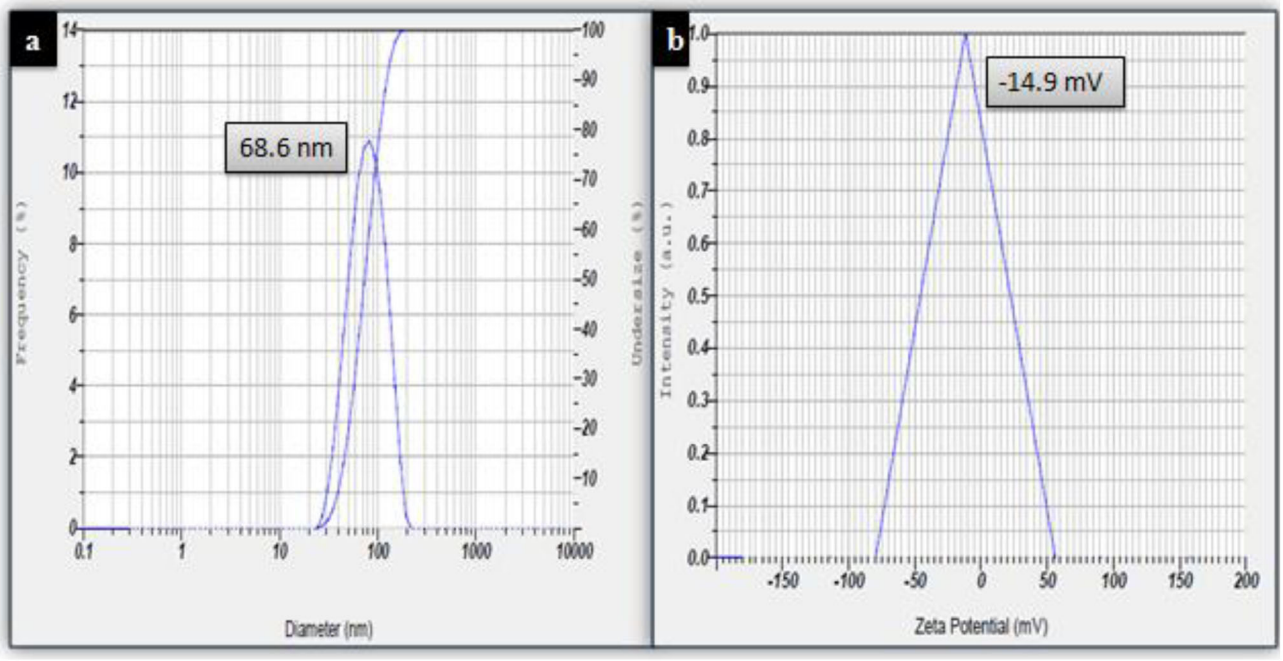

Figure 1. (a) DLS analysis for the isolated prostasomes, the DLS gave a wide peak from 20 to 110 nm with an average size represented as *Z* average of the prostasomes as 68.6 nm and the polydispersity index was 0.168; (b) Zeta potential analysis of the prostasomes from seminal sample for stability determination, the surface charge on the prostasomes reflects the stability which is measured through frequency difference of the laser beam. In this case, the prostasomes exhibited normal stability as the zeta potential value was 14.9 mV.

2.6.1. System preparation

Molecular dynamics (MD) simulations study on human Clusterin was carried out using GROMACS 4.5 package (Berendsen et al., 1995). Simple point charge (SPC21) water molecules of 0.9 nm were used for solvation of protein models in simulation box. The neutralization of the system was obtained by adding six sodium ions to replace initial SPC water molecule in all directions. Energy minimization of all systems was carried out by steepest descent energy minimization with tolerance limit 100 kJ/mol and GROMOS96 43a1 force field was used for the simulations of protein (Lindahl et al., 2001). A cutoff of 14 Å for van der Waals interactions and 12 Å for electrostatic interactions was used for the process. Electrostatic interactions were computed using the Particle Mesh Ewald method. The LINCS algorithm was used to constrain all bond lengths and the SETTLE algorithm was applied to constrain the geometry of water molecules in the system. The energy minimization was done two equilibration phases, NVT ensemble with constant temperature of 300 K and with a coupling constant of 0.1 ps for duration 100 ps and NPT ensemble with constant pressure of 1 bar was employed with a coupling constant of 5 ps for duration 100 ps. For both ensembles of equilibration, the coupling scheme of Berendsen was employed. Finally, the systems were subjected to production MD simulation for 40 ns run. MD trajectories of human Clusterin was analyzed by GROMACS utilities. The analysis included root mean square deviation (RMSD), root mean square fluctuation (RMSF), solvent accessible surface, radius of gyration (Rg) and number of hydrogens (NH). The stability analysis was performed by using utilities like g_ rms, g_rmsf, g_ sas, g_ gyrate and g_hbond, respectively.

2.6.2. Principal component analysis

Principal component analysis (PCA) or essential dynamics (ED), one of the critical analyses in the MD simulations (Amadei et al., 1993). PCA was more valuable technique in identification overall motion of protein. PCA was derived from reduction of the complexity of the data and extraction of the concerted protein motion during the respective simulations. PCA was performed in two steps (i) construction of variance/covariance matrix based on Cα atoms from MD simulations trajectories with the removal of rotational and translational movements (ii) diagonalization of the covariance matrix. During diagonalization of the covariance matrix, the

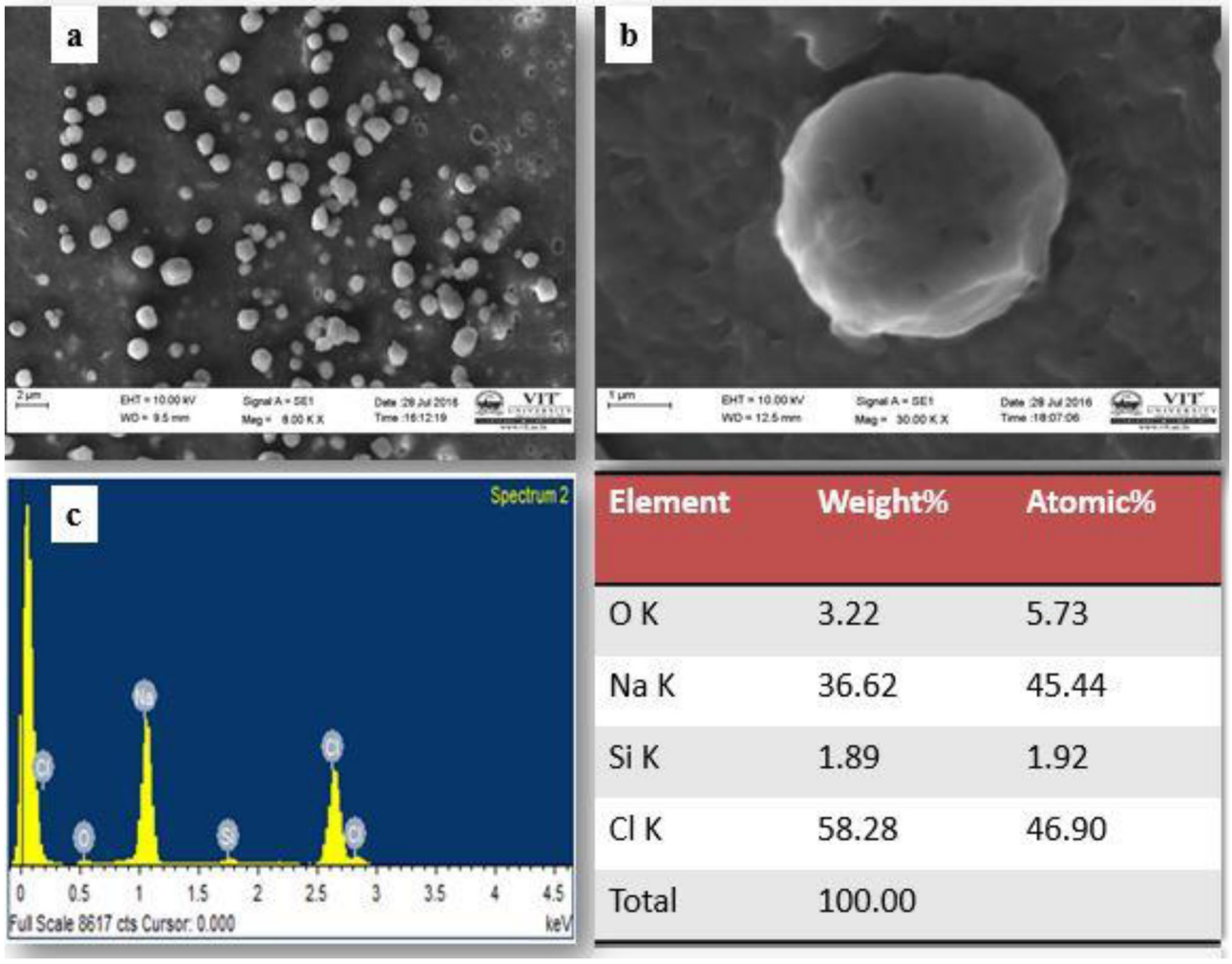

Figure 2. (a and b) Scanning Electron microscopy, the microscopic scanning was performed at magnification of 8000×. The obtained scanning electron microscope results showed bulged spherical like particles which fall under 1 μm; (c) The elementary composition making up the prostasomes which was analyzed by EDX showed Cl2+ and Na2+ as a mixture had higher percentage in the prostasomes composition and Si2+ and O2+ were contributing less percentage.

Table 2. Comparison of concentration of protein in different fractions with different categories of human semen samples.

Category	Spermatozoa (mg/ml)	Prostasomes (mg/ml)	Seminal plasma (mg/ml)
Oligospermia ($N = 35$)	1.82 ± 0.19	0.91 ± 0.08	19.1 ± 0.13
Oligoasthenospermia ($N = 35$)	0.89 ± 0.11	0.82 ± 0.19	21.10 ± 0.9
Asthenospermia ($N = 35$)	4.11 ± 0.18	0.49 ± 0.15	28.01 ± 0.14
Azoospermia ($N = 10$)	0.0	0.08 ± 0.15	12.12 ± 1.59
Normospermia ($N = 35$)	6.72 ± 0.35	0.18 ± 0.24	32.89 ± 1.99
Control ($N = 10$)	5.48 ± 0.48	2.45 ± 0.18	42.11 ± 4.22

All the values expressed as mean ± standard error of mean.

eigenvector and eigenvalues were derived from each trajectory. Eigenvector and eigenvalue described each component motion with direction and energetic contribution of each component to the motion, respectively. PCA was performed in GROMACS using protocol that evaluate the eigenvectors and eigenvalues. First two PCA (eigenvector 1 vs eigenvector 2) was projected in the conformational space for protein motion analysis.

3. Results and discussion

The semen samples collected was categorized based on the semen analysis report done based on the standard protocols designed by world health organization. Important parameters like volume, pH, total sperm concentration, sperm motility and normal morphology were measured. Based on the semen parameter values, categorization were done, oligoasthenospermia ($N = 35$), asthenospermia ($N = 35$), azoospermia ($N = 10$), normospermia (found to be normal as per semen standard values, but do not have child with regular unprotected sex for more than 2 years or so) ($N = 35$), oligospermia ($N = 35$) and control (who recently gave birth to a child) ($N = 10$). The established values for each parameter and category were tabulated (Table 1).

Recently smart phones with previously programmed features were in use for the preparation of semen analysis report like CASA, this could be used from home to know the

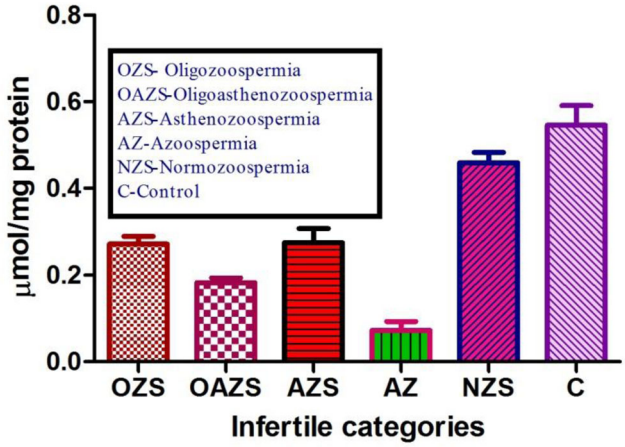

Figure 3. Comparison of cholesterol (µmol/mg of protein) content in different infertile and fertile category prostasomes phase.

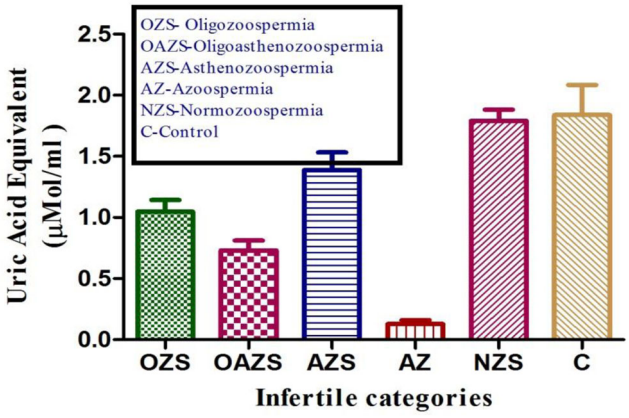

Figure 4. Comparison of antioxidant capacity of various infertile and fertile category prostasomes phase (seminal and spermatozoa free). Also Antioxidant evaluated in terms of uric acid equivalent.

basics of semen parameters (Kricka & Heyner, 2018). Prostasomes were isolated and confirmation was done by SEM. The mean size of the prostasomes was determined by DLS analysis which gives the *Z* average corresponds to the cumulative average and the polydispersity index (PI) represents the distribution width. In regards to this study, the DLS gave a wide peak from 20 to 110 nm with an average size represented as *Z* average of the prostasomes as 68.6 nm and the PI was 0.168 (Figure 1(a)). Ronquist also confirms the presence of isolated prostasomes by using SEM and DLS methods as well attached with EDX (Ronquist et al., 2016). The size range was in expected range. The zeta potential is the result of dynamic electrophoretic mobility of the colloidal suspension. The surface charge on the prostasomes reflects the stability which is measured through frequency difference of the laser beam. In this case, the prostasomes exhibited normal stability as the zeta potential value was 14.9 mV (Figure 1(b)). The stability results determine that the prostasomes falls under strict normal morphology (Chan et al., 2006). The fall in the zeta potential is after capacitation. The release of gp20-CD52 happens as the time is delayed after isolation of prostasomes which makes the zeta potential value less negative (Giovampaola et al., 2001).

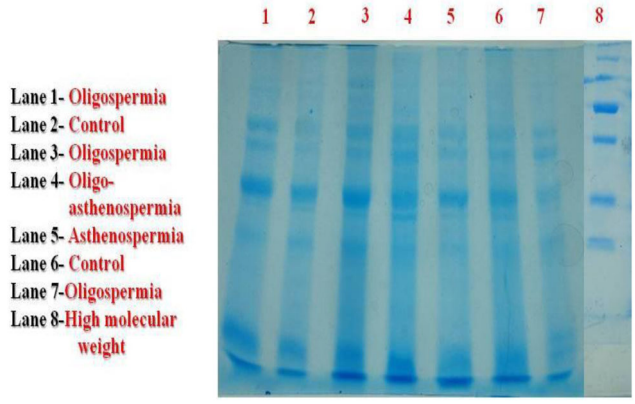

Figure 5. Comparison of protein profile for various infertile and fertile category prostasomes. More than 5 to 6 bands were expressed in all categories, but the band with 52 kDa proteins was found with very less expressed or the intensity of the band was very low. Image lab software was used to evaluate the intensity-based separation or measurement of protein in prostasomes in different categories. The loaded samples from Lanes 1 to 8 were mentioned in the figure.

The morphology of the isolated prostasome was analyzed by SEM and the elemental composition making up the prostasomes were obtained by EDX. Prostasomes are very rich in cholesterol and are highly responsible for sperm motility. When the elemental composition is affected, the function of the prostasomes which is protein transfer to specific sperm cell membrane domain will be affected (Saez et al., 2003). The microscopic scanning was performed at magnification of 8000×. The obtained SEM results showed bulged spherical like particles which fall under 1 µm (Figure 2(a,b)). This clearly indicates that the DLS results are validated and the sizes of the particles were under 100 nm showing normal stability. The elementary composition making up the prostasomes which was analyzed by EDX showed Cl^{2+} and Na^{2+} as a mixture had higher percentage in the prostasomes composition and Si^{2+} and O^{2+} were contributing less percentage (Figure 2(c)). These elements helps in the retaining the fertilization capacity of the sperm.

Once the presence of isolated prostasomes were confirmed with the help of SEM, the next step was to check for the protein concentration in different phases of semen like seminal plasma, spermatozoa and prostasomes. The protein content was checked with the help of standard protocol designed by Lowry's method, 1951. The protein content in each phase was tabulated (Table 2). It was found that on an average normospermia yields 32 mg of protein in ml for seminal plasma but yields only 6.5 mg in the case of prostasomes phase. This difference is because of prostasomes are rich only in cholesterol and phospholipids contents rather than protein contents. García-Rodríguez also confirms our results, that prostasomes protein content found will be very less when compared to cholesterol content of prostasomes (García et al., 2018). Even in azoospermia case, we are able to isolate 0.08 mg of protein in prostasomes phase.

The same set of samples was used for evaluation of cholesterol content present per mg of protein present in the prostasomes phase. On an average, the control and normospermia samples yield around 0.51 and 0.55 µmol of cholesterol content, respectively. The cholesterol content per mg of protein was calculated for each category samples

Table 3. MASCOT search results for MALDI results.

Variable modification	Fixed modification	Protein	Sequence coverage	Significance score
Carbamidomethyl (C)	Carbamidomethyl (N - term)	Clusterin (human)	81%	79
Carbamidomethyl (C) Carbamyl (K)	Carbamidomethyl (N - term) Carbamyl (N - Term)	Transmembrane serine protease 2	69%	56
Carbamidomethyl (C) Carbamyl (K)	Carbamidomethyl (N - term) Carbamyl (N - Term)	Epididymal protein	55%	52

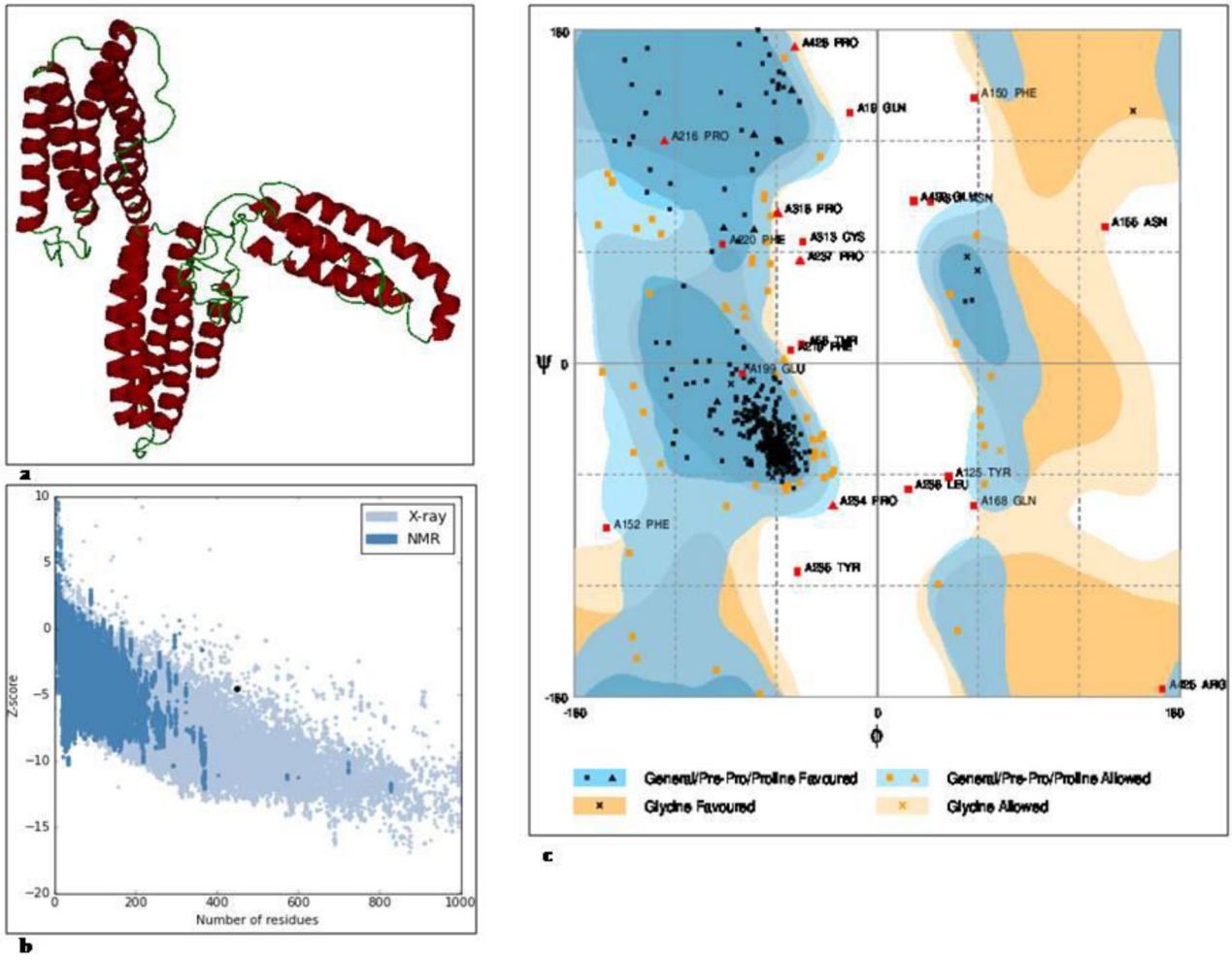

Figure 6. (a) Three-dimensional model of human Clusterin from prostasomes. (b) Quality index of Clusterin model from ProSA server. (c) Ramachandran plot for Clusterin model from Rampage.

prostasomes and content of cholesterol in various categories was showed (Figure 3).

The same set of samples was used to evaluate the total antioxidant capacity by using uric acid as a standard or equivalent. On an average, the total antioxidant capacity of prostasomes in control and normospermia was found to be 1.8 and 1.9 µMol/ml, respectively. When compared to oligospermia and asthenospermia, asthenospermia found with more antioxidant capacity (Figure 4). The functions of cholesterol in prostasomes were well studied and established (Pfrieger & Vitale, 2018).

The same set of samples was used to identify the fertility-associated protein in prostasomes (Clusterin); prostasomes isolated from different semen samples' categories were subjected to SDS PAGE analysis. Further, the protein band which was differentially expressed in different infertile categories was subjected to MALDI TOF MS analysis and then Mascot search for identification of protein. More than five to six bands were expressed in all categories, but the band with 52 kDa proteins was found with very less expressed or the intensity of the band was very low (Figure 5). Image lab software was used to evaluate the intensity-based separation or measurement of protein in prostasomes in different categories.

The differentially seen band from SDS PAGE gel with 52 kDa was excised and dehydrated with 50% 50 mM ammonium bicarbonate and 50% acetonitrile. The gel was rehydrated with 250 ng trypsin solution for 1 h. at 4 °C. After removing excess trypsin, 150 µL of 50 mM ammonium bicarbonate buffer was added and incubated at 37 °C overnight. Voyager-DE STR instrument in linear or reflectron mode was used to acquire MALDI TOF MS spectra. 2,5-Dihydroxybenzoic acid matrix was used throughout the procedure. Positive ions accelerated to 20 V were calculated. Both matrix and sample were dissolved in milliQ water and equal ratios of matrix and sample were mixed

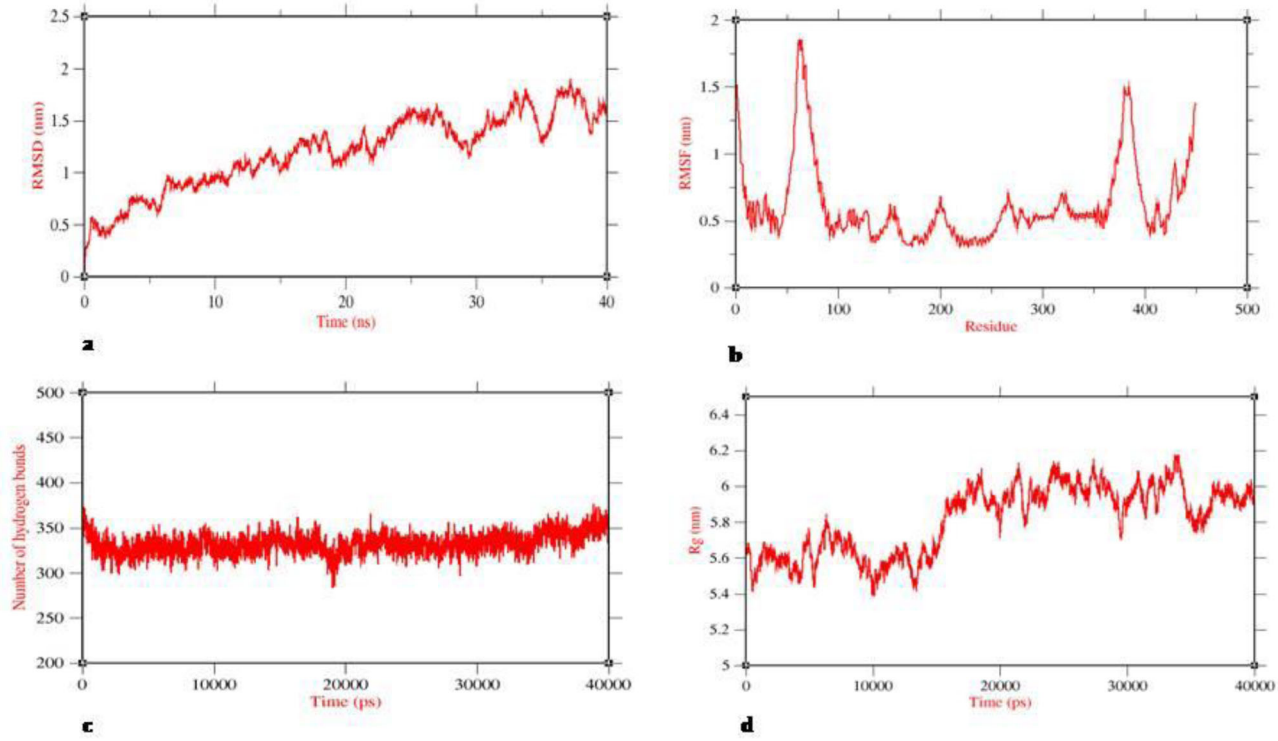

Figure 7. Molecular dynamics simulations of 3D structural model of Human Clusterin. (a) RMSD plot. (b) RMSF plot. (c) Inter-H bonds. (d) Rg.

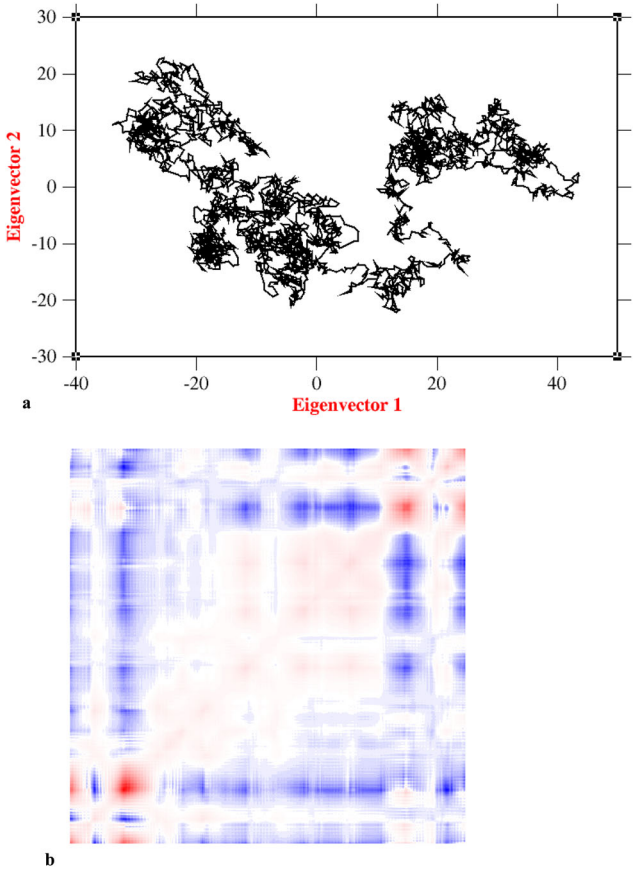

Figure 8. (a) Principal component analysis of human Clusterin. Projection of eigenvector 1 vs. eigenvector 2. (b) Covariance matrix.

and spotted onto MALDI plate for analysis. Tanase studied all the possible ways to prove the full representation of proteins present in human prostasomes by using MALDI TOF and mass spectroscopy (Tanase et al., 2017).

The *m/z* ion values were searched in mascot MALDI TOF MS ions search, database as SwissProt, taxonomy as humans and enzyme as trypsin. The search data for mascot search was tabulated (Table 3). The maximum hit was obtained against the Clusterin, which has functions related to capacitation and motility. The Clusterin protein plays a major role in enhancing the capacitation, but still, it has many functions; there is no structure for the protein. So we have done MD approach to characterize the protein. Based on the MALDI TOF results and in silico approach, Clusterin protein could act as a biomarker for diagnosis of human male infertility especially with motility issues (asthenospermia and oligoasthenospermia).

In order to analyze the structural insights on human Clusterin from prostasomes related to male infertility, 3D structure prediction was most critical in the study. Due to the absence of experimental 3D structure, human Clusterin was modeled using I-Tasser server based on *C* score of −1.49 and TM-score $= 0.53 \pm 0.15$ using 5LM1 (Crystal Structure of HD-PTP phosphatase in complex with UBAP1). The model contains 12 helices and connecting loops (Figure 6(a)). The model was verified with *Z*-score of −4.58 in ProSA server and confirmed the model accuracy was near to experimental structures (Figure 6(b)). Ramachandran plot showed 81% in favored regions, 14.3% allowed regions and few residues in disallowed regions (Figure 6(c)). Ramachandran plot was generated for the 3D structural model of Clusterin in Rampage. Based on the phi and psi angles, the amino acids residues occupy in various regions of the plot. More residues in the favored regions confirmed the protein model was stable and glycine/proline occupied in all regions of Ramachandran plot.

The convergence of protein system during simulations was measured by RMSD of all Cα atoms from the initial structure. The initial equilibration of native structure was done in 5 ns. After equilibration phase, the structure showed RMSD range in 1–1.5 nm during the course of 40 ns simulations (Figure 7(a)). The structure was well-converged and confirmed the protein stability of Clusterin at end of simulations and structure with stable trajectory in the dynamic system. The dynamics behavior of residues due to mutation was determined by RMSF deals with flexibility of backbone structure. RMSF plot was plotted based on protein residues (Figure 7(b)). The results showed maximum fluctuation range to 1.8 nm and most residues in range of 0.5 nm. The residue range 50–75 and 355–390 showed maximum fluctuation due to the existence of loops. RMSF plot confirmed the Clusterin structure with minimal flexibility and secondary structure elements was proper with minimal flexibility. Intramolecular NH bond was critical in the stability of protein. NH bond was calculated for human Clusterin in dynamic system (Figure 7(c)). The native structure showed maximum NH bonds 300–350 confirmed the rigid nature during the course of 40 ns simulations. Rg was property of overall dimension of protein during simulations. Rg termed as a measure of mass weighted root mean square distance of all atoms from center of mass. Rg of native structure started with 5.6 nm but gradually increase to equilibrate with 6 nm (Figure 7(d)). Thus, the overall protein folding pattern of human Clusterin protein was observed.

PCA or ED was investigated to study the large-scale motion of native Clusterin in phase space of 40 ns simulation. Diagonalization of covariance matrix for all Cα atoms showed the overall flexibility of protein. The eigenvalue of native structure was 632.062 nm^2. The projection of PCA1 versus PCA2 of first two eigenvectors of native Clusterin structure showed the motion (Figure 8(a)). The corresponding covariance matrix deciphered the motion of Cα atoms around the average position and red color represent to highly coordinated motion of the atom pair along the same direction, blue color represents a negative correlation motion in opposite directions (Figure 8(b)). The cluster pattern of Clusterin protein showed the overall motion and rigid nature with defined cluster.

4. Conclusion

The current research on human male infertility majorly focused on the isolation of the prostasomes from the semen by standard centrifugation methods. The confirmation of the prostasomes presence was done using advance techniques like DLS and SEM. The cholesterol level and antioxidant capacity was found as critical biochemical parameters in order to evaluate the quality and quantity of prostasomes. The critical finding was an average normospermia yields 32 mg of protein in ml for seminal plasma but yields only 6.5 mg in the case of prostasomes phase. Also, the presence of fertility associated proteins in prostasomes of 52 kDa protein namely Clusterin was identified and reported to play a key role in capacitation process and motility related parameters in boost up of male fertility or enhancement. The first 3D structure of Human Clusterin was predicted using *in silico* approach and used for further functional annotation. In addition, all atom MD simulations on Clusterin model evaluated the native structural behavior and stability of the protein in the dynamic system. In conclusion, the combined *in vitro* and *in silico* approaches shed the novel biomarker identification in the prostasomes and the presence or intensity of Clusterin can act as proper diagnosis method of Human male infertility.

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Author contributions

ASV designed, performed, supervised the *in vitro* section and wrote the manuscript. AK designed, performed *in silico* section and wrote the manuscript. GG, TS, NR and JP compiled the data, reviewed and wrote the manuscript.

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