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Analysis and Comparison of Human Prostasomes Amino Acid Content Variation in Normal Men and Infertile Men-A Clinical Relevance Study for Effective Diagnosis Method for Human Male Infertility

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Abstract

Aim: *The main objective of this study is to compare and analyse human prostasomes amino acid content variation in normal men semen samples and infertile men semen samples for identification of clinical relevance.* ***Materials and methods:*** *Semen samples were collected from normal men (N=32) and from infertile men (N=32) and by following the standard world health organisation protocol semen analysis was done. Amino acid quantification was done by using amino acid analyzer. Prostasomes were separated from spermatozoa and seminal plasma by using centrifugation technique at 95000 RPM for 90 mins.* ***Results:*** *Independent sample T-test was carried out and shows that proline and alanine amino acids concentration ($p<0.01$) statistically significant compared with fertile men and infertile men. High concentration of amino acids in prostasomes were found in fertile men samples (18.09 ± 0.20 µmoles/L) when compared with infertile men samples (15.12± 0.37 µmoles/L).* ***Conclusion:*** *Amino acid in prostasomes plays an important role in the fertilization; the change in the concentration of amino acid in prostasomes leads to infertility of men. Here we found that the concentration of amino acids is high in fertile men when compared to infertile men which could act as an innovative diagnosis method for infertility.*

Key-words: Amino Acids, Prostasomes, Amino Acid Analyzer, Infertility, Innovative Methods for Diagnosis, Semen, Centrifugation, Reproductive Medicine.

1. Introduction

This research is about to identify the clinical relevance of amino acid content variation in human prostasomes of fertile and infertile men. Male Infertility rate has been increased about 30% in the past 10 years. Male infertility is due to various reasons that occurs in the semen and its parameters. The importance of this research is to keep evaluating the amino acid contents in prostasomes for diagnosis of human male infertility. Worldwide these types of research become important as diagnosis of male infertility needs more attention (A.S. Vickram, Samad, et al. 2020). Seminal fluid components that present in the semen varies with people due to several conditions like temperature, climate and food habit (A.S. Vickram, Samad, et al. 2020). Prostasomes are the extracellular fluid which have gained a huge attention due to its sufficient cause such as clear access in the semen fluids (György et al. 2011). These prostasomes are termed as the extracellular membrane fluids which usually ranges from 40-5000mm in diameter. They are categorized based on their origin, size, morphology, and its mode of ejection (Witwer et al. 2013). This study results may lead to application in the area of andrology and reproductive medicine.

We looked for the most cited articles in the pubmed and science direct database whereas it ended with 425 articles published in this domain. Amino acid concentration in prostasomes and seminal plasma is one of the important parameters which plays a key role in deciding fertility status of men. The most cited article describes that variation in seminal fluid profile can be representative of genital tract dysfunctions and thus serve as an infertility biomarker (Herwig et al. 2013). In this regard, while exosomes isolated from seminal plasma of asthenospermia and azoospermic patients have similar form, scale, and expression of typical normospermic patients differs from the infertile men whereas 50% of infertility cases is due to content variation in seminal fluid (Candenas and Chianese 2020). Approximately 2/3 of infertile men have a sperm production problem, which includes a low sperm count, poor sperm parameters, and a high non-motile sperm count (Carrell et al. 2016). Infertility is on the rise with 25% of couples attempting but failing to conceive. The male factor is responsible for more than 40% (Sengupta 2015). To make the testing process simple here a novel technique is approached that amino acid concentration variation in prostasomes of normal men and infertile men.

Previously our team has a rich experience in working on various research projects across multiple disciplines (Sathish and Karthick 2020; Varghese, Ramesh, and Veeraiyan 2019; S. R. Samuel, Acharya, and Rao 2020; Venu, Raju, and Subramani 2019; M. S. Samuel et al. 2019; Venu,

Subramani, and Raju 2019; Mehta et al. 2019; Sharma et al. 2019; Malli Sureshbabu et al. 2019; Krishnaswamy et al. 2020; Muthukrishnan et al. 2020; Gheena and Ezhilarasan 2019; Vignesh et al. 2019; Ke et al. 2019; Vijayakumar Jain et al. 2019; Jose, Ajitha, and Subbaiyan 2020). Now the growing trend in this area motivated us to pursue this project.

There is no clinical relevance study for variation of amino acids content in prostasomes of fertile men and infertile men. We had already expertised in this field of research for over a decade. The major aim of this current study is to analyse and compare the prostasomes concentration in the seminal fluid of fertile men and infertile men. The concentration of amino acids in prostasomes is high in fertile men when compared to infertile men.

2. Materials and Methods

This study was conducted at biochemistry lab in saveetha school of engineering. Samples were collected in accordance with the world organization (WHO) standard procedure. Sample size was collected by using previous study results (García-Rodríguez et al. 2018) in clinicalc.com by keeping threshold 0.05 and G power 80%, confidence interval 95% and enrollment ratio as 1. Two different groups were taken for the analysis: one is a fertile men group (N=32) and the other one is infertile men group (N=32). Computer assisted semen analysis (CASA)- german made and amino acid analyser were used in this study for analysis.

The semen samples which are used for this research were obtained from the milan fertility center, bangalore, karnataka. The samples are collected from the people who are in abstinence time (about 4 to 7 days) and then recorded. The samples are collected through a mastrubation process in a clean and intoxic wide mounted plastic container in the sample collection room. The liquefaction of samples are done and time is noted (A.S. Vickram, Anbarasu, et al. 2020) Computer assisted semen analysis is a modern technique which differs from the manual semen analysis by the process of evaluation (Agarwal, Henkel, and Majzoub 2021) The modern CASA systems are designed in a way of measuring quantitatively the several aspects of prostasomes content such as sperm concentration, sperm motility, and its morphology through this CASA technique the semen parameters are identified for both the fertile group and infertile group.

The step-in separation of prostasomes from amino acid is centrifugation. Centrifugation operates on the idea that two particles in suspension (cells, organelles, or molecules) of different masses or densities can settle at different rates to the bottom of a tube (Li and Boix 2021). 800 RPM

separates the sperm cells in 8 minutes under 4°c, 1000 RPM separates the debris in 10 minutes under 4°c and 95,000 RPM for prostasomes in 90 minutes under 4°c.

Statistical Analysis

The statistical comparison of fertile men group and infertile men group was done through SPSS version 21. There are no dependent variables whereas the independent variables are prostasomes and amino acids volume, sperm motility. Analysis was done for mean, standard deviation, independent T-test.

3. Results

Semen analysis was performed and reported in Table 1 for major parameters like volume, pH, sperm concentration, total motility, rapid progressive motility, and normal morphology, all the parameters were shown with normal values as per world health organization in case of normospermia and the values where not compatible with the world health organization in case of all infertile conditions which reflected in Table 1.

Table 1- Represents the Mean ± Standard Error for major Semen Parameters between Fertile Men and Infertile Men. From the Table it can be Observed that the Infertile Conditions such as Oligoasthenospermia, Oligospermia, Azoospermia and Asthenospermia have very Low Volume (ml), low pH, Low Sperm Concentration (millions/ml) when compared to the Normospermia. The Normal Morphology of Infertile Group is very Low with 12.54 % in Oligoasthenospermia whereas in the Fertile Group it was High with 40.3 %

Semen category	Volume (ml)	pH	Sperm concentration (millions/ml)	Total motility (%)	Rapid progressive Motility (%)	Normal morphology (%)
Oligoasthenospermia (N=8)	2.9±0.8	7.7±06.2	4.6±0.4	5.3±1.4	2.02±0.6	17.2±2.3
Asthenospermia (N=8)	2.3±0.2	7.7±0.2	28.8±4.2	08.3±1.8	4.6±1.6	12.54±1.4
Azoospermia (N=8)	2.2±0.3	7.8±0.2	NIL	NIL	NIL	NIL
Normospermia (N=16)	3.3±0.7	7.8±0.3	85.7±7.3	42.31±10.2	28.4±5.4	22.3±3.9
Oligospermia (N=8)	2.6±0.5	7.6±0.2	7.3±0.9	19.9±2.4	20.5±5.7	22.6±3.2
Control (N=16)	3.6±0.9	7.8±0.1	89.4±15.1	48.7±6.6	31.1±4.9	40.3±4.4

Prostasomes content in mg/ml was evaluated for all the infertile conditions and fertile conditions and depicted in Table 2. In Table 2, it was observed that the mean value for prostasomes content was found to be 2.46 in control which was 3 times higher than oligoasthenospermia.

Table 2- Represent the Prostasomes Content (mg/ml) for various Infertile Conditions and Fertile Conditions. From this Table it was observed that Infertile Groups have Low Prostasomes Concentration when Compared with Fertile Men Semen Samples. The Control Group has a High Mean Value with 2.46±0.19 (mg/ml) whereas the Infertile Category Azoospermia have Low mean with 0.09±0.22 (mg/ml)

category	**Prostasomes (mg/ml)**
Oligospermia (N=8)	0.92±0.09
Oligoasthenospermia (N=8)	0.83±0.21
asthenospermia(N=8)	0.51±0.16
azoospermia(N=8)	0.09±0.22
normospermia(N=16)	2.12±0.25
control(N=16)	2.46±0.19

From Table 3, it was observed that the fertile group has high concentration of amino acid except serine when compared to the infertile group. 13 amino acids concentration are identified through the amino acid analyser which have shown a drastic difference in the fertile group and in infertile group that are depicted in Table 3. The mean amino acid content in the fertile group is 18.09 (μ moles/ L) and in infertile category it is about 15.12 (μ moles/ L), this shows the difference in between fertile and infertile categories shown in Table 4. Comparison of amino acid content in prostasomes was done in Table 5 between fertile and infertile men, the independent T test was done and found that amino acids proline and alanine was found with significant difference between fertile and infertile category.

Table 3- Represents the Amino Acids that are Quantified in the Prostasomes in Fertile Semen Samples and in Infertile Semen Samples. From this Table it was Observed that the Fertile Group has High Concentration of Amino Acid except Serine when Compared to the Infertile Group. 13 Amino Acids Concentration are Identified through the Amino Acid Analyser which have shown a Drastic difference in the Fertile Group and in Infertile Group. **NS** – difference in Mean is Insignificant,** Signifies $p<0.01$,*** signifies $p<0.001$

Infertile Group (μ moles/ L)	**Control Group (Fertile) (μ moles/ L)**	**Amino acid**
0.61 ± 0.04	4.52 ± 0.18 ***	Aspartic acid
2.17 ± 0.01	5.73 ± 0.18 **	Tyrosine
1.75 ± 0.29	1.53 ± 0.18 ^{NS}	Valine
2.84 ± 0.13	2.52 ± 0.10 ^{NS}	Asparagine
1.23 ± 0.07	1.54 ± 0.11 ^{NS}	Glutamine
18.03 ± 0.24	4.56 ± 0.38 ***	Serine
1.86± 0.15	9.41 ± 0.13 ***	Glycine
3.91 ± 0.07	1.14 ± 0.05**	Phenylalanine
0.92 ± 0.07	0.93 ± 0.0.4 ^{NS}	Amino butyric acid
1.11 ± 0.13	3.29 ± 0.06 ***	Glutamic Acid
5.05 ± 0.14	2.42 ± 0.12 ***	Alanine
0.92 ± 0.06	2.05 ± 0.02 ***	Histidine
0.721 ± 0.03	2.04 ± 0.07 **	Proline

Table 4- Represents the Total Amino Acid Content in Fertile and Infertile Prostasomes Fraction. In which each Category Contains 32 Samples. The Mean Amino Acid Content in Fertile Group is 18.09 (μ moles/ L) and in Infertile Category it is about 15.12 (μ Moles/ L)

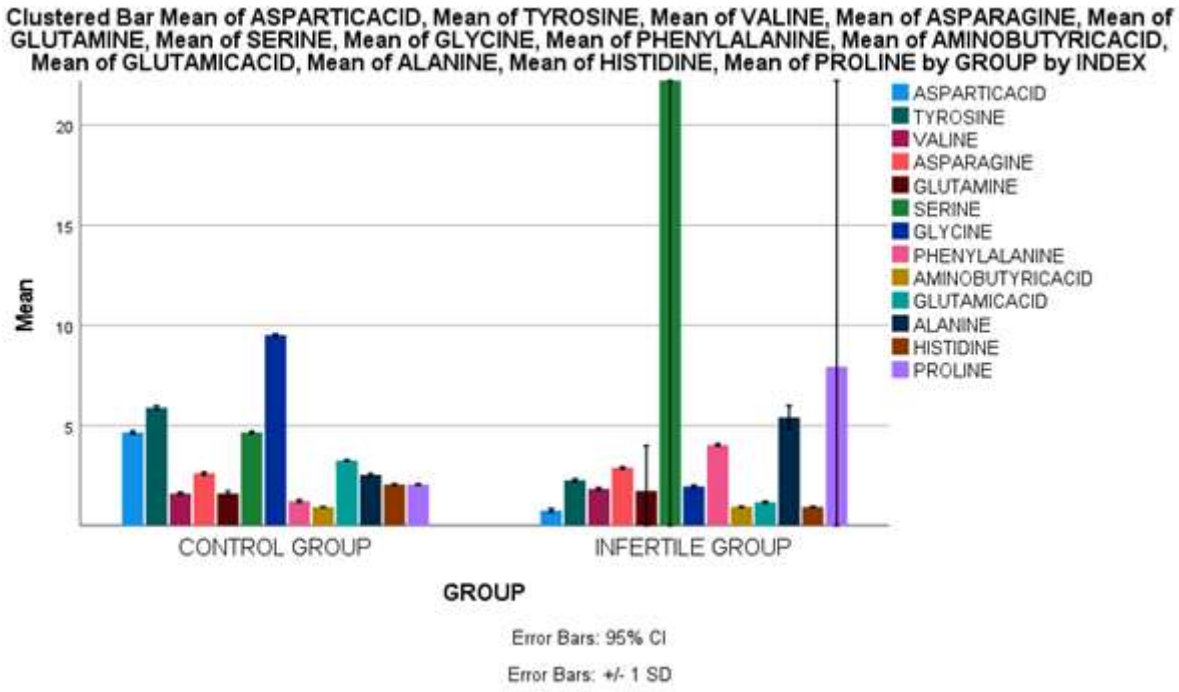
	Fertile category	**Infertile category**
No of samples	32	32
Amino acid content (μ moles/ L)	18.0±90.20	15.12±0.37

Table 5- Represents that Independent Sample T-Test which Shows the Significance in which the Amino Acids such as Alanine, Proline are found with Statistical Significance ($p<0.01$) when Comparing Fertile Groups with Infertile Groups

		Leven's test for equality of variances					**t-test for equality of means**		**95% confidence Interval of the difference**	
		F	**Sig**	**t**	**df**	**Sig [2-tailed]**	**Mean diff.**	**Std. Error diff.**	**Lower**	**Upper**
Aspartic Acid	Equal variances assumed	.497	.483	181.18	62	<.001	3.89	.021	3.85	3.93
	Equal variances not assumed			181.18	61.37	<.001	3.89	.021	3.85	3.93
Tyrosine	Equal variances assumed	4.48	0.38	172.49	62	<.001	3.62	.021	3.58	3.66
	Equal variances not assumed			172.49	56.55	<.001	3.62	.021	3.58	3.66
Valine	Equal variances assumed	.020	.889	-14.845	62	<.001	-232	.016	-.263	-.2
	Equal variances not assumed			-14.845	61.91	<.001	-.23	.016	-.263	-.20
Asparagine	Equal variances assumed	1.27	.262	-10.239	62	<.001	-.28	.015	-.310	-.24
	Equal variances not assumed			-10.239	59.59	<.001	-.28	.015	-.310	-.24
Glutamine	Equal variances assumed	3.46	.067	-.290	62	.773	-.11	.394	-.901	.673
	Equal variances not assumed			-.290	31.18	.774	-.11	.394	-.917	.689
Serine	Equal variances assumed	4.26	.043	-1.241	62	.219	-69.499	55.995	-181.431	42.434
	Equal variances not assumed			-1.241	31.00	.224	-69.499	55.995	-183.701	44.704
Glycine	Equal variances assumed	1.34	.25	452.41	62	<.001	7.56	.017	7.5	7.59
	Equal variances not assumed			452.41	60.58	<.001	7.56	.017	7.52	7.59
Phenylalanine	Equal variances assumed	1.92	.170	-168.89	62	<.001	-2.8	.017	-2.83	-2.7
	Equal variances not assumed			-168.89	60.76	<.001	-2.8	.017	-2.83	-2.7
Aminobutyric Acid	Equal variances assumed	11.2	.001	-1.354	61	.178	-.00	.006	-.019	.004
	Equal variances not assumed			-1.373	53.01	.176	-.00	.006	-.019	.004
Glutamic Acid	Equal variances assumed	5.05	.028	200.25	62	<.001	2.08	.010	2.06	2.10
	Equal variances not assumed			208.25	55.48	<.001	2.08	.010	2.06	2.10
Alanine	Equal variances assumed	16.2	<.001	-27.595	61	<.001	-2.8	.104	-3.08	-2.6
	Equal variances not assumed			-27.167	30.93	<.001	-2.8	.106	-3.09	-2.6
Histidine	Equal variances assumed	.001	.980	169.29	62	<.001	1.11	.007	1.10	1.13
	Equal variances not assumed			169.29	61.99	<.001	1.11	.007	1.10	1.13
Proline	Equal variances assumed	15.8	<.001	-1.454	62	.151	-5.6	3.887	-13.4	2.12
	Equal variances not assumed			-1.454	31.00	.156	-5.6	3.887	-13.5	2.27

13 different amino acids content were compared in Fig. 1 and found that proline and alanine yielded better results in case of fertile men, It shows that concentration varies in fertile men and infertile men in which control group mean is 5 (µmoles/L) in infertile group mean is (25 µmoles/L).

Fig. 1- The Bar Chart Represents the Comparison of Concentration of 13 Amino Acids in Fertile Men and Infertile Men. It Shows that Concentration Varies in Fertile Men and Infertile Men in which Control Group Mean is 5 (µmoles/L) in Infertile Group Mean is (25 µmoles/L). X Axis: Represents Control vs Infertile, Y Axis: Represents Mean Concentration of Amino Acids with ± 1SD



4. Discussion

Our overall results show that there are huge variations observed in the concentration of amino acids present in human prostatosmes between various infertile conditions and normospermia men. However the identification of the sperm and amino acid concentration as an ideal biomarker is a major challenge, our results were found to be in accordance with the studies conducted by Agrawal et al (Agarwal, Selvam, and Baskaran 2020). Due to the lack of alternative testing or the need for invasive diagnostic procedures, many clinical areas in the field of male fertility are primed for the production of seminal biomarkers, our results may lead to use of prostasomes as one of the biomarker for male infertility, and all our results in accordance with Bieniek (Bieniek, Drabovich, and Lo 2016). Prostasomes present less in infertile category in our study, more similar findings were observed in the study conducted by Garcia et al (García-Rodríguez et al. 2018).

We followed the standard protocol of the World Health Organization, 2010 very strictly, for the preparation of semen analysis report, we segregated the infertile groups only based on the standard values mentioned, in our study, got better results for semen analysis report. Amino acid analyzer and CASA instruments were calibrated completely before analysis for better results. Semen samples were collected in a toxic-free plastic container, so that the sperm could survive even after collection for correct analysis. Samples were analysed by already standardized protocol by vickram et al (S. Vickram et al. 2021) (A.S. Vickram, Anbarasu, et al. 2020) (S. Vickram et al. 2021). The prostasomes which are present in the semen is an excellent source of biomarker which makes it an innovative method of diagnosis for male infertility (Ebert, Kisiela, and Maser 2015). The analysis of amino acid level in the fertile group and infertile group from that we could identify that amino acids in the fertile group is high in concentration whereas in infertile group the concentration of amino acid is low. In 13 amino acids all amino acids have drastic variation when comparing them with other groups.

Our institution is passionate about high quality evidence based research and has excelled in various fields ((Vijayashree Priyadharsini 2019; Ezhilarasan, Apoorva, and Ashok Vardhan 2019; Ramesh et al. 2018; Mathew et al. 2020; Sridharan et al. 2019; Pc, Marimuthu, and Devadoss 2018; Ramadurai et al. 2019). We hope this study adds to this rich legacy.

We had some limitations in our study execution, while using amino acid analyzer turbidity is an issue due to the particulates in the samples, CASA had limitations in guaranteeing the identification of sperm parameters such as motility and concentration for continuous examination. Centrifugation for 1 hour to separate the prostomses from seminal plasma was another limitation as the machine got heat and not maintaining -4 degree celsius for continuous time, this may lead to denaturation of protein.

Still the relevance and few more properties of prostasomes and amino acids remains unknown. Vast development and multi omics approach on prostasomes will lead the researchers to focus on new findings on seminal fluid with unique properties that can be identified in a better way.

5. Conclusion

The concentration of amino acid present in prostasomes of fertile men is high about 18.09 ± 0.20 (μ moles/ L) when compared to infertile men 15.12± 0.37 (μ moles/ L). This identification led to the conclusion that drastic reduction in amino acids concentration of prostasomes will lead to

infertility. Prostasomes play an important role for infertility and this could be used for the diagnosis purpose.

Declarations

Conflict of interests

No conflict of interests in this manuscript.

Authors Contributions

Author DV was involved in data collection, data analysis, manuscript writing. Author VAS was involved in conceptualization, data validation, and critical review of manuscript.

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