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Correlation Studies on Human Seminal Plasma Proteins and Their Relation with Semen Freezablity

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ABSTRACT

Purpose of this research was to elucidate the protein profiles of the seminal plasma in various categories of male infertility, to scrutinize their correlation with seminal parameters. Oligoasthenospermia (N = 15), asthenospermaia (N = 17), azoospermia (N = 12), normospermia (N = 27), oligospermia (N = 12) and fertile (control subjects, N = 10) were collected. The samples were diluted by tris-egg yolk extender and were frozen. Plasma was separated from semen by centrifugation, underwent SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE). The mean values with its standard error of semen parameters of fresh sample were shown significant difference ($p<0.0001$) when compared to the post-thaw samples. Of the various fractionations, the protein with molecular weight 44.6 kDa shows high significant and positive correlation ($p<0.01$) with sperm concentration of freshly evaluated semen samples and low level significant ($p<0.00001$) with the frozen samples. Sperm motility was positively correlated ($p<0.029$) with the protein molecular weight 56.6 kDa in the freeze thawed semen samples. This reality could sustain the implication that seminal plasma proteins act on the sperm physiology and morphology and it found to act on strange ways. Supplementary studies are essential to define the mechanism of different proteins involved in the fertilization and their correlation.

Key words: Sperm motility, freezing, correlation, seminal plasma, extender

INTRODUCTION

Male Infertility can be distinct as the inability to fulfill pregnancy subsequent to a reasonable time of sexual intercourse without contraceptive measures taken (Nadeem *et al.*, 2012). Seminal plasma is a composite muddle that contains secretions of epididymis, testis prostrate and other sex glands. It is thought that, the seminal plasma hold certain aspect that can alter the fertilizing knack of the sperm (Henault *et al.*, 1995). Male infertility is accountable for more than 40% of infertility in the world (Tuzun *et al.*, 1998). No precise reason can be found in about 10-15% of infertile couples (Basar and Tuglu, 2009). Even though extensive development has been made toward understanding the biology and the sperm physiology, at rest additional job in molecular and

cellular stage is essential to reveal pathology and extend the pinpointing and treatment methods for the clinical studies, predominantly in infertile cases (Yeni *et al*., 2010). Sperm motility and morphology, the volume as well as the amount of spermatozoa per insemination and the amount of acrosome reacted sperms or its percentage has been broadly appraised as a suggestion of sperm's capacity to fertilize an egg. The freezing procedure produces thermal shock that exposes the spermatozoa, which in turns results in damage to acrosome and plasma membrane (Woelders *et al*., 1997; Celeghini *et al*., 2008). The molecular symphony of the seminal fluid is extremely multifarious and its plasma plays a major function in fertilizing capacity of the sperm (Cross, 1993). Seminal plasma helps in maintain sperm motility in ram and bull (Jobim *et al*., 2004) and is not yet proven in case of human. The seminal fluid may also helps and influencing the human fecundity and sperm storage, but its maximum function in case of freezing is unknown and in some cases, remains controversial (Jobim *et al*., 2004). A variety of extenders has been experienced in an effort to frontier cellular injury to the spermatozoa. Egg yolk is mainly dilapidated of these particular extenders by insemination research centres. Glycerol is used as a cryoprotectant that capable of annoying the cell membrane, but the foundation of its cryoprotective chattels is not entirely implicit (Aires *et al*., 2003). An extender should hold a vigour resource substrate fructose or glucose, to avert cold shock, non-ionic or ionic substances to uphold an appropriate pH and osmotic pressure, enzymes and antibiotics (Vishwanath and Shannon, 2000). Modern research reveal that unexpected temperature alteration such as warm and cold shocks, dissolution and ice formation during the freeze-thawing progression, affects the reliability of cells at the structural and sub structural levels (Watson, 1995; Parks and Graham, 1992; Quinn *et al*., 1969; Nath, 1972). In the past years, numerous seminal plasma proteins has been recognized and characterized. Data suggests that the protein composition is dissimilar among various species and that a few seminal plasma proteins are allied with fertility in a range of species. Bovine Seminal Plasma (BSP) contains a kind of major proteins elected with molecular masses ranging from 18.5-20.7 kDa and BSP associated protein 30 kDa with a projected molecular mass of 28-30 kDa (Manjunath and Sairam, 1987). Earlier research is usually connected to the assessment of seminal plasma composition flanked by different fertility or the isolation and characterization of explicit seminal proteins that could persuade sperm fertilization and capacitation (Cardozo *et al*., 2006).

On the other hand, there is petite information existing regarding human seminal plasma proteins. This research was carried out to evaluate the protein profile of the human seminal plasma by means of SDS-PAGE and to examine a possible relationship and correlation between these proteins and the freezability of the spermatozoa as well as with the semen parameters.

MATERIALS AND METHODS

Research population: This research was carried out with 93 male infertile patients (range, 25-45 years) who are attending the OPD (Out Patient Department) of Andrology, Bangalore Assisted Conception Centre, Bangalore, for the evaluation of male infertility. Patients suffering from any acute infection (Sexually Transmitted Diseases) were excluded from this research. The semen samples were classified into different infertile categories based on sperm count, motility and morphology.

Research ethics: This research study is basically a part of a major research project, for which the human ethical approval and clearance was obtained from Institutional Ethical Committee.

Semen assessment: Immediately after semen collection, the ejaculate was kept in a 37°C incubator and the volume was recorded prior to liquefaction with the help of a graduated plastic

tube. Its pH was anticipated with the help of pH meter. Sperm motility and total motile sperm was determined with microscopic examination. A drop of ejaculated semen sample was placed on the pre-warmed slide at 37°C and covered with a cover slip and then the percentage of the motile and normal spermatozoa was examined at 100X magnification. Eosin nigrosin was used to determine the percentage of the live spermatozoa. Fresh semen was diluted to 1:10 with the phosphate buffer at pH 7 and then placed a drop of mixture on the haemocytometer chamber, the sperm concentration was calculated and expressed as millions mL^{-1}. All the semen parameters were analyzed and the report was prepared and the inference was mentioned, based on the WHO protocols. Semen extenders were prepared and preserved at -25°C until or otherwise use. Tris-egg yolk extender, that contains 25 mL of egg yolk, 2.8 g Tris (buffer), 2 g citric acid, 2 g of fructose, 7 mL glycerol, 50 mg gentamicin, 60,000 IU penicillin, were mixed in 100 mL of miiliq distilled water. After analysis, the samples were preserved, frozen and kept at -40°C with the semen extender. After freezing, the semen sample when required, it is thawed at 37°C water bath for 30 sec. Semen parameters were then evaluated as mentioned previous and the results were compared with the fresh samples.

Seminal plasma preparation: The plasma was removed straight away after collection from the total ejaculate. Fresh semen was centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was transferred into centrifuge tubes and then centrifuged at 12000 rpm for 20 min at 4°C to eliminate the remaining sperm. The seminal plasma was kept at -20°C until further use, after the determination of total protein concentration by using the spectrophotometer at 280 nm. The sample is stored at -40°C until use.

Sample processing for SDS-PAGE: The stored samples were taken and processed for protein analysis by SDS-PAGE. Equal amount of gel loading dye (2.5 mL of 4X Tris Cl-SDS at pH 6.8, 2 mL of glycerol, 0.4 g of SDS, 200 µL of 2-mercaptoethanol, 0.1 g of bromophenol blue, dissolved in 10 mL of Milli Q water) was added, mixed and boiled at 100°C hot water bath for 10 min. Then the SDS-PAGE gel was prepared (Separating gel was prepared by mixing 2.5 mL of 30% Acrylamide and N, N-methyl bis-acryl amide, 1.8 mL of 4×Tris Cl at pH 8.8, 3.05 mL of Milli Q water, 80 µL of freshly prepared APS and 40 µL of TEMED. Mixed it well and poured in PAGE plates. It was allowed to polymerize for 30 min. Stacking gel was prepared by mixing 0.65 mL of 30% Acrylamide and N, N-methyl bis-acrylamide, 1.25 mL of 4× Tris Cl-SDS buffer at pH 6.8, 3.05 mL of Milli Q water, 40 µL of freshly prepared APS and 25 µL of TEMED. It was poured above the separating gel and allows it to polymerize for 20 min. The comb was inserted into the SDS plates without disturbing the gel volatility. This set up was allowed for 1 h. The set up was made ready to load the sample, after loading the gel was allowed to run in the buffer for 3 h. After electrophoresis, gels were stained with methanolic silver staining, the molecular weight of each band was calculated by using high and low molecular weight markers.

Data investigation: Data analysis was performed with the help of biostatistics tool, graph pad prism, version 5.03, for windows. All the results were represented as Mean±Standard Error. Analysis of Variance (ANOVA) was done to compare the mean values of each parameter and between the groups. Tukey's test was used subsequently to evaluate the parameters at a significance level of $p<0.05$. Pearson's correlation coefficient was examined to evaluate the

correlation of the semen parameters between the group and the correlation of semen parameters between the groups before and after freeze thaw. It is also used to evaluate the correlation between the relative protein content of the human seminal plasma and the semen parameters.

RESULTS

The results of the semen analysis of 93 human semen samples including all the categories of infertile and fertile (control), fresh samples were depicted in Table 1. The semen analysis for the same samples after freeze thaw was depicted in Table 2. The mean values with its standard error of the semen parameters including sperm concentration, sperm normal morphology, sperm motility, sperm progressive motility of fresh sample were shown significant difference ($p<0.0001$), when compared to the post-thaw of the same samples. Sperm concentration (spearman $r = 0.9383$, $p = 0.0001$) and sperm motility (spearman $r = 0.678$, $p = 0.0001$) of frozen and post thaw semen samples were highly correlated with the fresh semen sample. The normal morphology was not shown any higher level significance between the fresh and frozen samples. Seminal plasma proteins of different categories were fractionated with the help of poly acryalamide gel. The gel resulted with 16 bands starting from 14 to 205 kDa shown in Fig. 1. Of the various fractionations, the protein with molecular weight 44.6 kDa shows high significant correlation ($p<0.01$) with sperm concentration of freshly evaluated semen samples and low level significant ($p<0.00001$) with the frozen samples. Sperm motility was positively correlated ($p<0.029$) with the protein molecular weight 56.6 kDa in the frozen thawed semen samples. The 205 kDa protein band is found to be

Table 1: Evaluation of semen parameters immediately after collection (fresh)

	Infertile groups					
Semen parameters	Oligoastheno spermia (n = 15)	Astheno spermia (n = 17)	Azoo spermia (n = 12)	Normo spermia (n = 27)	Oligo spermia (n = 12)	Fertile group (control) (n = 10)
Volume (mL)	2.49±0.43000	1.89±0.1690	1.960±0.32	2.56±0.22	2.01±0.12	3.0±0.240
pH	7.75±0.08000	7.58±0.0740	7.51±0.108	7.58±0.03	7.73±0.10	7.6±0.090
Sperm concentration (mil mL^{-1})	9.60±1.70100	44.40±4.1530	NIL	86.6±11.20	9.24±1.40	107.1±13.20
Total motility (%)	16.56±3.53000	36.07±4.9110	NIL	68.8±2.360	38.5±2.300	65.61±6.30
Rapid progressive (%)	1.880±0.9181	5.800±1.022	NIL	18.2±1.520	30.8±4.600	29.01±2.68
Normal morphology (%)	9.700±2.2470	18.90±0.7210	NIL	24.9±0.880	34.63±2.13	17.04±1.50

All values represented as Mean±Standard error of mean

Table 2: Evaluation of semen parameters after freeze and thaw (post-thaw)

	Infertile groups					
Semen parameters	Oligoastheno spermia (n = 15)	Astheno spermia (n = 17)	Azoo spermia (n = 12)	Normo spermia (n = 27)	Oligo spermia (n = 12)	Fertile group (control) (n = 10)
Volume	2.19±0.410	1.74±0.19	1.85±0.310	2.44±0.230	1.880±0.10	3.03±0.23
pH	7.7±0.1000	7.5±0.130	7.53±0.111	7.59±0.050	7.86±0.090	7.73±0.10
Sperm concentration (mil mL^{-1})	7.89±1.850	41.9±4.100	NIL	87.47±14.30	3.44±1.170	103.4±13.50
Total motility (%)	15.6±3.6000	32.87±4.80	NIL	60.19±2.850	27.72±3.200	64.4±6.290
Rapid progressive (%)	1.71±0.916	5.09±1.05	NIL	17.59±1.525	18.50±2.200	25.9±2.100
Normal morphology (%)	9.40±2.142	18.2±0.850	NIL	24.33±0.860	26.7±2.0100	16.13±1.71

All values represented as Mean±Standard error

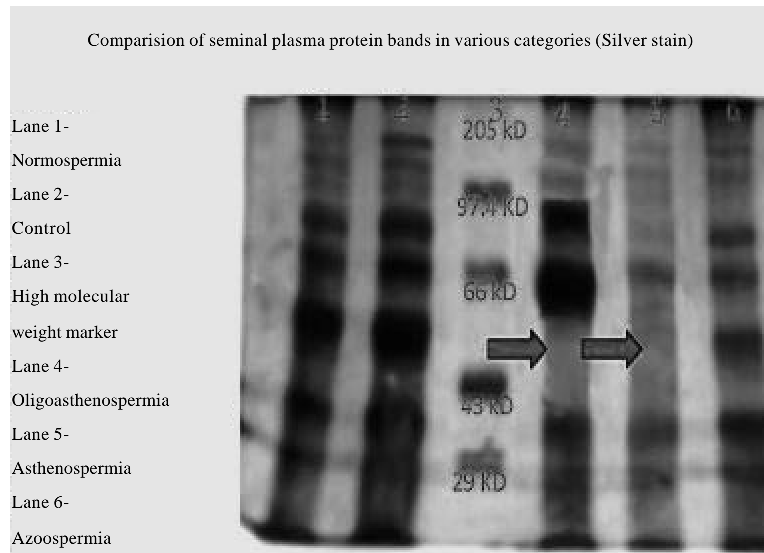

Fig. 1: Seminal plasma protein profile of various categories and their correlation with semen parametrs and freezeablity

missing in all the infertile categories except normospermia. Sperm normal morphology was not correlated (positively) with any of the protein bands in our research. Sperm progressive motility was correlated with 56.6, 83.4, 105 kDa proteins with the p-value 0.0001. The pH and volume of the samples were not showing any significant difference when compared with freeze thaw as well as infertile categories and fertile samples.

DISCUSSION

Even though sperm count is an imperative reason in prominent fecundatory potential of men, it is not the solitary factor in shaping infertility (Fadiloglu *et al*., 1996). The fertility of the male is correlated with its seminal plasma proteins in many species stallion (Amann *et al*., 1987), boar (Strzezek *et al*., 2005), bull (Liberda *et al*., 1998), ram (Bergeron *et al*., 2005), buffalo (Asadpour *et al*., 2007), canine (De Souza *et al*., 2007), human (Chiu and Chamley, 2003), human male (Davalieva *et al*., 2012) and infertile men (Kosanovic and Jankovic, 2010). Nevertheless, a little data is available regarding the human seminal plasma proteins with different categories of male infertility. This research was conducted to get some information about the correlation of human seminal plasma proteins of the human with different infertile categories and the results were compared with the control (fertile) samples. All the samples were evaluated freshly (immediately after collection) and after frozen (post-thaw). SDS-PAGE was used to detect the protein bands in human seminal plasma. The molecular masses ranging from 14.2-205 kDa were detected in this research. Most of the samples especially asthenospermia, the 205 kDa protein band were missing. The protein fractions were separated, for human seminal plasma proteins were below 40 kDa and this band is with most frequent appearance (32%). Also, it has been optional that egg yolk could diminish post thawing practicality and acrosome reliability of spermatozoa in a variety of species such as goat (Ritar and Salamon, 1991), buffalo (Kumar *et al*., 1993). Salamon and Maxwell (1995a, b) found that the sperm motility and viability diminished following

by freeze-thawing in contrast with that of the pre-freezing situation. Some researchers used Bonferroni tests for evaluating the parameters (Kisa *et al*., 2008). In our studies also, the sperm concentration, total motility and progressive motile sperms were found to be in decreased level, in case of freeze thaw samples, when compared to fresh samples, irrespective of categories. Nevertheless in other studies counting couples with inaccessible male factor infertility, processed total motile sperm count was not allied with pregnancy (Iltemir Duvan *et al*., 2009). In the study conducted by Alavi, the semen characteristics and the proceedings of the freezablity of the spermatozoa and whole semen, 27.5 kDa protein was significantly correlated with progressive motility in the fresh ($p<0.002$) and also with viability for freeze thawed semen samples ($p<0.19$) (Alavi-Shoushtari and Babazadeh-Habashi, 2006). In our research, of the various fractionations the protein with molecular weight 44.6 kDa shows highly significant and positively correlated ($p<0.01$) with sperm concentration of freshly evaluated semen samples and low level significant and negatively correlated ($p<0.00001$) with the frozen samples. It is effortless to say "prevention is better than cure", but the complexity with infertility is that the existing awareness on pathogenesis is on the breadline (Jayachandra, 2005).

CONCLUSION

In this research, divergence in the seminal plasma protein profile of different categories of male infertility patients with low and high semen quantity and quality were elucidated. The semen parameters were found to be decreased in case of frozen samples when compared to the fresh samples. Some of the seminal plasma proteins, irrespective of categories, were correlated with semen parameters before and after freezing. This reality could sustain the implication, that seminal plasma proteins proceed on the sperm morphology and physiology and it acts on strange ways. Supplementary studies were essential to define the mechanism of different proteins involved in the fertilization.

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