

Full Length Research Paper

Spermogram and biochemical approach in evaluating male infertility in South Karnataka

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Infertility is a disorder of the reproductive system which affects both men and women with almost equal frequency. Male factors alone constitute around 50% of all cases of infertility. Human male fertility is normally assessed on the basis of a semen profile, with respect to spermatozoa. In this study, an attempt has been made to evaluate the semen profile of 132 infertile male subjects which include physical examination, microscopic studies, sperm function tests and assessment of the functional status of accessory reproductive organs according to the WHO protocol. The functional status of the seminal vesicle and prostate gland are studied by estimating the levels of fructose and citric acid respectively. Values are recorded and subjected to statistical analysis. In this study ten different infertile conditions are observed of which azoospermia is predominant and asthenoteratozoospermia is the least reported condition. All these infertile conditions show varied response to sperm function tests and biochemical analysis of semen. All these analyses are simple and cost effective that can help in the precise diagnosis and effective treatment at the earliest for affected individuals.

Keywords: Infertility, semen analysis, sperm function tests, biochemical markers

INTRODUCTION

Infertility can be defined as failure to conceive after 12 months of unprotected sexual intercourse with the same partner (Rubenstein, 2006). The prevalence of infertility in India is not yet clear, however, according to a study by WHO, the incidence of infertility is estimated to be 10 % to 15% and approximately 13 to 19 million couples are infertile (Rowe and Farley, 1988; Sharma et al., 2005). Male factors play a significant role in about 50% of infertile couple (Schill, 1981; Sharma et al., 2005), where the causes are multifactorial and their management and treatment depend on the precise diagnosis. Generally, clinical diagnosis depends on semen analysis for the assessment of male infertility and also for evaluating efficacy of male fertility regulating agents. Apart from the routine semen analysis, specific investigations are required to assess the functional capacity of spermatozoa. The most important mechanisms of fertilization such as capacitation, acrosome reaction and building

of spermatozoa to the egg surface depend on the functional integrity of the sperm membrane (Jayendran et al., 1984). Acrosomal enzyme reflects the sperm fertilizing capacity (Amin et al., 1996; Sharma et al., 2005), chromatin condensation shows the morphological as well as functional status of the spermatozoa, hypo-osmotic swelling (HOS) test is to know the physical integrity and also the chemical changes in the sperm plasma membrane. Biochemical analysis of semen such as fructose and citric acid helps to evaluate the functional status of accessory reproductive organs. The secretions of the organs contributing to the ejaculate differ in composition. It was a longstanding interest in evaluating the composition of semen from a diagnostic point of view (Eliasson, 1982). The prostate gland is the main source of the acid phosphatase, citric acid, inositol, calcium, zinc, and magnesium found in the ejaculate. Seminal vesicles secretions are rich in fructose, ascorbic acid, and prostaglandins. While L-carnitine and neutral alpha-glucosidase are indications of epididymal function (WHO, 1999).

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Hence, in the present study, efforts have been made to describe the significance and assessment of semen parameters, sperm function tests with the importance of fructose and citric acid as biochemical markers of the seminal vesicle and prostate gland.

MATERIALS AND METHODS

The present study was carried out in the Department of studies in Zoology, Human Genetics Research Lab., University of Mysore, Mysore. A total of 132 infertile subjects were recruited from Mediwave IVF and Fertility research center, Mysore, Karnataka, India. On the basis of microscopic examination of the semen, the study subjects were classified into different infertile conditions based on the count motility and morphology of the sperm. Normal healthy donors with proven fertility (n=20) were recruited as a control population for this study. Institutional ethical clearance was taken from University ethical clearance committee and informed written consent letter were obtained from subjects and a control group before including them into the study. Infertile males are interviewed to collect information about lifestyle factors, family, medical and reproductive history.

Semen collection and preservation

Semen samples were collected after 3-5 days of ejaculatory abstinence in a sterile plastic container by the process of masturbation (WHO, 1999). The collected samples were allowed to liquefy at 37°C for 30 minutes and analyzed within one hour of collection.

Semen analysis

Physical examinations such as liquefaction time, odor, color, pH and viscosity were recorded after liquefaction. Microscopic examinations were carried out to record the count, density, motility and morphology of the sperm according to the WHO protocol (1999). Vitality assessment was carried out through eosin and nigrosine method. The functional capacity of the spermatozoa was examined by sperm function tests through Hypo-osmotic Swelling test (HOS) (Misro and Chaki, 2008), Acrosome Reaction test (AR) and Nuclear Chromatin Decondensation test (NCD) (Gopalkrishnan, 1995). The semen samples were centrifuged at 3,000 rpm for 10 minutes and the seminal plasma was separated and stored at -20°C for further biochemical analysis. Results were recorded and subjected to statistical analysis (one way ANOVA) using statistical software Prism (Version 3).

Estimation of fructose

The level of fructose was estimated by indole method. 20µl of seminal plasma was added to 220µl of distilled water and mixed thoroughly the solution were deproteinized by adding 50µl of ZnSO₄ and 50µl NaOH. After 15 minutes of incubation at room temperature this mixture were centrifuged at 2500 RPM for 15 minutes and 200µl of the cleared supernatant was used for analysis. Blank solution consists of 200µl of distilled H₂O, 200µl indole reagent; 2ml of 32% HCl. Standard consist of 200µl of standard solution, 200µl indole reagent, 2ml of 32% HCl. The sample consists of 200µl of sample solution, 200µl indole reagent, 2ml of 32% HCl. These solutions were incubated for 20 min at 60°C and then cooled in ice water and readings were taken at 470nm (Karvonen and Malm, 1955).

Estimation of citric acid

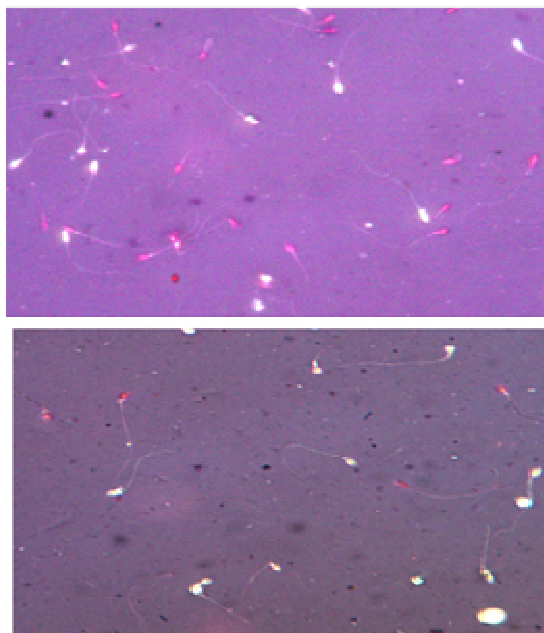
For this analysis, 100 µl of seminal plasma was added to 100 µl 50% TCA (trichloro acetic acid) and cooled in ice bath. This mixture is centrifuged at 2000 rpm for 15 min and 100 µl of supernatant is used for further analysis. Blank was prepared with 100 µl of water, 100 µl of trichloroacetic acid and citric acid standard solution were taken in separate tubes. 800 µl of anhydrous acetic anhydride were added to sample, blank and standard tubes and heated at 60°C for 40min and cooled in ice bath for 5 min and absorbance was measured at 400nm against blank (Polakoski et al., 1977).

RESULTS

In the present study, 132 men diagnosed with different infertile conditions are grouped into 10 different conditions, in which the percentage of azoospermic condition (28.7%) was high followed by oligozoospermia (13.16%) and idiopathic (12.8%) cases. Asthenoteratozoospermic was observed as a least reported condition (2.2%) which is furnished in the table 1. The results of different physical semen parameters in infertile conditions are shown in the table 1. In asthenoteratozoospermia the average semen pH shows the higher range (8.4), but other conditions are in normal range. The sperm vitality was normal (75%) in Asthenoteratozoospermia but the decreased vitality rate was observed in the rest of the cases (table 3). Figure 1 shows the response of spermatozoa for vitality test. Coagulation in the ejaculated semen was normal in oligoteratozoospermia, asthenoteratozoospermia and in idiopathic cases but decreased coagulation was

Table 1: Prevalence of different infertile conditions and physical semen parameters (NR=Normal, LT=Liquefaction Time, AB= Absent and ABR=Abnormal).

Sl. No	Condition	Number of samples	In percent (%)	Coagulation Normal (present)		L.T (30minutes)		Color (Grayish white)		pH (7.5-8)
				NR %	AB %	NR %	AB %	NR %	ABR %	
1	Oligozoospermia	18	13.16	77.7	22.2	83.33	16.66	8.88	11.1	8.2±0.3
2	Oligoteratozoospermia	07	5.03	100	0	71.9	28.1	71.4	28.5	8.1±0.2
3	Teratozoospermia	13	9.8	84.6	13.3	69.2	30.7	84.6	15.8	8.2±0.2
4	Oligoasthenozoospermia	12	9.09	75	25	75	25	58.3	61.7	8.2±0.19
5	Asthenozoospermia	09	6.8	88.8	12.2	77.7	22.3	88.8	12.2	8.2±0.3
6	Asthenoteratozoospermia	03	2.2	100	0	66.6	33.4	100	0	8.4±0.1
7	Azoospermia	38	28.7	78.9	21.1	63.1	36.9	63.1	36.9	8.1±0.2
8	Oligoasthenoteratozoospermia	08	6.06	75	25	87.5	12.5	87.5	12.5	8.2±0.2
9	Aspermia	07	5.03	-	-	-	-	-	-	-
10	Idiopathic	17	12.8	100	0	58.8	41.2	88.2	11.8	8.17±2.5
11	control	20		100	0	80	20	90	10	7.8±0.3

**Figure 1:** Response of spermatozoa for vitality test. Pink stained spermatozoa indicate the non viable and unstained (white) spermatozoa indicate the viability.

observed in oligoasthenoteratozoospermic condition (25%) followed by oligozoospermia (22.2%). Liquefaction time was varied from the normal range in idiopathic cases (41.2%) followed by azoospermic cases (36.9%). Variation in semen color was observed in

oligoasthenozoospermia (61.7%) followed by azoospermic (36.95%) and oligoteratozoospermic conditions (28.5%).

Table 2 shows the results of sperm function tests such as NCD, HOS and AR. In all cases, NCD

Table 2: Distribution of different infertile conditions with respect to different semen parameters including functional tests for spermatozoa (NCD= Nuclear chromatin decondensation test, HOS=Hypo-osmotic swelling test, AR= Acrosomal reaction test).

Sl. No.	Condition	Sperm count (20millions/ml)	Vitality (75%)	Motility (50%)	AR (>50%)	HOS (>60%)	NCD (>70%)
1	Oligospermia	12.1±5.2	58.4±14	46.6±26	59±3.0	54.3±17	62.6±12.1
2	Oligoteratozoospermia	11.85±5.1	49.1±2.17	42.5±20.9	47.1±22.14	42.1±20	56.8±27
3	Teratozoospermia	52±0.4	68.2±16.6	58±17	45.15±5	49.1±21	65.2±16
4	Oligoasthenozoospermia	8.5±5.1	53.7±22	18.5±13	45±15.5	50±21	51.5±25.07
5	Asthenospermia	47.37±11	62.1±13	22.4±18	41.7±16.8	64.2±12	66.7±17
6	Asthenoteratozoospermia	27.65±10	75±10	31.6±2.8	50.5±10.7	36±25.4	56.15±21
7	Azoospermia	-	-	-	-	-	-
8	Oligoasthenoteratozoospermia	11±3.7	43.8±2.15	17.25±11	41.7±15	45±25.4	40.3±21.2
9	Aspermia	-	-	-	-	-	-
10	Idiopathic	68.4±44	74.5±10	59.1±23.	63±15.9	63.0±18	62.9±18.0
11	Control	83.12±35.2	74.9±10.8	59.9±21.8	69.9±7.4	65.7±18	75.5±9.5

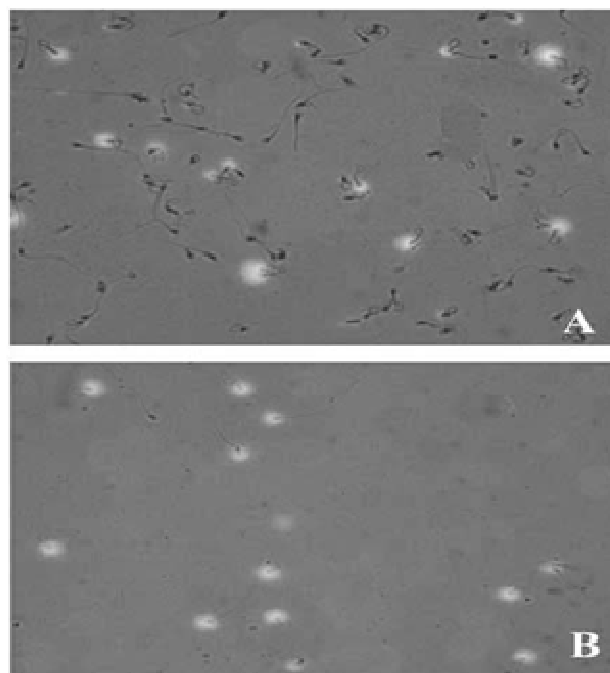


Figure 2: Response of spermatozoa for Acrosomal reaction (AR). Halos in the sperm head indicate normal sperm acrosome and absence of halos indicates absences or inactivation of acrosomal enzymes. Plate 'A' shows the negative response to AR, plate 'B' shows positive response (Control) to AR.

test showed decreased value. Asthenozoospermia and idiopathic cases showed a normal response for HOS and the rest of the cases showed low values from the normal range. Oligozoospermia, asthenoteratozoospermia and idiopathic cases showed a normal response to AR test and the rest of the cases showed values below normal. The response of sperm for different sperm function tests

namely, AR, HOS and NCD test were shown in the figures 2, 3 and 4 respectively. Table 3 depicts ANOVA analysis and shows significant value ($P<0.05$) for semen parameters.

The results of fructose value in all the cases lie in the reference range and the seminal volume is also in the normal range. The value of citric acid is

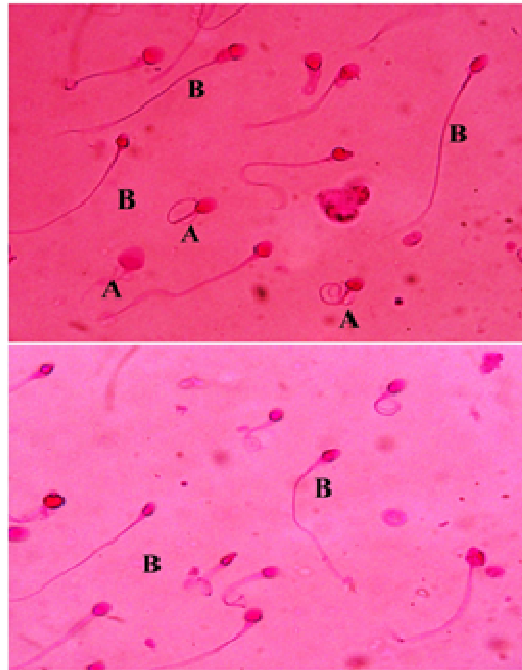


Figure 3: Response of spermatozoa for Hypo-Osmotic Swelling (HOS) test. 'A' indicates positive response by spermatozoa which show coiled tails; 'B' indicates negative response by spermatozoa which show uncoiled tails.

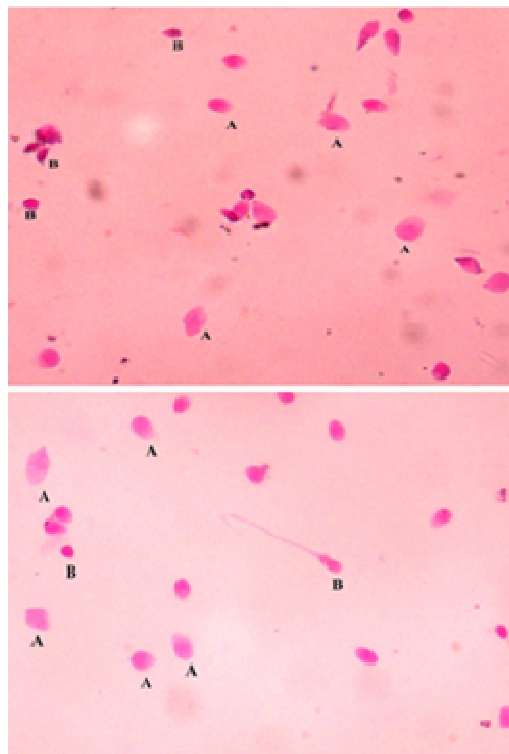


Figure 4: Response of spermatozoa for Nuclear Chromatin Decondensation test (NCD). 'A' indicates positive response by spermatozoa which show enlarged head; 'B' indicates negative response by spermatozoa which show condensed head.

Table 3: Comparison of semen parameters among control and infertile group.

SI No.	Semen parameters	P value	F	R squared
1	Sperm count	0.0001*	16.61	0.58
2	Vitality	0.0008*	5.453	0.32
3	Motility	0.0001*	12.24	0.50
4	NCD	0.0012*	3.568	0.23
5	HOS	0.0370*	2.158	0.15
6	AR	0.0010*	7.023	0.38

*P significant <0.05

Table 4: Comparison of Fructose concentration with seminal volume and Citric acid with pH in different infertile condition.

SI. No.	Conditions	Volume	Fructose 13µmole/ejaculation	pH (7.5 – 8)	Citric acid ≥13mg/ejaculate
1.	Oligospermia	1.6±0.8	17.49±15	8.2±0.3	9.9±5.4
2	Oligoteratozoospermia	2.5±0.8	16±10	8.1±0.2	13.9 ± 4.3
3.	Teratozoospermia	2.2±1.19	15.4±7.3	8.2±0.2	8.5±3.4
4.	Oligoasthenozoospermia	2.18±1.3	17.1±15	8.2±0.19	9.4±3.8
5.	Asthenospermia	1.7±1.1	20.2±11	8.2±0.3	12.4±2.4
6.	Asthenoteratozoospermia	2.42±1.8	20.2±12	8.4±0.1	7.9±3
7.	Azoospermia	2.5±0.5	26±5.6	8.1±0.2	10.4±2.2
8	Oligoasthenoteratozoospermia	2.12±0.8	13.3±12	8.2±0.2	8.6±4
9	Aspermia	-	-	-	-
10	Idiopathic	2.3±1.1	19±3.4	8.17±2.5	15.9 ± 4.3
11	Control	2.9±1.2	18.9 ± 1.1	7.8±0.3	18 ± 4.1

observed to decrease in all the cases may be due to alkaline pH of semen (table 4).

DISCUSSION

Semen analysis is the most commonly advised tests to estimate fertility rates in men. In many studies, the reported incidence of azoospermia was 12.32% to 16% (Khan et al., 1992; Rikhasor et al., 2001). In this study the azoospermic condition was found to be predominant and the incidence of oligozoospermia is not deviating from other studies (Hermanns and Hafez, 1981; Matilsky et al., 1993; Mehta et al., 2002). In our study, we found varied semen parameters in different infertile conditions which indicate multiple etiologies namely pH indicates functional status of prostate, color acts as an indicator of infections and internal bleeding, volume is a marker of seminal vesicle, liquefaction time indicates infection prostate gland function

Sperm function tests reveal functional status of sperm with reference to acrosomal enzymes, sperm DNA and sperm plasma membrane. Acrosin and hyaluronidase are the two main acrosomal enzymes, which play an important role in penetration of oocyte during fertilization.

Assessment of acrosin gives ample indications for fertilizing potential of the sperm. Normal acrosin activity is observed in the sperms of males with high fertilization capacity, whereas the halo diameters and halo formation rates are decreased in most cases of poor fertilization capacity (Fenichal et al., 1989; Gopalkrishnan, 1995). In the present study, oligozoospermia, asthenoteratozoospermia and idiopathic cases exhibited a normal response to AR test and the rest of the cases showed below normal value. Therefore, this method may give the information about the fertilizing potential of the spermatozoans, especially in the case of teratozoospermia where more of abnormal spermatozoa exists with reference to the structure of the sperm.

In this study, all infertile conditions showed the decreased value for the NCD test. Our studies are supported by many other earlier studies where a high proportion of morphological abnormal spermatozoa were shown to be associated with increased heterogeneity of chromatin structures (Fawcett et al., 1971; Meistrich et al., 1976; Sharma et al., 2005). Nuclear shape, which is species specific, depends on chromatin condensation during spermatogenesis as well as on a precise organization of DNA within the nucleus (Fawcett et al., 1991; Highland et al., 1991; Taddei A et al., 2004).

Chromatin condensation is achieved by gradual substituting of lysine rich somatic histones by testis specific histones. It has been suggested that a low DNA content in spermatozoa of sub fertile individual may be responsible for inducing alternations in sperm shape (Baccetti et al., 1977; Terquem and Dadoune 1983; Hammadeh et al., 2001). Nuclear chromatin decondensation is very important for fusion of male pronuclei with female pro nuclei any subtle change may alter motility and shape of spermatozoa.

Hypo-osmotic swelling test evaluates the integrity of the sperm membrane and can be used as routine clinical investigations. This is based on the semi permeability of the intact cell membrane, which causes spermatozoa to swell under hypo osmotic conditions when an influx of water which results in an expansion of cell volume (Liu et al., 1989; Misro and Chaki, 2008). The plasma membrane may be physically intact but chemically nonfunctional which may impair the ability of spermatozoa to undergo capacitation, acrosome reaction and bind to the zona pellucida. Through HOS test the ability of the plasma membrane to transport water by subjecting the spermatozoa to hypo-osmotic conditions can be measured. If water transport has occurred, the cytoplasmic volume of the sperm tail will greatly increase, causing the tail fibers to coil within the expanded plasma membrane. Tail coiling can be seen under phase contrast microscope. The test is also useful for detection of damage during cryopreservation, toxic effect of drugs and chemicals to assess the quality of spermatozoa. In the present study, asthenozoospermia and idiopathic cases showed a normal response for HOS and the rest of the cases showed lesser value from the normal range. This indicates that less motility of the spermatozoa in asthenozoospermic condition in the present study is not due to defect in the plasma membrane but might be due to mitochondrial damage and the presence of cytoplasmic residues.

The biochemical constituents of seminal plasma routinely studied are fructose and citric acid. Fructose a readily glycolysable sugar is produced in humans mainly by the seminal vesicle with a small contribution from the ampulla of the ductus deferens and is essential for spermatozoa metabolism and motility as an energy source (Schoenfeld et al., 1979). Absence of fructose indicates congenital absence of seminal vesicle mainly in case of azoospermia. In an individual with a low volume ejaculate, the absence of fructose indicates ejaculatory duct obstruction, seminal vesicle dysfunction or hypoplasia (Aumuller and Riva 1992). In the present study, fructose value and semen volume is normal for all infertile cases. This indicates neither of seminal vesicle agenesis in azoospermic cases nor ejaculatory duct obstruction in other infertile conditions.

As semen owes high calcium ion buffering capacity to citrate is probably the major regulator of ionized calcium levels in seminal plasma (Fong et al., 1986; Magnus et

al., 1990). Citric acid is a reliable indicator of the prostatic function where the absence or low concentration suggests the dysfunction of prostatic glands and decrease in its concentration is observed in cases of inflammation or prostate cancer (Kammer et al., 1991; Costello and Franklin 1998). In the present study we observed decreased citric acid level intern increase in the alkaline nature of semen which indicates some extent of infection, inflammation and prostate gland malformation in all the infertile cases.

CONCLUSION

Clinical diagnosis of male infertility is usually based upon very few basic routine analyses in many parts of the developing countries. This leads to ineffective treatment procedures. Analysis of functional status of sperm function is essential to plan the treatment and also to envisage the possible outcomes. Thus the work carried out helps us to predict the chances of natural pregnancy and also to consider the option of assisted reproductive techniques (ART).

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