Andrology-Open Access

Shivaprasad et al., Andrology 2015, 4:2
DOI: 10.4172/2167-0250.1000137

Research Article Open Access

A Comprehensive and Systematic Study of Semen Quality and Sperm Functional Status in Normozoospermic Controls and Infertile Males from South India

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Abstract

Objective: Despite, several global studies indicate the variations in semen characteristics that accounts for male infertility, the association of specific changes in semen quality and fertility status among different Indian communities are poorly investigated. With wide range of geographical locations, diverse lifestyle patterns, seasonal variations combined with heterogeneous population, India offers an excellent system to study genotype-to-phenotype correlation. Hence, the current study has been initiated in South Karnataka region of India, in order to examine the variations in semen quality and sperm functional status in infertile individuals compared with normozoospermic controls.

Methods: WHO strict guidelines are followed for systematic semen analysis of 239 infertile and 244 normozoospermic control subjects.

Results: Interestingly, compared to normozoospermic controls, higher percentage of physical abnormalities such as, low semen volume and reduced sperm count are observed in infertile men. Additionally, semen characteristics namely, vitality and motility values are significantly reduced in infertile than controls. Further, in sperm function test the lower scores are documented for hypo-osmotic swelling assay, but not for sperm chromatin decondensation and acrosome intactness examination, suggesting loss of sperm plasma membrane integrity in infertile men. Moreover, the observed changes in semen parameters and sperm function are also evident in different infertile sub-conditions with varied responses. Surprisingly, age wise analysis revealed reduction in sperm morphology scores, whereas, vitality, count, motility and volume remain unchanged with increasing age of infertile males. However, we recorded inverse relationship between age and sperm vitality as well as motility in normozoospermic control men. Together, though the scores for different semen parameters in normozoospermic control group are in accordance with WHO reference range, the infertile men displayed poor semen quality.

Conclusion: Thus, our data establishes basic differences between infertile and normozoospermic control group in terms of semen characteristics and sperm functional status, but the cause may be attributable to genetic or environmental factors or interaction of the two, which necessitates further detailed examination in larger cohort among heterogeneous population.

Keywords: Male infertility; Semen features; Sperm function; Normozoospermic; Age group

Abbreviations: WHO: World Health Organization; NCD: Nuclear Chromatin Decondensation Test; HOS: Hypo-osmotic Swelling Test; AIT: Acrosome Intactness Test; OA: Oligoasthenozoospermia; OAT: Oligoasthenoteratazoospermia; NSC: Normozoospermic Control; IF: Infertile; ANOVA: Analysis of Variance.

Introduction

Despite the extensive research in the field of human reproductive biology, the causes of infertility in different population/communities are relatively less understood. The main reason for this lack of information is attributable to multifactorial etiology and in general, infertility is regarded as a heterogeneous disorder. Considering infertility affects approximately 15% of the couples in their middle of reproductive age [1], it is important to diagnose the causes in order to provide clinical assistance to the couples. In country like, India infertility is a socially traumatic condition associated with social stigma along with parental and family member's pressure on the infertile couples to attain biological parenthood [2]. Surprisingly, in this situation most commonly female partners are held responsible for the condition and are subjected to clinical investigation. Given that, the infertility is credited towards the male factors in 50% of the cases [3], it is necessary to examine the male counterpart for various clinical conditions such as, low sperm count, defects in sperm morphology, altered motility and/or other abnormal sperm characteristics that adversely impact on sperm production, thereby impairs fertilization [4]. Thus, in principal the basic information gathered during the semen collection, along with physical examination of semen and systematic functional evaluation of the spermatozoa may assist a clinician to provide medical assistance to the couples based on the observed infertility condition.

As a general rule, an event of successful fertilization depends on the healthy sperm that swims through female genital tract to reach the oocyte and further, enters through the investment of oocyte. Later,

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Received June 09, 2015; **Accepted** July 08, 2015; **Published** July 20, 2015

Citation: Shivaprasad HS, Sreenivasa G, Kavitha P, Malini SS (2015) A Comprehensive and Systematic Study of Semen Quality and Sperm Functional Status in Normozoospermic Controls and Infertile Males from South India. Andrology 4: 137. doi:10.4172/2167-0250.1000137

sperm nuclear chromatin decondenses to form a male pronucleus in order to fuse with female pronucleus, resulting in embryo formation [5]. Standard semen analysis including physical examination of the semen features and microscopic investigation of the sperm has been considered as a primary laboratory test to screen the infertile couples. However, this routine semen analysis fails to provide insights into the functional competency of spermatozoa, thereby this approach be unsuccessful in predicting the maturation process of sperm, which is an essential step to achieve successful fertilization with an ovum [6-8]. For example, Hyaluronidase is an important acrosomal enzyme required for the dissolution of the cumulus oophorus matrix containing hyaluronic acid surrounding oocyte. Deficiency or absence of this acrosomal enzyme leads to infertility in men [9]. Similarly, the subtle defect in the sperm plasma membrane modifies the functional integrity as well as the sperm morphology, which may be a contributing factor for male infertility [10,11]. Taken together, the routine semen analysis reveals basic information for initial evaluation, but not a critical test for screening infertility.

Having said that the semen analysis has to be accompanied with sperm functional assays, which indirectly measure the capability of a spermatozoa to fuse with an egg [12]. For this process, the spermatozoa has to be produced in adequate numbers with normal motility, count and morphology, and then it has to pass through the cervical mucus, uterus, and ampullae of the oviducts wherein it undergoes capacitation, acrosome reaction, zona pellucida binding, and nuclear decondensation. Defects in any of one of the above mentioned events results in male infertility [13,14]. Thus, it is extremely important to understand the sperm functional tests and rationale behind them, before declaring the fertility status of an individual and providing reproductive assistance to the infertile couples.

In the last two decades, several global reports from various world populations have successfully drawn attention towards the decline in the semen quality in terms of sperm count, motility and normal sperm morphology [15-21]. Surprisingly, individuals with <40 million/mL of total sperm count are implied to experience reduced fecundity [22]. On contrary, the semen volume and sperm count remain significantly unaltered in the healthy volunteers of Sapporo, Japan [23]. While, the global inconclusive temporal trend in semen quality is still on debate, the regional differences in the semen quality are reported from parts of USA, Europe, Japan, India and China [24-30]. For instance, the sperm concentration of Danish fertile men is 74% of Finnish men and 82% of Scottish men [26]. Further, the semen quality of Japanese fertile men is similar to Danish men, which has lowest values among the European males [24]. Meanwhile, a number of studies indicate the association of increased age with decline in the semen characteristics [31-34] whereas, a few reports showed no such association [35-36]. Altogether, there is a possibility that combination of genetic and environmental factors including nutritional, geographical, life style, and their interactions accounts for variations observed in the semen parameters that are studied across different populations.

Most importantly, in many instances authors have not strictly followed the WHO guidelines while grouping their study subjects and in few cases, results are solely interpreted based on the data obtained from routine semen analysis. Considering all these issues, in order to establish the infertility condition in an individual based on semen characteristics it is extremely important to perform sperm functional assays along with routine semen analysis and compare the observed phenotypes to normozoospermic control individuals residing in the same geographical area. Thus, the current study has been initiated in the South Karnataka region of India, where no such systematic study reports are available till date.

Here, we examined (i) the quality of semen and variations in semen parameters such as, sperm count, motility, vitality and morphology (ii) functional status of sperm by employing a specific sperm functioning tests, and (iii) finally, association between the subject's age and the quality of semen in clinically proven infertile and normozoospermic control males are analysed. Our findings suggest that the values for semen parameter and sperm function test performed in normozoospermic control males are in accordance with WHO reference values and compared to control samples, higher percentage of abnormalities in semen are observed in infertile males. Interestingly, age wise analysis showed inverse relationship for sperm vitality and motility among normozoospermic control and morphology in infertile men.

Materials and Methods

Study design and the recruitment of subjects: Before conducting the current study, the institutional ethical committee clearance (IHEC-UOM No.50/Ph.D/2011-12) was obtained from the University. 247 infertile men and 244 individuals with proven fertility having normal semen characteristics (normozoospermic control), aged between 20-45 years were recruited respectively as a case and control. Due to ejaculatory failure and sample issues, eight cases excluded from infertile group and restricted to 239 samples for the analysis. Initially, all the subjects were clearly instructed about the purpose of the study as well as the sample collection procedure and its usage, subsequently informed written consent letters were obtained. Following the careful clinical examination from an experienced male infertility specialist at the respective hospitals/clinics in Mysore from where the subjects were enrolled for the study, the semen samples were collected after determining the fertility status. In addition, a detailed family history, occupation, health related details and other necessary information that were gathered by means of pre-designed Perfoma. In this study the individuals with known causes of spermatogenic failure such as, hypo gonadotropic hypogonadism, bilateral cryptorchidism, congenital absence or surgery of the vas deferens were excluded, in addition to individuals with retrograde ejaculation, obstructive azoospermia, age issues, health problem and individuals not volunteering for this study, subjects who had a history of any contagious infections/allergies or any other disease and disorders were also excluded.

Conventional semen analysis: Prior to semen collection, individuals were recommended 3 to 5 days of sexual abstinence. By means of masturbation the semen sample was collected in a sterile container and allowed it to liquefy at 37°C for 30 minutes. Furthermore, according to the WHO guidelines [46-49] the semen analysis was performed within one hour after ejaculation. The analysis includes physical examination of semen such as, colour, odour, pH, liquefaction time and total semen volume, whereas microscopic investigation comprises of sperm concentration, sperm morphology, viability and sperm counting using Neubauer counting chamber. By employing eosin-nigrosin staining method sperm viability was assessed, while by documenting the rapidly progressive motile spermatozoa (a) and the sluggish progressive motile spermatozoa (b), the percentage of total motile spermatozoa were calculated (a+b) that accounts for total sperm motility.

Sperm function tests

i. **Nuclear chromatin decondensation (NCD) test:** With minor modifications, previously described NCD test was adopted, which enables the visualization of in vitro decondensation of spermatozoon nuclear material. Briefly, the semen pellets were washed with 0.05M borate buffer [49]. Later, 1:9 volumes of the semen sample and EDTA-SDS containing glutaraldehyde, was incubated for an hour at 37°C. Using a pipette, 10 µL of the incubated sample was placed on a clean glass slide and sealed with a cover slip. The slides were visualized under the phase contrast microscope (Olympus, USA) with 40X objective. Approximately 200 spermatozoa were examined for the presence of condensed or decondensed heads. If more than 70% of the spermatozoa displayed decondensed nuclear chromatin network, then it was considered as normal.

ii. Hypo-osmotic swelling (HOS) test: In order to analyze the integrity of the sperm plasma membrane HOS assay was performed as illustrated by previous studies [10]. Initially, the semen sample was diluted in an equal volume of aqueous fructose and sodium citrate solution, was incubated at 37°C for 30 minutes. Subsequently, 10 µL of the incubated sample mixture was placed on a clean glass slide and covered with a cover slip. Approximately 200 spermatozoa were observed for the nature of their plasma membrane. If the observed values were more than 60%, then the condition was considered as normal.

iii. Acrosome intactness test (AIT): The quality for the enzymes present in the spermatozoan acrosome was examined by employing AIT [49]. Primarily, 1:5 ratio of liquefied semen sample and phosphate buffer saline- D-glucose was incubated at 37°C for 10 minutes. A drop of incubated semen sample smeared on gelatin slide (NIHFW, New Delhi) was placed inside a petridish containing a moistened filter paper. After 2 hours of incubation at 37°C, the slides were examined under phase contrast microscope with 40X magnification. The presence of halos surrounding the spermatozoa head was investigated and if the observed values were more than 50%, then it was regarded as normal.

Statistical analysis

In the present case-control study, to evaluate the significant values, SPSS statistical software version 20 (IBM, SPSS Statistics) was employed. For continuous variables, mean, standard deviation and standard error values were calculated. Further, Chi-square test and independent sample t-test analysis was performed for different sperm parameters among case and control group. In addition, one way ANOVA test was applied between different infertile conditions and normozospermic control males to observe any deviation in the sperm characteristics. Also, the relationship between different age groups and the observed semen parameters within the case and control groups, as well as between the two groups were independently analysed. p value ≤ 0.05 was considered as statistically significant.

Results

Clinical evaluation of various infertile conditions

The current study involves a systematic assessment of 239 infertile and 244 normozoospermic control men for various semen parameters. Primarily, based on clinical examination and semen analysis, the infertile group has been categorized into 54 asthenozoospermia (22.5%), 54 azoospermia (22.5%), 41 idiopathic (17.5%), 14 oligoasthenoteratazoospermia (OAT) (5.8%), 37 oligoasthenozoospermia (OA) (15.4%), 34 oligozoospermia (14.2%) and 2 teratazoospermia (2.09%) cases (Figure 1A).

Further, the study subjects from both infertile and normozoospermic control groups have been classified into 20-30 years, 31-40 years and 41-45 years age groups. Average age documented in each subgroup for infertile and normozoospermic control categories are comparable. For instance, 26.5 ± 2.61 years and 27.9 ± 2.02 years are mean age of normozoospermic control and infertile individuals respectively in 20-30 years category. Similarly, 35.1 ± 2.91 years (normozoospermic control) and 35.1 ± 2.72 years (infertile) for 31-40 years age group, and 43.4 ± 2.11 years (normozoospermic control) and 43.1 ± 2.14 years (infertile) among 41-45 years. This age wise classification accounts for any changes observed among infertile and normozoospermic control men of similar age and also, allows the comparison as well as the assessment of semen parameters across different age groups. Thus, in the current study in addition to broader analysis of infertile and control group, we have narrowed the investigation to different age groups as well.

Physical assessment of the semen characteristics

Semen sample from infertile and normozoospermic control subjects are compared for various physical features that include colour, odour, volume, pH and liquefaction time (Table 1). As expected, higher percentage of abnormality in odour, volume, pH and liquefaction time are observed in infertile males than controls. Independent t-test showed a statistical significance for all the semen characteristics that are examined among the two groups (Table 1). Interestingly, although both the groups displayed similar average values for total semen volume in different categorized groups (Figure 1B), the number of normozoospermic control individuals with more semen volume are higher in comparison to infertile men (Figure 1C). To our surprise, compared to 18.44% of controls, 29.14% of infertile men exhibited lesser than 1.4mL of semen volume and in contrast to 27.45% of controls, who showed more than 2.5ml of semen than infertile individuals (12.14%) (Figure 1C). Taken together, the physical examination of semen suggests that the semen collected from infertile subjects is relatively of poor quality than controls.

Microscopic examination of the semen sample

Further, variations in the physical features of the infertile semen sample prerequisite for microscopic examination, which unravels the changes in the motility, vitality, count and the morphology of the semen, if any. Though, the sperm morphology of infertile is indistinguishable from the controls, the average scores for vitality (44.3 ± 23.4) and motility (34.9 ± 21.4) are significantly reduced in infertile individuals compared with normozoospermic control men as well as with WHO reference values (Figure 2A). Notably, as opposed to controls (0%), 49.7% of the infertile individuals displayed less than 10 million/mL of sperm count. In addition, we observed a linear decrement in the number of infertile men with an increasing sperm count (Figure 2B). Thus, the reduced potential nature of spermatozoa may results in the failure of fertilization and thereby, attribute for infertility. This is evident from variations in the sperm characteristics in different infertile conditions (Table 2). For instance, subjects with OAT condition showed severe reduction in the sperm vitality, count and motility, but with no modifications in the sperm morphology. Similarly, diminished sperm motility has been documented in the asthenozoospermia and OA cases, whereas, oligozoospermia and OA individuals showed decreased sperm count compared to normozoospermic control subjects (Table 2). Statistical significance values calculated by ANOVA test for morphology, vitality,

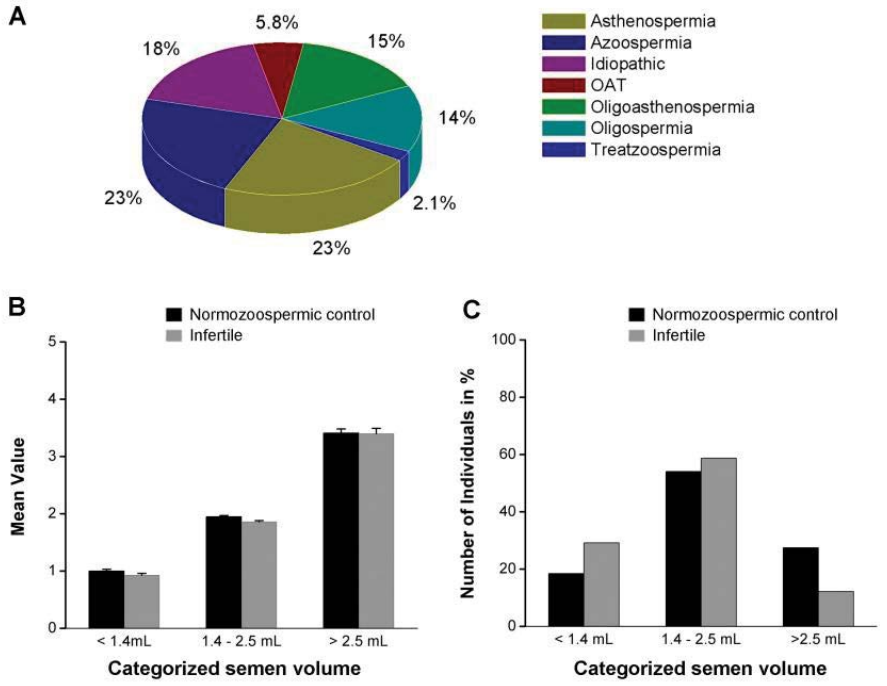

Figure 1: Illustration of various clinical conditions and assessment of semen volume. (A) Pie chart shows the incidence of different infertile subconditions in the present study subjects. (B) Comparison of semen volume among normozoospermic controls and infertile individuals. Error bars indicate mean ± SD. (C) Histogram shows the number of individuals (in percentage) belongs to different categorized semen volume groups in case and control groups. Black and grey bars correspond to Normozoospermic and infertile groups, respectively

Sl. No.	Semen Variables	Normozoospermic controls		Infertile		*t* value	*p* value*
		Normal	Abnormal	Normal	Abnormal		
1	Colour	78.2%	21.7%	87.8%	12.1%	2.80	0.005
2	Odour	95.9%	4.09%	80.9%	19.2%	5.35	0.001
3	Volume	81.1%	18.8%	69.8%	30.1%	5.18	0.001
4	pH	41.3%	58.6%	23.0%	77.9%	4.45	0.001
5	Liquefaction	87.2%	12.7%	64.4%	35.5%	5.98	0.001

**p* values were determined by independent *t*-test between normozoospermic controls and infertile men $p<0.05$ considered as statistically significant.

Table 1: Independent *t*-test analysis for physical examination of semen among normozoospermic controls and infertile males.

count and motility between subjects with infertile sub-conditions and normozoospermic control males demonstrated a significant difference between the groups (Table 2).

As stated before, we have assessed the correlation between the semen parameters in different age groups of the study subjects (Table 3). For this purpose, in addition to comparison between the same age group of infertile and control individuals, assessment among the categorized age groups of both case and controls are performed. Across all the age groups the semen vitality, count and motility are significantly reduced in the infertile cases than normozoospermic control individuals. However, the sperm morphology remains unchanged in both the groups (Table 3 and Figure 2C and 2D). Additionally, ANOVA test conducted within the different groups of infertile demonstrated statistical significant differences for vitality p=0.001 and motility p=0.03 within the normozoospermic control group, whereas in the infertile group significant difference are recorded for morphology p=0.007, but not for other semen parameters. In specific, 41-45 years of infertile age group showed an increase in 9.5 million/mL of mean sperm count, 10.5% of vitality and 1.65% increase in motility as compared to 20-30 years individuals. However, the total mean value for vitality and motility remain lower weighing against the WHO reference values. Similarly, the same age group (41-45 years) in normozoospermic control demonstrated 12.4 million/mL increase in the mean sperm count, whereas 10% and 9.05% decrement in the vitality and motility are respectively documented. Given that there no change in the morphology scores, all the observed values for remaining semen parameters are above the WHO reference values.

Assessment of the sperm function tests

About mentioned approaches clearly demonstrate the alteration in the semen characteristics as well as establishes the difference between

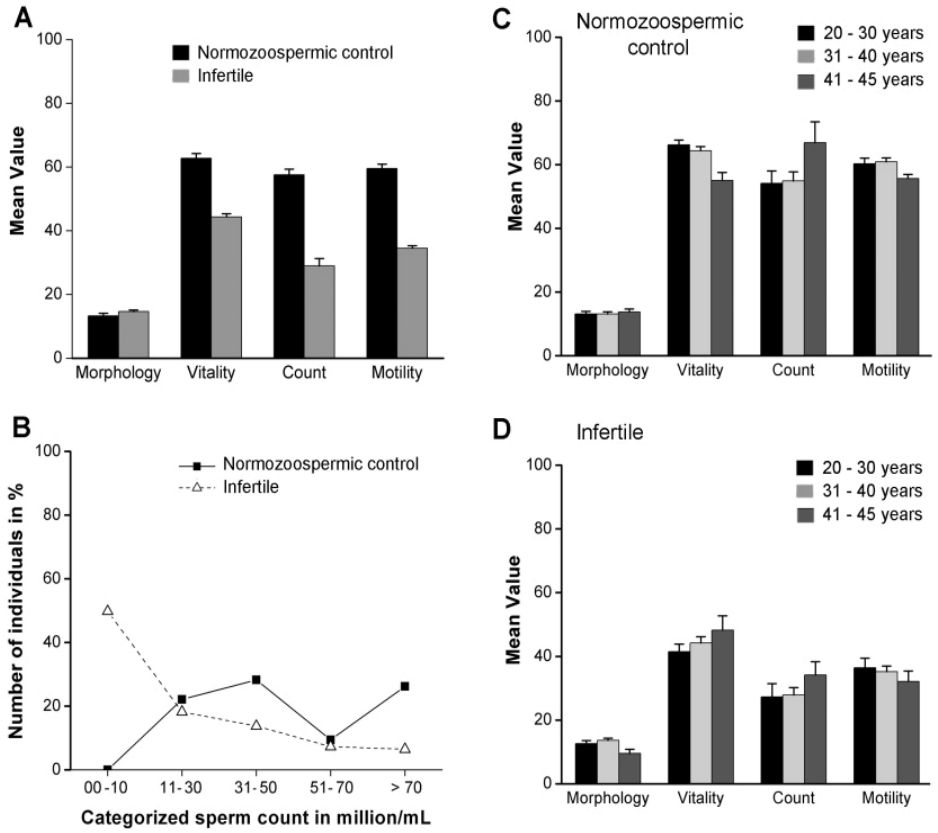

Figure 2: Microscopic examinations of sperm characteristics. **(A)** Histrogram shows the average scores for different sperm parameters in normozoospermic controls and infertile groups. *p* values are calculated using Independent test and $p<0.05$ considered as statistically significant. **(B)** Classified subgroups for total sperm count in case and control males. Straight and dotted lines respectively represents the control and case values. **(C)** and **(D)** Assessment of different semen parameters in categorized age group of normozoospermic controls and infertile men. Black, light grey and dark grey bars correspond to 20-30 years, 31-40 years and 41-45 years age groups, respectively. Error bars indicate mean ± SD. *p* values are calculated using ANOVA test, $p<0.05$ considered as statistically significant. Azoospermic cases were excluded from this analysis

Sl. No.	Semen Variables	NSC Mean ± SD	AS Mean ± SD	IP Mean ± SD	OAT Mean ± SD	OA Mean ± SD	OS Mean ± SD	TS Mean ± SD	F	*p* value*
1	Morphology	13.2 ± 7.3	12.4 ± 12.3	15.6 ± 9.2	8.28 ± 15.3	16.8 ± 14.0	18.2 ± 12.9	2.6 ± 1.1	4.12	0.001
2	Vitality	62.7 ± 15.3	42.9 ± 21.9	61.9 ± 17.7	14.0 ± 19.5	38.1 ± 25.2	45.3 ± 15.2	62.4 ± 20.8	32.8	0.001
3	Count	57.5 ± 36.7	46.0 ± 30.9	51.1 ± 21.1	6.89 ± 4.4	7.78 ± 3.5	7.95 ± 3.3	40.2 ± 21.6	27.7	0.001
4	Motility	59.5 ± 12.9	24.0 ± 11.4	55.4 ± 12.4	13.7 ± 18.3	18.4 ± 14.6	53.7 ± 12.8	51.8 ± 5.8	112.8	0.001
5	NCD	66.2 ± 15.2	71.0 ± 15.4	74.2 ± 11.6	63.5 ± 12.9	60.3 ± 16.4	64.9 ± 15.9	48.0 ± 23.9	4.89	0.001
6	HOS	65.2 ± 14.2	54.4 ± 20.1	63.4 ± 14.3	39.3 ± 16.0	45.1 ± 18.0	55.0 ± 18.1	44.8 ± 19.8	16.4	0.001
7	AIT	51.5 ± 15.9	50.9 ± 16.1	57.0 ± 9.5	44.2 ± 17.9	42.9 ± 16.3	51.0 ± 13.8	30.0 ± 16.5	4.74	0.001

NSC: Normozoospermic controls, AS: Asthenospermia, IP: Idiopathic, OAT: Oligoasthenoteratazoospermia, OA: Oligoasthenospermia, OS: Oligospermia, TS: Teratazoospermia and Azoospermic cases were excluded from this analysis. **p* values were determined by ANOVA test, $p<0.05$ considered as statistically significant.

Table 2: Showing the mean ± SD values for normozoospermic controls and among different infertile sub-conditions.

infertile and normozoospermic control subjects. Furthermore, in order to examine the functional status of the semen following assays are performed for all the study samples, which illustrate the condensation ability of the nuclear material, integrity of the plasma membrane and quality of the acrosome (Figure 4B,4C and 4D). NCD assay unravels the decondensation ability of the sperm DNA, which is essential for fusion of male pro-nuclei with female pro-nuclei and any subtle change in this process may alter the motility and morphology of spermatozoa. While, the HOS is an inexpensive functional test for examining the integrity of sperm plasma membrane, which is demonstrated by a functionally active and intact membrane in the mid-piece and tail region of spermatozoa that allows the entry of hypo osmatic solution/water inside the cell resulting in typical tail swelling. Further, assessment of acrostic using AIT assay indicates the fertilizing ability of the sperm. Normal acrostic activity increases the chances of fertilization, whereas the halo diameters and halo formation in most cases causes decreases in

Study Variables	20-30 Years		*p* value*	31-40Years		*p* value*	41-50 Years		*p* value*
	NSC	IF		NSC	IF		NSC	IF	
Subjects in %	28.27%	25.1%	_	48.36%	58.29%	_	23.36%	16.59%	_
Mean age	26.5 ± 2.6	27.9 ± 2.0	_	35.1 ± 2.9	35.1 ± 2.7	_	43.4 ± 2.11	43.1 ± 2.1	_
Morphology	13.1 ± 6.7	9.28 ± 8.4	0.005	13.1 ± 7.6	9.50 ± 9.2	0.001	13.7 ± 7.3	8.28 ± 8.3	0.001
Vitality	66.2 ± 12.6	30.4 ± 24.3	0.001	64.4 ± 13.5	32.2 ± 28.3	0.001	55.0 ± 18.9	41.8 ± 30.6	0.001
Count	54.1 ± 32.6	20.0 ± 29.9	0.001	54.9 ± 30.9	20.4 ± 26.4	0.001	66.9 ± 49.6	29.7 ± 26.3	0.001
Motility	60.3 ± 14.5	26.7 ± 25.6	0.001	69.0 ± 12.9	25.8 ± 23.9	0.001	55.6 ± 10.1	27.9 ± 21.9	0.001
NCD	69.7 ± 13.7	65.6 ± 15.9	0.15	64.1 ± 15.8	68.3 ± 14.8	0.04	66.2 ± 15.2	67.7 ± 16.1	0.65
HOS	67.8 ± 12.4	55.1 ± 18.4	0.001	65.0 ± 14.6	51.1 ± 18.9	0.001	62.5 ± 15.1	57.5 ± 17.7	0.16
AIT	52.9 ± 16.7	52.0 ± 12.8	0.76	50.8 ± 16.0	48.9 ± 16.2	0.38	51.4 ± 14.8	49.9 ± 15.6	0.63

Azoospermic cases were excluded from this analysis. *$p<0.05$ considered as significant.

Table 3: Illustration of mean and standard deviation values along with independent t-test for different categorized age group among normozoospermic controls (NSC) and Infertile (IF) males.

fertilization capacity of the sperm. Given that these functional features are essential requirement of sperm for successful fertilization with an egg, these functional assays suggest the fitness of the spermatozoa and may indicate the possibility of fertilization.

Compared to normozoospermic control semen and WHO reference values, the infertile mean values for both NCD and AIT test are indistinguishable (Figure 3A) and thereby, rule out the changes in the nuclear material decondensation and alteration in acrosome enzyme activity, respectively. However, HOS average values (52.9 ± 19) of infertile males are significantly different from controls (Figure 3A), suggesting changes in the integrity of the plasma membrane. Surprisingly, though the total mean values for NCD test in infertile are comparable to controls, the reduced average scores are observed in the OAT, OA, oligozoospermia and teratazoospermia cases. Similarly, OAT, OA and teratazoospermia samples showed lower mean scores for AIT, whereas except idiopathic cases all the infertile subconditions displayed lower mean scores for HOS test. One way ANOVA test between various infertile subgroups and normozoospermic control males demonstrated significant difference (p=0.001) for NCD, HOS and AIT tests (Table 2).

Analogous to age group analysis performed after microscopic examination of the semen sample, subsequent to functional assays association between the subject's age and functional status of the sperm are investigated (Table 3; Figure 3B and 3C). In comparison with NCD reference values, the observed NCD values for infertile and controls across all the age groups are within the subnormal range. However, the HOS test in infertile individual's showed 12.7% (20-30 years), 13.9% (31-40 years) and 5% (41-45 years) decrease in the values compared to their respective age controls in the normozoospermic control group (Table 3; Figure 3B and 3C). In addition, similar to NCD results AIT values are also remains unchanged among infertile and control samples. Taken together, independent t-test analysis determined statistical significant differences for HOS (p=0.001) but not for NCD and AIT among infertile and normozoospermic control groups (Table 3). Finally, age wise study within the infertile and normozoospermic control group using one way ANOVA fails to determine any statistical difference for all the sperm function analysis tests.

Discussion

Here by employing systematic approaches the quality of semen and the functional status of sperm in infertile and normozoospermic control individuals are investigated. Although, several studies have made an attempt to address these issues in the heterogeneous population of South Karnataka [29,37-38], the lack of complete profiling of semen characteristics necessitates the importance of current study. Thus, after characterising the semen quality and its functional status in the study subjects, we have analysed as well as discussed the observed results in the light of previously published Indian as well as global reports. A comparative analysis of different semen parameters that are studied in an assorted population of infertile and fertile men has been summarized in the Tables 4 and 5 respectively.

In this context it is worth mentioning that unlike earlier reports, by recruiting approximately same number of infertile and normozoospermic control subjects the current study rule out the possibly introduction of sampling error. Furthermore, as mentioned before the average age of study cohorts in both the groups are comparable, which is in contrast to mean age ranging from 20 to 40 years for fertile individuals and 31 to 40 years among infertile that are reported previously [37]. In addition to characterization of semen features in both groups, we have analysed the observed results across the categorized age groups among infertile and controls. Finally, WHO guidelines are strictly followed while considering the individuals to normozoospermic control group, whereas the reported studies have followed different criteria while grouping individuals to fertile group.

Importantly, the observed variations in the mean values of different semen parameters between the case and control groups are in consistence with earlier reports [8,24,35,37-40]. Additionally, the values recorded in our study for various semen parameters in infertile subjects are mostly comparable to studies conducted in other populations such as, USA, Europe, China, Japan and Korea, with inconsequential deviations. For instance, sperm count of the infertile individuals is in agreement with values reported in the South India study [29] and weighing against our lab reports, there are no deviations observed in the mean values [37-38]. However, lowest (10.5 million/mL) and highest (96.1 million/mL) average values for sperm count are respectively documented in Austrian and Tunisian men [21,40]. Similarly, compared to WHO reference values, USA, Scotland and Tunisian population, we have recorded lower sperm motility that fall in between Riyadh population and previous reports from our lab [37,38,40]. Further, lower averages are documented in our study for total semen volume in comparison with any other population except, Tunisia and Riyadh population, wherein higher scores are authenticated. Finally, the percentage of normal sperm morphology displayed intermediate scores between Austria and South Indian population [21,29,37-40].

Normozoospermic control individuals in our study showed higher sperm count than the WHO reference values and conversely correspond

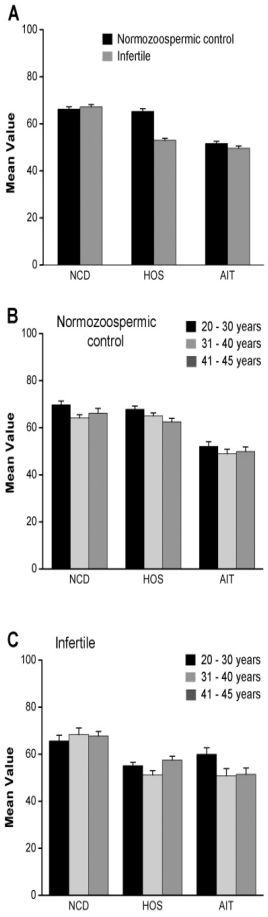

Figure 3: Functional status of the spermatozoa. **(A)** Illustration of total mean ± SD scores for NCD, HOS and AIT functional tests in normozoospermic controls and infertile males. Black and grey bars correspond to normozoospermic controls and infertile groups, respectively. *p* values are determined by Independent t-test and $p<0.05$ considered as statistically significant. **(B)** and **(C)** Total mean ± SD values for different sperm function test in categorized age group among normozoospermic controls and infertile men. Black, light grey and dark grey bars correspond to 20-30 years, 31-40 years and 41-45 years age groups, respectively. Error bars indicate mean ± SD. *p* values are calculated using ANOVA test, $p<0.05$ considered as statistically significant. Azoospermic cases were excluded from this analysis

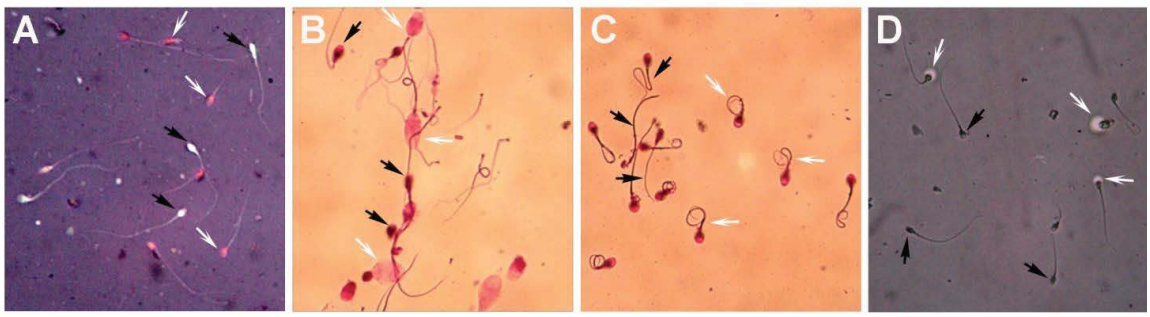

Figure 4: Representative images for sperm vitality and sperm function tests. (A) Image shows the sperm vitality by using eosin-nigrosin staining technique. Spermatozoa that are stained with pink colour (white arrow) indicate dead sperms, whereas white color (black arrow) corresponds to live sperms. (B) NCD test for spermatozoa. Black and white arrows respectively indicate the negative (condensed head) and positive (decondensed head) response for the assay. (C) Image for HOS test. Spermatozoa with curled tail represent positive response (white arrow) and uncurled tail spermatozoa indicate negative response (black arrow). (D) Representation of AIT test. Spermatozoa with halo formation near the head region represent positive reaction (white arrow), while absence of halo formation shows negative response (black arrow). All the images are acquired using phase contrast microscope with 40X magnification.

Sl. No.	Authors	Country	Published year	Number of subjects	Mean age in years	Mean semen volume in mL	Mean count in million/mL	Mean motility in %	Mean morphology in %	Study Subjects
1	Seo et al. [36]	Korea	2000	22,249	32	N/A	60.5	N/A	N/A	Infertile males
2	Guzick et al. [8]	USA	2001	765	34.7	N/A	52	49	11	Infertile males
3	Marimuthu et al.	North India	2003	1176	31.2	2.6	60.6	N/A	N/A	Infertile males
4	Lackner et al. [39]	Austria	2005	7780	31.6	N/A	10.25	21	15	Infertile males
5	Sripada et al [50].	Scotland	2007	4832	34	N/A	61	49	N/A	Infertile males
6	Adiga et al. [29]	South India	2008	1610	N/A	2.64	26.61	47	19.7	Infertile males
7	Feki et al. [21]	Tunisia	2009	2940	36	3.2	96.1	44	25	Infertile males
8	Kavitha et al. [37]	South India	2010	447	39.4	2.30	24.94	33.9	N/A	Infertile males
9	Sreenivasa etal. [38]	South India	2011	132	N/A	N/A	29.85	36.9	N/A	Infertile males
10	Nadia and Aleisa [40]	Riyadh	2012	160	35.65	2.97	39.38	39.28	29	Subfertile males
11	Present Study	South India	2015	239	34.6	1.77	28.96	34.49	14.67	Infertile males

N/A: Data Not Available.

Table 4: Comparative analysis of various semen variants in infertile individuals reported by different global and Indian studies.

Sl. No.	Authors	Country	Published year	Number of subjects	Mean age in years	Mean semen volume in mL	Mean count in million/ mL	Mean motility in %	Mean morphology in %	Study Subjects
1	Auger et al. [17]	Paris	1995	1351	32	3.8	60	66	45	Fertile men
2	Irvine et al. [18]	Scotland	1996	577	27	3.4	104.5	61.3	N/A	Unknown fertility
3	Bilotta et al. [19]	Italy	1999	1068	N/A	N/A	61	66	63	Fertile men
4	Guzick et al. [8]	USA	2001	696	33.5	N/A	67	54	14	Fertile men
5	Jørgensen et al.	Denmark	2001	349	31.5	3.8	77	60	49	Fertile men
6	Jørgensen et al [26].	Paris	2001	207	32	4.2	94	56	50	Fertile men
7	Jørgensen et al [26].	Finland	2001	275	30	4.1	105	66	52	Fertile men
8	Jørgensen et al [26].	Edinburgh	2001	251	32.5	3.9	92	67	50	Fertile men
9	Iwamoto et al [24].	Japan	2006	324	32.5	3.7	53	62	42	Fertile men
10	Nallela et al [41]	USA	2006	56	N/A	N/A	69.9	72.5	37.7	Fertile men
11	Li et al. [34]	China	2009	1346	20-40	2.3	77.8	70.9	N/A	Fertile men
12	Kavitha et al. [37]	South India	2010	100	39.1	1.48	53.11	57.5	N/A	Fertile men
13	Srinivasa et al [38].	South India	2011	20	N/A	N/A	83.12	59.9	N/A	Proven fertility
14	Nadia and Aleisa [40]	Riyadh	2012	49	37	3.03	116	57	45	Fertile men
15	Present Study	South India	2015	244	34.6	2.19	57.54	59.3	14.67	Normozoospermic control males

N/A: Data Not Available.

Table 5: Compilation of different semen parameters that are studied in fertile individuals.

to reports from Paris and Italian population [17,19]. Finland, Scotland and Edinburgh inhabitants displayed highest score for sperm count, meanwhile the lowest values are documented in Japan and South Indian community [18,24,26-27,37,50]. Besides, the observed motility values strongly corresponds to Riyadh and our previous lab reports, whereas the highest scores are reported in USA and Chinese community and lowest in Cleveland, USA [8,34,37-38,40-41]. Further, the mean values of semen volume match up Chinese reports, with higher volume observed in Paris, Finland and Edinburgh population and lower semen volume documented in South India [17,26-27,37]. In comparison to Italy, Paris and Finland men, our subjects displayed lower average scores for sperm morphology [17,19,26]. Taken together, the semen characteristics are observed in infertile and fertile individuals appears to be similar across different population, however, geographical, nutritional, environmental, ethnicity and lifestyle factors (occupation, smoking and alcoholism) along with genetic polymorphism may contribute for observed variations, thereby impact on individual's fertility status.

Several reports demonstrated the inverse relationship between age and the semen parameters [33,35], on contrary other studies showed the negligible influence of age on various semen characteristics [34-36,42]. For example, Kidd et al., (2001) suggested that advanced age is associated with a decrease in semen volume, sperm motility, and sperm morphology, but has no effect on sperm count [30]. Berling and Wolner- Hanssen (1997) study that analysed 718 infertile Swedish men for a period of 10 years reports insignificant decrease in semen volume and increase in morphology and motility, thereby fails to establish a correlation between age and semen parameters [43]. A most recent study showed an inverse effect of age on semen volume, but not on other semen parameters [42]. This ambiguous relationship between age and changes in semen features are also observed in our study subjects. Normozoospermic control males showed statistical positive correlation between age and decline in vitality and motility, but not for other parameters. Among infertile men, except morphology that showed decrement with respect to age, sperm count, vitality, motility and volume scores remain unaltered. Surprisingly, in comparison with 20-30 years and 31-40 years, 41-45 years age group demonstrated increase in sperm count among both infertile and normozoospermic control males (Table 3). This remark necessitates further verification, as the reduced sample number possibly account for the current observation in 41-45 years age group.

Furthermore, to our surprise the sperm functional assay reveals that decondensation of sperm nuclear material and acrosome contents of spermatozoa are normal, however the integrity of the sperm plasma membrane in the infertile individuals are significantly different from normozoospermic control males. Interestingly, previous studies have established that increased heterogenecity of chromatin or low DNA content of sperm results in high occurrence of morphological defects in spermatozoa [44-47]. These findings are further evident from our observation that minimal incidence of morphological defective sperms in both the groups as well as statistically insignificant NCD values between the case and control groups. Besides group investigation, even age wise analysis also showed the variations in the HOS assay, suggesting the defects in the functional integrity of sperm plasma membrane, which in turn affects the in vitro fertilization ability of the spermatozoa.

Conclusion

In summary, compared to WHO reference values, increased abnormalities in physical features of semen and defects in sperm functions are observed in infertile individuals, whereas the values are in accordance with WHO recommendations for normozoospermic control group. Thus, the outcome of this study suggests that clinical diagnosis of male infertility condition can be clearly determined by simple basic semen analysis combined with sperm functional tests. However, in country like India, which exhibits world's largest heterogeneous population with varied geographic patterns, seasonal climatic conditions, diverse life style practices and different socioeconomic status, it is necessary to consider individual's background prior to detailed semen examination, as these aspects also influence the individual's fertility status. In addition, by this standard approach if there are any variations in the semen characteristics across different communities can also be determined. Taken together, we suggest that meticulous clinical investigation along with demographic details facilitate the appropriate diagnosis of infertile condition, thereby envisage the chances of natural pregnancy among the couples or else to consider the option of Assisted Reproductive Techniques (ART) for healthy offspring.

Acknowledgment

SHS was supported by senior research fellowship from Indian Council of Medical Research (ICMR), Government of India, New Delhi (grant number: 1/2/28/12-RCH, Dated: 04.06.2013). Authors are thankful to Madhumala K. S., National Centre for Biological Sciences - TIFR, Bangalore and lab members of MRHGL, University of Mysore, for their valuable inputs and comments during manuscript preparation. We acknowledge Institution of Excellence (IOE), University of Mysore, Mysore for providing Central Instrumentation facility. We thank Prof. M. R. Gangadhar, Department of Studies in Anthropology, and University of Mysore for his kind support.

Author Contributions

Project conception and experimental analysis: SHS, SG and KP; Data and statistical analysis: SHS and SG; literature review and manuscript preparation: SHS; Project supervision, manuscript review: SSM and SHS.

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