FINAL PROGRESS REPORT

(2011-2014)

UGC ORDER NO.F.40-286/2011SR, DATEED 29.6.2011

AN ANALYSIS OF THE EFFECT OF OBESITY ON MALE FERTILITY RATES IN KARNATAKA

Submitted

by

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ANNUAL REPORT OF THE WORK DONE FOR THE PERIOD

09-11-2011 to 30-06-2014

1. Project title: **An Analysis of the Effect of Obesity on Male Fertility Rates in Karnataka**

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4. Date of start: **09-11-2011**

5. Duration**: 29. 09. 2011 to 01. 07. 2014**

DECLARATION

I, **Dr. M. R. Gangadhar** hereby declare that the final progress report submitted by me to the UGC, New Delhi, encompass the findings of the work carried under the UGC sanctioned Major Research Project titled "**An Analysis of the Effect of Obesity on Male Fertility Rate in Karnataka**", and the same has not been submitted elsewhere for any other purpose.

Dr. M. R. Gangadhar

ACKNOWLEDGEMENTS

I am extremely grateful to the University Grants Commission for sanctioning the project and providing the necessary funding to implement and sustain the undertaken work.

I thank University of Mysore for providing the necessary infrastructure at the department for the conduct of the research work.

I thank Prof. M. Annapurna, present Chairman, as well as the former Chairman's Prof. N. Ningaiah and Prof. H. K. Bhat, DOS in Anthropology for providing the necessary support for the smooth conduct of the research work.

I also thank the Registrar, Finance Officer and Auditor for rendering necessary support and feedback regarding the official formalities concerned with the expenditures made during the conduct of the project work.

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INTRODUCTION

OBESITY

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have an adverse effect on health, leading to reduced life expectancy and/or increased health problems (WHO, 2000; Haslam and James, 2005). Body mass index (BMI) is a statistical measurement that compares a person's weight and height. Though it does not actually measure the percentage of body fat, but it is a useful tool to estimate a healthy body weight based on a person's height. Due to its ease of measurement and calculation, it is a widely used diagnostic tool to identify obesity problems within a population. However, it is not considered as a final indication for diagnosis. BMI was invented between 1830 and 1850 by the Belgian polymath, Adolphe Quetelet during the course of developing "social physics". BMI is defined as the individual's body weight divided by the square of the height. The WHO considers persons with a BMI over 25 kg/m^2 as overweight (pre-obese), and as obese when BMI is over 30 kg/m^2. Another way to express the severity of overweight/ obesity is by determining waist circumference and waist/hip ratio (Fejes et al., 2005). Although it has been suggested that these may be even better markers for overweight and obesity, the majority of the studies uses BMI as the marker. We therefore use BMI as the indicator of overweight and obesity in the present study. The International Classification of adult underweight, overweight and obesity according to BMI (kg/m^2)

WHO Classification	BMI	Risk of Death
Underweight	Below 18.5	Low
Normal weight	18.5-24.9	Average
Overweight (grade 1 obesity)	25.0-29.9	Mild increase
Obese (grade 2 obesity)	30.0-39.9	Moderate/severe
Morbid/severe obesity(grade 3)	40.0 and above	Very severe

(WHO, 2010)

Historical Account

The dynamic of obesity is changing, but its origins can be traced back 30,000 years, to our prehistoric ancestors including the famous Venus of Willendorf, depicted anatomically accurate abdominally obese women. The function of these caricatures is hotly debated. They may have been fertility symbols – ironic in view of current knowledge that obesity causes infertility. Acceptance of obesity as a medical phenomenon has been slow. For thousands of years, overweight and obesity were exceptional, rarely seen and never studied. In some cultures, indeed, obesity was prized, indicating status and wealth. Hippocrates understood that obesity led to infertility and early death.

Another great physician, Galen (130-200 AD), wrote on food and diet, recounting one of the earliest case studies of obesity management. Morgagni (1765) recognized that obesity was not only linked to disease, but also, the position of the fat was crucial through anatomical dissection. William Banting, who wrote the first commercially available diet programme (Banting, 1864), knew of the breathlessness caused by obesity, and also described the joint pains, often then generically known as ‘gout’; but the co morbidity of deafness led him to seek help from surgeon William Harvey, who identified the cause of his hearing loss as pressure from the fat around his neck compressing the airways.

Prevalence of obesity

Currently more than 1 billion adults are overweight and at least 300 million of them are clinically obese (WHO, 2003). Current obesity levels range from below 5% in China, Japan and certain African nations, to over 75% in urban Samoa. But even in relatively low prevalence countries like China, rates are almost 20% in some cities (WHO, 2003).Obesity has reached epidemic proportions in India in the 21st century, affecting 5% of the country's population (Hindu, 2007). Unhealthy processed food has become much more accessible following India's continued integration in global food markets. Indians are genetically susceptible to weight accumulation especially around the waist and it is essential to aware society regarding the side effects to prevent further complications.

Etiology and risk factors for obesity

Body weight is determined by an interaction between genetic and environmental factors. Although genetic predisposition is important in gaining weight, the marked rise in the prevalence of obesity is best explained by behavioural and environmental changes (Hill and Peters, 1998). A sedentary lifestyle with decreased physical exercise promotes obesity in children, adolescents and adults (Esparza et al., 2000; Maffeis, 2000). Of dietary factors that have been associated with weight gain and obesity, the most important are high fat content, energy density, pleasantness of taste and dietary variety (Bray and Popkin 1998; McCrory et al., 2000). With respect to behavioural aspects, overweight subjects have been reported to have more difficulties in controlling eating, a stronger feeling of hunger, a tendency to engage in emotional eating and binge eating (Wilson et al., 1993; Lindroos et al., 1997; Hakala et al., 1999). Socioeconomic factors such as low levels of education and income and social isolation are also associated with weight gain and predictive of the development of obesity (Kahn and Williamson 1990; Rissanen et al., 1991; Kuczmarski et al., 1994; Pietinen et al., 1996; Rahkonen et al., 1998).

Male Obesity

The obese male is generally characterized as having greater than 25 percent body fat of total body mass with a BMI in excess of 30 kg/m^2 (Phillips and Tanphaichitr, 2010). While obesity affects both men and women, male obesity is seen to be more on the rise. Male obesity poses a risk to general health, leading to infertility, type II diabetes, heart diseases, and a host of other diseases related to eating disorders. The United States is facing a major health problem with the tremendous rise in male obesity in the past few years. It is known that the incidence of obesity is reaching epidemic levels in the western world. For example, in the United States, the incidence of obesity increased from 12% to 17.9% between 1991 to 1999. In the same period, the incidence of male obesity increased from 11.7% to almost 18% (Mokdad et al., 1999). According to male obesity reports in the year 2004, 30.6% of the male population in the U.S was Obese.

Male infertility

Male infertility refers to the inability of a male to achieve a pregnancy in a fertile female within one year after marriage is described as male infertility (Campbell and Irvine, 2000). In humans it accounts for 40-50% of infertility. Male infertility is commonly due to deficiencies in the semen, and semen quality is used as a surrogate measure of male fecundity (Cooper et al., 2009). It is a major cause of concern as 11% of men attending clinics are diagnosed as being oligo or azoospermic with no known reason (Comhaire et al., 1987). It can occur either as an isolated disorder or syndrome.

According to Bhasin et al. (1981), male infertility is a heterogeneous syndrome and therefore it is likely that a multitude of genes and loci will be implicated in different infertility subsets. Although male infertility is associated with impotence, many infertile men have a perfectly normal and happy sexual relationship. Sub fertility is another condition which is characterized by sperm concentration less than 15×10^6/ml, sperm forward progressive motility less than 50%, and normal morphology less than 20% (WHO, 1999).

Semen has two major characters:

1. The total number of spermatozoa: This reflects sperm production by the testes and the patency of the post-testicular duct system.
2. The total fluid volume contributed by the various accessory glands: this reflects the Secretary activity of the glands.

Based on the semen quality like sperm count, morphology, and motility the abnormalities are classified follows. (WHO, 2010)

1. Aspermic: No semen/retrograde ejaculation/ ejaculation failure
2. Asthenozoospermia: Percentage of progressively motile (PR) spermatozoa is lower with respect to reference value.
3. Asthenoteratozoospermia: Percentage of both Progressive motile (PR) and morphologically normal spermatozoa below the lower reference limits.
4. Azoospermia: No spermatozoa in the ejaculate.
5. Oligoasthenozoospermia: Total number of spermatozoa and percent of progressively motile (PR) spermatozoa, below the lower reference limits.

6. Oligoasthenoteratozoospermia: Total number of spermatozoa, and percent of progressively motile (PR) spermatozoa and morphologically normal spermatozoa, below the lower reference limits.
7. Oligozoospermia: Total number of spermatozoa below the lower reference limit.

States	Male (%)	Male rank	Female (%)	Female rank
Punjab	30.3	1	37.5	1
Kerala	24.3	2	34	2
Goa	20.8	3	27	3
Tamil Nadu	19.8	4	24.4	4
Andhra Pradesh	17.6	5	22.7	10
Sikkim	17.3	6	21	8
Mizoram	16.9	7	20.3	17
Himachal Pradesh	16	8	19.5	12
Maharashtra	15.9	9	18.1	13
Gujarat	15.4	10	17.7	7
Haryana	14.4	11	17.6	6
Karnataka	14	12	17.3	9
Manipur	13.4	13	17.1	11
India	12.1	14	16	15
Uttarakhand	11.4	15	14.8	14
Arunachal Pradesh	10.6	16	12.5	19
Uttar Pradesh	9.9	17	12	18
Jammu and Kashmir	8.7	18	11.1	5
Bihar	8.5	19	10.5	29
Nagaland	8.4	20	10.2	22
Rajasthan	8.4	20	9	20
Meghalaya	8.2	22	8.9	26
Orissa	6.9	23	8.6	25
Assam	6.7	24	7.8	21
Chattisgarh	6.5	25	7.6	27
West Bengal	6.1	26	7.1	16
Madhya Pradesh	5.4	27	6.7	23
Jharkhand	5.3	28	5.9	28
Tripura	5.2	29	5.3	24

State-wise Incidence of Obesity in India

- (Karla and Unnikrishnan, 2012)

Annual estimates of Total Fertility rate by residence, India and bigger States, 2005-10

India & Bigger State	Total						Rural						Urban					
	2005	2006	2007	2008	2009	2010	2005	2006	2007	2008	2009	2010	2005	2006	2007	2008	2009	2010
India	2.9	2.8	2.7	2.6	2.6	2.5	3.2	3.1	3.0	2.9	2.9	2.8	2.1	2.0	2.0	2.0	2.0	1.9
Andhra Pradesh	2.0	2.0	1.9	1.8	1.9	1.8	2.2	2.1	2.0	2.0	2.0	1.9	1.7	1.6	1.6	1.6	1.6	1.6
Assam	2.9	2.7	2.7	2.6	2.6	2.5	3.1	3.0	2.9	2.8	2.8	2.7	1.6	1.6	1.5	1.5	1.6	1.6
Bihar	4.3	4.2	3.9	3.9	3.9	3.7	4.4	4.3	4.1	4.0	4.0	3.8	3.2	3.0	2.9	2.8	2.8	2.7
Chhatisgarh	3.4	3.3	3.1	3.0	3.0	2.8	3.7	3.6	3.4	3.2	3.2	3.0	2.2	2.2	2.1	2.0	2.0	1.9
Delhi	2.1	2.1	2.0	2.0	1.9	1.9	2.2	2.1	2.1	2.1	2.0	2.1	2.1	2.1	2.0	1.9	1.9	1.9
Gujarat	2.8	2.7	2.6	2.5	2.5	2.5	3.1	3.0	2.9	2.8	2.8	2.7	2.3	2.3	2.2	2.2	2.1	2.1
Haryana	2.8	2.7	2.6	2.5	2.5	2.3	3.0	2.9	2.8	2.7	2.6	2.5	2.3	2.4	2.3	2.2	2.2	2.0
Himachal Pradesh	2.2	2.0	1.9	1.9	1.9	1.8	2.2	2.1	1.9	2.0	1.9	1.9	1.5	1.4	1.4	1.4	1.3	1.3
Jammu & Kashmir	2.4	2.3	2.3	2.2	2.2	2.0	2.6	2.5	2.5	2.4	2.4	2.2	1.6	1.6	1.5	1.5	1.4	1.4
Jharkhand	3.5	3.4	3.2	3.2	3.2	3.0	3.9	3.7	3.5	3.5	3.4	3.2	2.3	2.2	2.2	2.1	2.2	2.1
Karnataka	2.2	2.1	2.1	2.0	2.0	2.0	2.5	2.3	2.3	2.2	2.2	2.1	1.8	1.7	1.7	1.8	1.7	1.7
Kerala	1.7	1.7	1.7	1.7	1.7	1.8	1.7	1.7	1.7	1.7	1.7	1.8	1.7	1.7	1.7	1.7	1.8	1.8
Madhya Pradesh	3.6	3.5	3.4	3.3	3.3	3.2	4.0	3.9	3.7	3.6	3.6	3.5	2.5	2.4	2.3	2.2	2.3	2.2
Maharashtra	2.2	2.1	2.0	2.0	1.9	1.9	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.8	1.8	1.7	1.8	1.7
Odisha	2.6	2.5	2.4	2.4	2.4	2.3	2.7	2.6	2.5	2.5	2.5	2.4	1.7	1.7	1.7	1.6	1.6	1.6
Punjab	2.1	2.1	2.0	1.9	1.9	1.8	2.2	2.1	2.1	2.0	1.9	1.8	1.9	1.9	1.8	1.8	1.7	1.7
Rajasthan	3.7	3.5	3.4	3.3	3.3	3.1	4.0	3.8	3.7	3.6	3.6	3.3	2.7	2.7	2.6	2.5	2.5	2.4
Tamil Nadu	1.7	1.7	1.6	1.7	1.7	1.7	1.8	1.8	1.7	1.7	1.8	1.8	1.6	1.6	1.5	1.6	1.7	1.6
Uttar Pradesh	4.2	4.2	3.9	3.8	3.7	3.5	4.5	4.4	4.2	4.0	3.9	3.7	3.3	3.2	3.1	3.0	3.0	2.7
West Bengal	2.1	2.0	1.9	1.9	1.9	1.8	2.4	2.2	2.1	2.1	2.1	2.0	1.4	1.3	1.4	1.3	1.3	1.3

Source : Registrar General of India, SRS-2010

MATERIALS AND METHODS

For the purpose of studying in detail the role of several etiologies leading to effect of obesity on male fertility in Karnataka, the following experimental protocols were adopted

- Establishment of genetic register, pedigree analysis
- To analyze the physical and microscopic semen parameters
- To analyze the functional status of the sperm
- To analyze sperm DNA degradation through apoptotic process
- Biochemical analysis of seminal plasma
- Hormone analysis namely FSH, LH, Prolactin and Inhibin B.

Study area

The present study was conducted at Karnataka by recruiting obese individuals from various gyms, camps organize by us in different parts of Karnataka's, obesity clinics in and around Mysore and also from dietician clinic.

Study Group

In the present study 200 obese men of age group between 20-45 years were recruited from different camps, clinic and gyms as well as by interacting with random population in parks when they come for morning walk or evening walk

Control Group

Apart from this 50 samples were collected from control individuals with proven fertility. Control group individuals are those who had BMI- 15-25, and are free from any kind of diseases

Ethical clearance

Ethical clearance has been taken for handling human sample (IHEC- UOM NO.33 RES/2013-14). A written consent was obtained from the subjects before their inclusion in the study (Annexure I).

Establishment of genetic register and pedigree analysis

Genetic registry was established after collecting all the information pertaining to the individual along with pedigree. Minimum of two rounds of interaction was carried out with the subjects to collect the necessary information and the same was recorded by using a standard questionnaire (Annexure II). The genetic register provided lots of important information about their family history, medical history, reproductive history, life style and any other information related to the problem of both proband and the control individuals .

Pedigree is important for helping families to identify the risk of transmitting an inherited disorder and as starting point for gene searches. Very large pedigrees are helpful in gene hunt because they provide researchers with information on individuals with a particular disorder. Therefore, pedigree of suspected subjects were constructed and analyzed.

Sample collection

The subject was given a clear, written or oral instruction concerning the collection of the sample and if required transport of the sample. The semen samples were collected in a sterilized, wide mouthed plastic container by means of masturbation, after recommending three days of sexual abstinence. Two milliliter of venous blood sample was collected in EDTA vacationer tubes.

Basic semen analysis–physical and microscopic examination

1.1. Physical examination

Physical examinations such as liquefaction time, odour, colour, pH and viscosity were recorded after liquefaction according to WHO guidelines, 2010.

a) Volume

The volume of the ejaculate is contributed mainly by the seminal vesicles and prostate gland, with a small amount from the bulbourethral glands and epididymides. Precise measurement of volume is essential in any evaluation of semen, because it allows the total number of spermatozoa and non-sperm cells in the ejaculate to be calculated. The volume of the ejaculate was measured by using a graduated cylinder. **The lower**

reference limit for semen volume is 1.5 ml (5th centile, 955 confidence interval CI 1.4 - 1.7)

b) Liquefaction

Immediately after ejaculation into the collection vessel, semen is typically a semisolid coagulated mass. Within a few minutes at room temperature, the semen usually begins to liquefy (become thinner), at which time a heterogeneous mixture of lumps will be seen in the fluid. As liquefaction continues, the semen becomes more homogeneous and quite watery, and in the final stages only small areas of coagulation remain. The complete sample usually liquefies within 15 minutes at room temperature, although rarely it may take up to 60 minutes or more. The liquefaction time was recorded after observation. Normal liquefied semen sample may contain jelly like granule which do not liquefy. The presence of mucus strand, however, may interfere with semen analysis.

c) Viscosity

The viscosity of the liquefied sample was measured by gentle aspiration into a 5ml pipette and allowing the semen to drop by gravity to observe the length of the thread.

d) Appearance of the ejaculate

A normal liquefied semen sample has a homogeneous, grey-opalescent appearance. It may appear less opaque if the sperm concentration is very low; the colour may also be different, i.e. red-brown when red blood cells are present (haemospermia), or yellow in a man with jaundice or taking certain vitamins or drugs. The appearance of the semen was recorded accordingly after observation.

e) Semen pH

The pH of semen reflects the balance between the pH values of the different accessory gland secretions, mainly the alkaline seminal vesicular secretion and the acidic prostatic secretion. The pH should be measured after liquefaction at a uniform time, preferably after 30 minutes, but in any case within 1 hour of ejaculation since it is influenced by the loss of CO2 that occurs after production. For the samples, pH paper in the range 6.0 to 10.0 was used. The semen sample was mixed and a drop of semen was evenly spread

onto the pH paper. Once the colour of the impregnated zone became uniform the colour was compared with the calibration strip to read the pH.

Physical characteristic of Semen (WHO, 2010)

Characteristic	**Normal(Fertile)**	**Abnormal(Sub fertile)**
Coagulation	Present	Absent
Liquefaction	20 – 30 min	> 60 min
Odour	Musky/ spicy	Malodorous
Colour	Greyish / white	Reddish brown / yellow
Volume	1.5ml to 4.5ml	<1.5ml, > 4.5ml
pH	7.2 to 7.8	Variable

1.2. Microscopic Examination

During the microscopic investigation of the sample estimates were made of the sperm concentration, motility, count and morphology. A bright field microscope was used for observations. The nature of the liquefied ejaculate makes taking a representative sample of semen for analysis problematical. If the sample is not well mixed, analysis of two separate aliquots may show marked differences in sperm motility, vitality, concentration and morphology. To be certain of obtaining reproducible data, the sample was thoroughly mixed before aliquots were taken for assessment.

a) Sample preparation for analysis

A fixed volume of 10µl semen was delivered into a clean glass slide with a positive displacement pipette and covered with a cover slip. The weight of the cover slip spreads the sample for optimum viewing and care was taken to avoid forming and trapping bubbles between the cover slip and the slide. The freshly made preparation was left to stabilize for 1 minute. Initial evaluation at 100xtotal magnification provides an overview for determining the mucus strand formation, sperm aggregation and the evenness of spread of spermatozoa on the slide.

Motility

A simple system for grading motility is recommended that distinguishes spermatozoa with progressive or non-progressive motility from those that are immotile. The motility of each spermatozoon was graded as follows:

- Progressive motility (PR): spermatozoa moving actively, either linearly or in a large circle, regardless of speed.
- Non-progressive motility (NP): all other patterns of motility with an absence of progression, e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed.
- Immotility (IM): no movement.

Within a given field under the microscope all spermatozoa with grade a and b are counted first. Subsequently spermatozoa with non-progressive motility and immotile spermatozoa are counted in the same area.

c) Sperm vitality

Sperm vitality, was estimated by assessing the membrane integrity of the cells, may be determined routinely on all samples, but is especially important for samples with less than about 40% progressively motile spermatozoa. This test can provide a check on the motility evaluation, since the percentage of dead cells should not exceed (within sampling error) the percentage of immotile spermatozoa. The percentage of viable cells normally exceeds that of motile Cells.

Vitality test using eosin–nigrosin

This one-step staining technique uses nigrosin to increase the contrast between the background and the sperm heads, which makes them easier to discern. It also permits slides to be stored for re-evaluation and quality-control purposes (Bjorndahl et al., 2003).

Preparing the reagents

- Eosin Y: dissolve 0.67 g of eosin Y (colour index 45380) and 0.9 g of sodium chloride (Na Cl) in 100 ml of purified water with gentle heating

- Eosin–nigrosin: add 10 g of nigrosin (colour index 50420) to the 100 ml of eosin Y solution.
- Boil the suspension, and then allow to cool to room temperature.
- Filter through filter paper (e.g. 90 g/m2) to remove coarse and gelatinous sprecipitates and store in a sealed dark-glass bottle.

Procedure

- The semen sample was mixed well.
- 50µl aliquot of semen was taken and mixed with an equal volume of eosin–nigrosin suspension.
- The semen samples were remixed well before removing a replicate aliquot and mixed with eosin–nigrosin.
- A smear of the suspension is made on a glass slide and is allowed to dry in air.
- The slides were examined after drying with bright field optics at ×1000magnification and oil immersion.
- The number of stained (dead) or unstained (vital) cells was noted with the aid of a laboratory counter.
- 200 spermatozoa were evaluated in each replicate, in order to achieve an acceptably low sampling error.

The lower reference limit for vitality (membrane–intact spermatozoa) is 50% (5th centile, 95%Cl 55-63)

d) Sperm and germ cell morphology by Papanicolaou staining:

Papanicolaou staining is the widely used procedure for examination of germ cell morphology since it distinguishes clearly between basophilic and acidophilic cell components and allows a detailed examination of the nuclear chromatin pattern. This method gives a optimal results for analysis of sperm morphology and immature male germ cell.

(Stains used: Orange-G, EA-36, Haematoxylin)

Procedure

Wet smear of the semen sample was prepared in such a way that all the sperms lie in a single focal plane. Slides were air dried and fixed in ether-alcohol (1:1) mixture for 20 min.

Staining Method

95% Alcohol - 1x 10 dip

50% Alcohol - 1x 10 dip

Running water - 2min

Haematoxylin - 2 min

Running water - 2min

Alcoholic ammonia - 1x 10 dip

70% Alcohol - 2x 10 dip

95% Alcohol - 2x 10 dip

Orange G - 4 min

95% Alcohol - 2x 10 dip

EA 36 - 4 min

95% Alcohol - 2x 10 dip

100% Alcohol - 3x 10 dip

Xylene - 1x 10 dip

After 30minutes slide was cleared in Xylene and mounted in DPX. Slides were analyzed under optical microscope using an oil immersion x100 objective and enumerated different types of sperm morphological abnormalities.

Microscopic characteristic of Semen (WHO, 2010)

Characteristic Normal (fertile) Abnormal (sub fertile)

Sperm count 20 millions/ejaculation < 20 millions/ejaculation

Motility (%)> 50 < 50

Vitality (%) > 50 < 50

Morphology (%) > 20 < 20

e) Count

The number of spermatozoa in the ejaculate was calculated from the concentration of spermatozoa, which is measured during semen evaluation. For normal ejaculates, when the male tract is unobstructed and the abstinence time short, the total number of spermatozoa in the ejaculate is correlated with testicular volume and thus is a measure of the capability of the testes to produce spermatozoa and the patency of the male tract. The concentration of spermatozoa in the semen, while related to fertilization and pregnancy rates, is influenced by the volume of the secretions from the seminal vesicles and prostate and is not a specific measure of testicular function.

Determination of sperm number

A well-mixed, undiluted preparation of liquefied semen on a glass slide under a cover slip was examined, to determine the appropriate dilution and appropriate chambers to use. This is usually the wet preparation used for evaluation of motility.

- Semen is mixed well and appropriate dilutions are prepared with sperm diluent.
- The preparation is loaded into haemocytometer chamber allowing spermatozoa to settle in a humid chamber.
- The samples are assessed within 10–15 minutes.
- A minimum of 200 spermatozoa are counted per replicate.
- The replicate counts are compared to see if they are acceptably close and then proceeding with calculations; if not new dilutions are prepared.
- The concentration is calculated in spermatozoa per ml.
- The total number of spermatozoa per ejaculate was further calculated.

- The total number of spermatozoa per ejaculate was recorded as this parameter provides a measure of the capability of the testes to produce spermatozoa and the patency of the male tract. This was obtained by multiplying the sperm concentration by the volume of the whole ejaculate.

2. Sperm function tests

Sperm function tests were carried out to understand the extent of severity of the infertile conditions. Kits obtained from National Institute of Health and Family Welfare was used to carry out these function tests. Accordingly the following function tests were carried out for all the semen samples.

- Acrosomal Intactness Test
- Nuclear Chromatin Decondensation Test
- Hypo-Osmotic Swelling Test

2.1. Acrosome intactness test [AIT]

- Acrosome intactness test [AIT] was carried out by the protocol described by Gopalkrishnan et al. (1995).
- Semen samples were diluted with PBS – d – glucose solution and left for incubation at 37 ºC.
- These diluted semen samples were gently smeared on gelatin coated slides and left in room temperature for 5 to 15 minutes.
- The slide was then transferred to a moist chamber and incubated at37º C for 2 hrs.
- The slides were examined under bright field microscopy using at 40X objective.
- Spermatozoa having intact acrosome will show halo which indicates the acrosome reaction, and acrosome intactness.

2.2. Nuclear Chromatin Decondensation (NCD) test

- NCD test was carried out by the modified method of Gopal Krishnan.
- Semen samples were diluted with EDTA-SDS mixture.
- The mixture was then incubated with equal volume of glutaraldehydeborate buffer in 37 0 C for 60 minutes.
- 10 µl of the incubated mixture was placed on a clean glass slide.

- 200 spermatozoa were examined under phase contrast microscope using a 40X objective.
- Sperms with swollen head indicate the positive response and un-swollen indicates the negative response.

2.3. Hypo Osmotic swelling (HOS) test

- To assess the plasma membrane integrity, 10µl of liquefied semen sample was mixed with 50µl of fructose (1.47%) and sodium citrate (2.7%).
- The mixture was incubated at 37 °C for 15 min.
- After the incubation period, 5µl of stop solution was added.
- A drop of the incubation mixture was placed clean glass slide and observed under the microscope.

3. Biochemical analysis of seminal plasma

3.1) Estimation of seminal fructose

Biochemical analysis of seminal plasma is useful to identify the functional status of accessory reproductive organ. Estimation of seminal fructose was done by Karvonen, and Malm (1955) method.

Reagents required

1. Deprotenizing agent: 1.8% (18g/l) ZnSo4.7H2O, 0.1M NaOH.

2. Indole-reagent: 200mg of benzoic acid was added to 100 ml of distilled water and it was dissolved by repeated shaking in a hot water bath (about 600 C). After dissolving the benzoic acid, 25 mg of Indole was added. The obtained solution was filtered and stored at 40C. Stock Fructose standard (2.24mM): 40.32 mg of fructose was dissolved in 100 ml of distilled water and it was store at 40C

Method

2.24 mM standard were diluted with water and four additional standards of 1.12, 0.56, 0.28 and 0.14mM were prepared.

Preparation of Seminal plasma

1. Liquefied semen samples were centrifuged for 10 minutes at 1500rpm. The separated seminal plasma was stored at-200 C.
2. 20µl of seminal plasma was diluted with 220 µl of distilled water and it was gently mixed.
3. In the next step diluted semen samples were deprotinized by adding 50 µl of ZnSo4 and 50 µl NaOH. (Total dilution of seminal plasma was 20 µl: 320 µl = 1:16). This mixture was incubated for 15 minutes and then centrifuge at 2500 rpm
4. 200 µl of clear supernatant was used for analysis. The following table explains the protocol for estimation of seminal fructose.

Following table shows the assay procedure

Description	d H2O	STANDARD	SAMPLE (SUPERNATANT)	INDOLE REAGENT	HCL 32%	INCUBATION
BLANK	200 µl	-	-	200 µl	2 ml	20 min at 600C
STANDARD	-	200 µl	-	200 µl	2 ml	20 min at 600C
SAMPLE	-	-	200 µl	200 µl	2 ml	20 min at 600C

After incubation the mixture was cooled in ice water and takes the reading at 470nm.

Calculation

Read fructose concentration from standard curve, multiply it both by dilution factor (16) and ejaculate volume/ml and expressed as µmol /ejaculation or µmol/ml.

3.2) Estimation of seminal citric acid

Reagents required

1. 50% Trichloroacetic acid (TCA) in distilled water.

2. Anhydrous acetic acid.

3. Dry reagent grade pyridine.

4. Citric acid solution: 0.4mg of citric acid in 1ml of 25% TCA.

Method: Seminal plasma was separated by centrifugation at 2000 rpm for 20 minutes. One ml of seminal plasma was added to 1ml 50% TCA and subjected for centrifugation at 7000 rpm for 15min. After 15 min the supernatant was collected. Blank solutions were prepared by adding 1ml of distilled water with 1ml of TCA solution. 1ml of supernatant blank and citric acid standard solution were taken in the separate tubes. 8ml of anhydrous acetic anhydride was added and heat at 600C for 10 minutes, then 1ml of dry reagent grade pyridine was added and again incubation was continued at 600C for 40 minutes. After completion of incubation the incubated solution was kept in ice cool bath for 5

minutes and readings of seminal plasma was measured at the absorbance of 400nm against blank.

Calculations

A standard curve was constructed by using the above method with 25mg of citric acid in 1ml 25% TCA. The amount of citric acid present in the seminal plasma was estimated from the standard curve.

Inference: Normal value 52µl or 10mg per ejaculate.

3.3) Estimation of seminal Acid Phosphatase

Reagents Required

1. Citrate buffer 0.09M (mol/l) pH 4.8: Dissolve 1.891g of anhydrous free citric acid in distilled water. Adjust pH to 4.8 and 1M NaOH, make up to 100ml with water, store at 40 C.

2. 0.1M NaOH

3. p-Nitrophenol phosphate (substrate) solution: Prepare the solution freshly. Calculate the volume of substrate needed and dissolve the required amount of p-nitro phenol phosphate disodium salt in citrate buffer to a concentration of 4mg/ml.

4. p-Nitrophenol (PNP): 5mM (mMol/L) stock solution for standard curve. Dissolve 0.0695g of PNP in distilled water, warm the solution and make upto 100ml, stored at 40 C.

5. Sodium bisulphate.

Procedure

After liquefaction, semen was centrifuged at 3000rpm for 10 minutes to separate spermatozoa. 20µl of seminal plasma was pipette into 20 µl of sodium bisulphate solution in eppendorf tube; pH was stabilized between 5-6. The sample was stored at -200 C. Seminal plasma was diluted 10,000 times for assay i.e., for stabilized sample (already diluted 1:1) dilute 10µl into 0.5ml citrate buffer and further diluted to 10µl of this ratio to 1ml. 0.1ml of substrate solution was added in each assay tube and warmed

for 5 minute at 370 C for 30 minutes. Tubes without samples were incubated as blanks. Reaction was stopped by adding 2ml NaOH solution. The absorbance was read at 405nm.

Standard:400 µl of 5mM stock solution was added into 20ml volumetric flask and made up to 20ml using 0.1M NaOH (this solution is 100µM i.e., µmol/l). 45 This is diluted into 100µM solution and 0.1M NaOH to give standards of 0, 6, 12, 18, 24, 30 µg and read absorbance at 405nm.

Inference: Normal range is 200U per ejaculate or more.

3.4) Estimation of L-Carnitine in Seminal Plasma

The level of L-Carnitine in human seminal plasma was estimated by colorimetric method. Carnitine is a quaternary ammonium compound biosynthesized from lysine and methionine. It is required for fatty acid transport into the mitochondrial matrix via the carnitine/actylcarnitine shuttle, where β-oxidation occurs, acetate is generated and the acetate utilized in the TCA cycle for the generation of energy.

L-carnitine was estimated using calorimetric kit obtained from the BioVision Company, catalogue number #K642-100.

Carnitine standard Curve:

10 µl of the 100mM Carnitine Standard was diluted with 990 µl dH2O to generate 1mM Standard Carnitine. 0,2,4,6,8,10 µl of diluted carnitine standard were added into 96-well plate to generate 0,2,4,6,8,10 nmol/well standards. Made up the volume to 50 µl with assay buffer.

Sample Preparation: 10-50µl of deprotinized (Deprotenization is done by using Zinc sulphate and NaOH solutions) serum samples can be directly diluted in Assay buffer. Made up the sample wells to 50 µl/well with assay buffer in 96 well plates.

Reaction Mix

For each well prepare a total 50µlreaction mix which contain.

	L-carnitine measurement	**Back ground control***
Assay Buffer	40 µl	42 µl
Carnitine converting enzyme	2 µl	-
Carnitine Developmental Mix	2 µl	2 µl
Carnitine Substrate Mix	4 µl	4 µl

*Perform background control if high levels of acyl-CoA's or Free Coenzyme A are suspected to be in our samples. Choline in samples will give a positive signal but is present at ~10% of the carnitine concentration.

OD was measured at 570nm.

Calculation

Correct background by subtracting the value derived from the 0 carnitine control from all samples and standard readings. Later carnitine standard curve was plotted. Sample reading were applied to the standard curve. Carnitine concentrations of the test samples can be calculated using the formula.

C=Sa /Sv (nmol/ µl, or mM)

Where, Sa is the Carnitine content of unknown samples in std. Curve.

Sv is sample volume µl added into the wells.

(L-Carnitine Molecular Weight is 161.2g/mol)

4 MOLECULAR ANALYSIS OF SEMEN

4.1) DNA fragmentation by DNA ladder assay

DNA fragments of oligo-nucleosomal size (180-200 bp) are a hall mark of apoptosis in many cells. In apoptotic cells specific DNA cleavage becomes evident in electrophoresis analysis as a typical ladder pattern due to the multiple DNA fragments. Assessment of sperm DNA fragmentation will be done using standard protocols for modified alkaline single cell gel electrophoresis assay.

- The semen plasma treated with different doses of DEXA for 3 hr at 37^0C.
- Cells were centrifuged at 5000rpm for 2 minutes at 4^0C.
- Stored at -20^0C for further analysis.
- 500 µl of lysis buffer proteinase K added fresh to cell pallet and kept at 4^0C for 15 minutes.
- Centrifuged at 12K for 10 minutes at 4^0C.
- Supernatant collected and to that equal volume of phenol, chloroform, isoamyl alcohol (25:24:1) added and centrifuged at 12K for 5 minutes .
- The upper aqueous layer extracted with equal volume of chloroform and centrifuged at 12K for 5 minutes.
- The upper aqueous layer treated with 3M sodium acetate and absolute ethanol for 1 hour at -20^0C.
- Centrifuged at 16K at 4^0C for 20 minutes and wash with 0.5 ml of 70% ethanol.
- Acid dry the pallet and reconstitute in TE Buffer containing R Nase and incubated for 30 minutes.
- Equal volume of each sample loaded to 1.5% agarose gel containing 0.5µg/ml Etbr
- Run the gel in 1X TAE AT 60V and visualized the DNA fragment using gel documentation system.

Gel electrophoresis

Reagents preparation

1X TAE buffer:

- Dissolved 12.1g of Tris, in about 30 ml of double distilled water.
- After the salt was completely dissolved added 10ml of 0.5M EDTA.
- To this solution added 5.7ml of glacial acetic acid was added.
- The volume was made up to 100ml with double distilled water.

50X TAE buffer:

To 1ml of 1X TAE buffer, added 49ml of double distilled water to get the working solution of 50X TAE buffer.

Gel casting:

- To 50ml of TAE buffer added 0.5g of Agarose.
- Boiled over hot plate till a clear solution, indicating complete dissolving of the agarose is obtained.
- The solution was brought down to 50 C and 5µ of TBR (10mg/ml) was added.
- The solution was stirred for thorough mixing.
- Cooled down further and poured the gel onto the casting tray.
- The comb was inserted into the block and left the set up undisturbed for the gel to solidify.

Electrophoresis

- Once the gel is solidified, the combs were carefully removed.
- The gel was transferred into the electrophoretic unit tank filled with 50x TAE buffer.
- 15 µ of the product was mixed with 1 µ of gel loading dye using a pipette.
- The mixture was loaded on to the well carefully without rupturing the gel or spillage.
- The same was repeated for all the samples.

- The samples were electrophoresed at 80volts and 230mA for 45 minutes.
- Once the run was completed as indicated by the tracking dye the power supply was shut off.
- The gel along with the casting tray was removed from the tank and viewed on a UV transilluminator.

4.2) DNA fragmentation by Comet assay:

It is an uncomplicated and sensitive technique for the detection and evaluation of DNA damage and genotoxicity testing. It involves the encaptaulation of the cell in a low melting agarose suspension, lysis of the cells in neutral or alakaline (Ph>13) conditions, and electrophoresis of the suspended lysed cells. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage. This can be performed by manual scoring or automatically by imaging software

- Fully frosted clean slide were covered with 1% normal melting point agarose and immediately covered with large microscopic cover slide.
- After solidification cover clip removed and
- 10µl of human sperm in Ca^{2+} and Mg^{2+} free phosphate buffer saline were mixed with 85µl of 0.5% low melting point agarose
- From the mixed solution 10µl of sample repeated pipette over the first agarose layer and covered with cover slide and for solidification for 5minutes at -4^{o}C
- 0.5% low melting point agarose added to the slide after removing cover slip and left for solidification for 5minutes at -4^{o}C
- The slide kept in freshly prepared cold lysis buffer solution for 1 hour at -4^{o}C.
- The slide incubated for overnight at 37 ^{o}C with proteinase K.
- After overnight incubation slide was taken out and kept in horizontal gel electrophoresis tank filled with cold lysis buffer solution for 20 mint at 4 ^{o}C
- The gel run for 20 minutes at 25V adjusted to 300Ma at 4 ^{o}C
- After electrophoresis the slide was flooded with two change of neutralizing buffer for 5 minutes each.

- The slide was drained and treated with two changed of fixing solution for 5 mint each.
- The slide was then stained with silver staining solution for 10 minutes each for two wash.
- Coded slide were viewed under bright field microscope.
- For each sample 100 randomly selected sperm nuclei were evaluated by an image analysis system using software attached to the computer provided by leica microscope.

5) HORMONAL ANALYSIS OF BLOOD SERUM

Standard Kits was utilized for the hormonal assays of FSH, LH, Prolactin, Inhibin B.

Peripheral blood samples were collected from the subjects and centrifuged at 4000 rpm for 10 minutes. The serum was than separated and stored for future analysis. A total of 150 serum samples were subjected for hormone analysis employing ELISA Kits (CALBIOTECH) for LH, FSH, Inhibin B and prolactin. The readings were obtained using Biotek ELx 800 microplate reader.

Material required

- A micro titer plate calibrated reader (eg., the DRG instruments micro titer plate reader).
- Calibrated variable precision micropipettes
- Absorbent paper
- Distilled water
- Semi logarithmic graph paper or software for data reduction

5.1. Estimation of serum follicle stimulating hormone (FSH)

Human follicle-stimulating hormone (FSH, follitropin) is a glycoprotein produced and secreted by the basophilic cells of the anterior lobe of the pituitary gland. Secretion of FSH is stimulated by gonadotrophin-releasing hormone (GnRH). Gonadal steroids like progesterone, estrogens and androgens, exert both positive and negative feedback on FSH function. In men, FSH stimulates semeniferous tubules and testicular growth and is

involved in the early stages of spermatogenesis. FSH is use full in the diagnosis infertility, hypogonadism, gynaecomastia and tumours. In children, assessment of FSH is a important in investigating delayed or precocious puberty.

Principle

The FSH is solid phase direct sandwich ELISA method. The samples and diluted anti-FSH-HRP conjugate were added to the wells coated with MAb to FSH beta subunit. FSH in the patient's serum binds to anti-FSH MAb on the well and the anti-FSH-HRP second antibody the binds to FSH. Unbound protein and HRP conjugate are washed off by wash buffer. Upon the addition of the substrate, the intensity of colour is proportional to the concentration of FSH in the sample.

Sample Collection and Handling

- Only Serum or Plasma samples were used in this procedure.
- Grossly hemolysed, lipemic or microbially contaminated samples may interfere with the performance of the test and was not used.
- Neither Bilirubin nor Hemolysis has significant effect on the procedure. Store sample at 2º-8º C for up to a maximum of 5 days at (2-8^{o} C). For longer storage, sample was frozen. Avoid repeated freezing and thawing of sample.
- Prior to assay, frozen sera should be completely thawed and mixed well.

Preparation of Sample

Usually no dilution necessary, dilute samples with concentrations above 135mlU/mL 1:1 with test distilled water

Procedure

5 standards and 1 blank was included

- Remove the required micro wells from pouch and return unused stripes in the sealed pouch to refrigerator. Securely place the micro wells into the extra provided holder.
- Pipette 25µl of calibrators and patient samples into the wells and incubate 15 minutes at room temperature
- Add 100µl of Enzyme conjugate to the wells except for blank well and incubate 15minutes at room temperature.
- Add app. 300µl of distilled water, decant or aspirate. Repeat 4 additional times for a total of 5 washes.
- Pipette 100µl of Substrate solution into each micro well using the same order and timing as for the addition of the substrate solution.
- Read absorbance of each micro well at450 nm against blank using a micro plate reader. The developed color is stable for at least 30 minutes. Read optical densities during this time.

5.2. Estimation of serum Lutenizing hormone (LH)

The LH IEMA TEST is based on simultaneous binding of human LH to two monoclonal antibodies, one immobilized micro well plates, the other conjugate with horseradish peroxidase (HPR). After incubation the bound separation is performed by a simple solid-phase washing, and then the substrate solution (TMB) is added. After an appropriate time has elapsed for maximum

color development, the enzyme reaction is stopped and the absorbance are determined. The color intensity is proportional to the LH concentration in the sample.

Sample collection and Handling

- Serum or plasma sample is used in this procedure. The patient need not to be fasting and no special preparations are necessary.
- Grossly hemolyzed, lipemic or microbially contaminated sample may interfere with the performance of the test and was not be used. Neither Bilirubin nor Hemolysis has significant effect on the procedure.

- Store sample at 2° - 8°C for up to a maximum of 2 days. For longer storage, sample was frozen. Avoid repeated freezing and thawing of samples.
- Prior to assay, frozen sera should be completely thawed and mixed well.

Preparation of samples

Usually no dilution is necessary, for samples with concentration over 200mlU / ml, dilute the sample 1:1 with standard A.

Test procedure

5 samples and 1 blank was included,

- Remove the required micro wells from pouch and return unused strips in the sealed to refrigerator. Securely place the micro wells into the extra provided holder.
- Pipette 25µl of standards and 25µl patient samples into the wells. Incubate 10 minutes at room temperature
- Add 100 µl Enzyme conjugate to the well except for blank well and incubate 60 minutes at room temperature.
- Add approximately 300µlof distilled water, decant (tap or blot) or aspirate. Repeat four additional times for a total of five washes.
- Pipette 100µl of substrate solution into each micro well in the same order and timing as for the enzyme conjugate, Blank well included.
- Incubate 10 minutes at room temperature in the dark.
- Add 100µl of stop solution into each micro well using the same order and timing as for the addition of the substrate solution.
- Read absorbance of each micro well at 450 nm against blank using a micro plate reader. The developed colour is stable for at least 30 minutes. Read optical densities during this time.

5.3. Estimation of Prolactin

Prolactin is a glycoprotein with a molecular weight of approximately 23,000 Daltons consisting of 198 amino acids, synthesized by the anterior lobe of the pituitary gland; prolactin is secreted in a pulsating manner (every 20 minutes) and follows a circadian

rhythm with highest levels occurring during sleep. It can be present in different forms: monomeric and dimerous.

Many factors control the secretion of prolactin physiologically prolactin levels are controlled by the hypothalamus. Dopamine and GABA are the main inhibitory factors. TRH (thyrotropin-releasing hormone) and VIP (Vaso active intestinal peptide) stimulate prolactin secretion. Exogenous factors such as exercise, Stress, diet and hypoglycemia can cause an increase in prolactin levels. In males, prolactin affects gonadal function. Hyper prolactinemia has been recognized as a cause of infertility problems in men and women. The three etiological forms of hyperprolactinemia are latrogenic hyperprolactinemia associated with the use of certain medications. Primary hyperprolactinemia associated with tumours and secondary hyperprolactinemia (hypothyroidism, renal insufficiency etc.)

Sample collection

Serum or plasma

None of the following factors have been found to be influence this assay

- Hemolysis
- Lipemia
- Bilirubinemia

Procedure

- Only remove the required reagents from the refrigerator and allow them to come to room temperature for at least 30 minutes.
- Use one “PRL” strip and one “PRL” SPR for each sample, control or calibrator to be tested. Make sure the storage pouch has been carefully resealed after required SPRs have been required.
- The test is identified by the “PRL” code n the instrument. The calibrator must be identified by “S1” and tested in duplicate. If the control is to be tested, It should be identified by “C1”.
- If necessary, clarify the samples by centrifugation.
- Mix the calibrator, control, and samples using a vortex type mixer.
- For this test, the calibrator, control, and sample test portion is 200 µl.

- Insert the “PRL” SPRs and “PRL” strips into the instrument. Check to make sure the color labels with the assay code on the SPRs and the reagent strips match.
- Initiate the assay as directed in the user’s manual. All the assay steps are performed automatically by the instrument.
- The vials should be reclosed and return them to the required temperature after pipetting.
- The assay will be completed within approximately 40 minutes. After the assay is completed, remove the SPRs and strips from the instrument.
- Dispose the used SPRs and strips into an appropriate recipient.

5.4. Estimation of serum Inhibin-B

Assay procedure

- The quantity of the strips depends on the quantity to-be-tested samples and the standards. It is suggested to duplicate each standard and blank well. Every sample should be made according to the required quantity or in duplicate l.
- Set blank wells, standard wells, and test sample wells respectively:
- ✓ Blank well: do not add samples and horseradish peroxidase (HRP), other operations are the same.
- ✓ Standard wells: Add standard 50µl to Standard wells.
- ✓ Test sample wells: Add 40µl of Special diluent and then add 10µl of sample. (Thefinal sample dilution is five times and the final result calculation should be multiplied by five times).
- ✓ Add 50µl of horseradish peroxidase (HRP) into each well, except blank well. Then seal the plate, and gently shake, then incubate 60 minutes at 37°C.
- Discard Liquid excess, drying, fill each well with diluted washing liquid, mix and shake for 30 seconds, discard the washing liquid and tap the plate into absorbent papers to dry.

Repeat five times, and then pat dry.

- Add 50µl of chromogen solution A to each well, and then add 50µl of chromogen solution B to each well. Gently shake and incubate for 10 minutes at 37℃ away

from light.

- Stop: Add Stop Solution 50μl into each well to stop the reaction (the blue changes into yellow immediately).
- Final measurement: Set blank well zero, measure the optical density (OD) at 450 nm wavelength which should be carried out within 15 minutes after adding the stop solution.
- According to standards' concentration and the corresponding OD values, calculate out the standard curve linear regression equation, and then apply the OD values of the sample on the regression equation to calculate the corresponding sample's concentration. It is acceptable to use a variety of software to make calculations.

Prepare reagents, samples and standard

- Add prepared sample and standard and HRP-Conjugate Reagent, incubate for 60 minutes at37 ℃
- Wash plate five times and add Chromogen Solution A and B, incubate for10 minutes at 37 ℃
- Add stop solution

RESULTS

The present study shows six different infertile conditions (**Table 1**) wherein asthenozoospermic cases were found to be more and oligozoospermia individuals recorded the least in number recruited for the present study. Based on semen profile 200 obese overweight individuals were categories into different conditions as shown in the **Figure-7** where in 68% were normozoospermia followed by Asthenozoospermia (10%), Oligoasthenozoospermia (5.5%), Oligoastenozoospermia (5%), Oligospermic conditions (4%) and (1.5%) cases with Azoospermia. **Table 2** depicts the different conditions of obese and overweight condition according to different semen parameters. In all the conditions the average semen pH shows higher range (8.2) compare to control group (7.9) and mean vitality value of cases were lower (58.66±11.82) than control case (77.68±6.42). Semen count, sperm motility and vitality average results were significantly lower compared with control group whereas no significant results observed in sperm volume, sperm morphology. The live and dead sperms were analyzed using sperm vitality test as shown in F**igure 1**. The mean percentage of progressively and rapid motile sperm showed higher values (68±109) in fertile control males than obese and overweight males (31.51±6.19). Sperm function tests **Figure 2** showed the least values of HOS (57.58±7.33) and NCD (60.66±7.04) test in obese and overweight group and highest values in control group NCD 79.7±6.08 and HOS 79.0±7.5**.** The AIT value lies above the reference value both for obese and overweight (61.51±8.16) and control group (84.12±7.12) shown in **Table 3 and 4**.

Biochemical markers

The result of fructose value is lower in Oligospermia, Asthenosteratozoospermia conditions when compared with normal range. The values of citric acid were decreased in Oligoteratozoospermia. oligozoospermia and Asthenoteratoozoospermia. The results of Acid phosphatase assay in all the cases lie within the reference range**(Table 5)**where as carnitine level is normal value were 8.31± 0.75elevatedvalue were 87.86±5.78 and decreased value were8.09±1.65as described in **Table 6 and Figure 15.**

Hormonal Assay

The frequency of endocrine abnormalities observed in the present study is depicted in

Table 6 and Figure 16. LH and FSH recorded 38.75%, 30.05% of abnormalities. Prolactin levels were found to be 32.5%. Inhibin-B level was found to be 37.85% with more variations.

DNA ladder assay and commet assay for Semen Samples:

The isolated samples of both cases and control are loaded on 1% TAE gel along with 100bp DNA ladder and negative result came for control semen samples**.** The DNA fragmentation can be seen in the semen pellet in obese and overweight. For commet assay the DNA fragmentation were not observed both for control and cases represented in the **Table 7**.

TABLE 1: Prevalence of different conditions and physical semen parameters in obese/overweight and control group

Sl no.	Condition	In percent (%)	Coagulation		Liquification		Colour		pH (7.5-8)
			NR	AB	NR	AB	NR	AB	
1	Normozoospermia	68	100	-	100	-	100	-	7.9±0.4
2	Asthenozoospermia	10	70	30	80	20	100	-	7.78±0.4
3	Asthenoteratozoospermia	06	83.3	16.6	66.6	33.3	50	50	7.96±0.29
4	Azoospermia	1.5	74.4	24.6	78.9	21.1	84.9	15.1	7.4±0.0
5	Oligoasthenoteratozoospermia	5.5	70	30	66.6	33.3	100	-	8±0.28
6	Oligoastenozoospermia	05	80	20	83.3	16.6	80	20	7.66±0.41
7	Oligozoospermia	04	75.6	24.4	69.2	30.7	63.2	36.8	7.8±.6
8	Control	100	100	-	100	-	100	-	7.9±0.37

Table 2: Distribution of obese subgroups and control group based on BMI(body mass index),sperm count, volume, pH, vitality and motility

Sl. No.	Conditions	No. of Cases (200)	BMI	(Mean) Sperm Count (>20million/ml)	(Mean) Volume (1.5-4.5 ml)	(Mean) pH(7.5-8)	(Mean) Vitality 75%	(Mean) Motility A+B=50%
1	Normozoospermia	136	28.74±3.19	57.37±35.62	2.03±.75	7.9±0.4	79±13.46	57.37±12.40
2	Asthenozoospermia	20	28.79±3.25	42.77±22.75	1.68±0.87	7.78±0.4	66.8±14.9	22±8.23
3	Asthenoteratozoospermia	12	28.98±3.60	35.0±31.7	1.75±0.86	7.96±0.29	71.0±9.8	38.75±17.5
4	Azoospermia	03	51.76±0.0	00	1.5±0.0	7.4±0.0	0.0±0.0	0.0±0.0
5	Oligoasthenoteratozoospermia	11	28.75±1.48	8±5.65	1.9±0.2	8±0.28	68±19.8	23.5±12.0
6	Oligoastenozoospermia	10	29.36±1.65	14.0±0.81	1.7±0.43	7.66±0.41	74.0±13.0	27.0±15.0
7	Oligozoospermia	08	29.68±2.81	8.6±4.93	2.01±0.34	7.8±.6	77.0±8.0	52.0±6
8	Control	50	20±1.2	71.05±24.15	2.48±.73	7.9±0.37	77.68±6.42	68±10.9

Table 3: Conditions and sperm function test (NCD=nuclear chromatin decondensation, HOS=hypo-osmotic swelling, AIT= acrosome intactness test) results in obese individuals. (NR=Normal AB=Abnormal) (azoospermia=absence of sperm in the ejaculation)

No.	Condition	Number of sample (N-150)	HOS ≥60(normal)	NCD ≥75(normal)	AIT ≥50(normal)
1	Normozoospermia	136	72.11±8.95	76.35±11.03	66.10±9.55
2	Asthenozoospermia	20	68.9±8.89	66.±14.4	64.5±16.7
3	Asthenoteratozoospermia	12	64.7 ±13.84	67.25±9. 74	61.2±8.53
4	Azoospermia	03	Nil	Nil	Nil
5	Oligoasthenoteratozoospermia	11	82.5 ±3.53	845±2.12	56.5 ±19.1
6	Oligoastenozoospermia	10	68.67±10.02	71.33±5.68	65.0±4.0
7	Oligozoospermia	08	67.8±10.2	64.6±10.8	55.8±21.0
8	Control	50	79.7±6.08	79.0±7.5	84.12±7.12

Table 4: Comparisons of Mean semen parameters among control and cases

Sl. No.	SEMEN PARAMETER	CASE (N=250)	CONTROL (N=50)
1	Volume (1.5-4.5 ml)	1.75±0.45	2.48±.73
2	Motility (A+ B= 50%)	31.51±6.19	68±10.9
3	pH	7.78±0.29	7.9±0.37
4	Vitality (75%)	58.66±11.82	77.68±6.42
5	Count(> 20 million/ml)	23.69±14.49	71.05±24.15
6	NCD (75%)	60.66±7.04	79.7±6.08
7	HOS (60%)	57.58±7.33	79.0±7.5
8	AIT (60%)	61.51±8.16	84.12±7.12

Table 5: Biochemical analysis of control and Obese / Overweight men with different conditions

Sl No.	Condition	Fructose ≥13mmol	Citric acid ≥13mg/ejaculation	Acid Phosphotase ≥200U/ejaculation
1	Normozoospermia	13.8±0.23	16.46±3.20	419.35±12.9
2	Asthenoteratozoospermia	11.2±0.19	12.44±0.23	334.56±21.28
3	Asthenozoospermia	20.2±12	13.37±11.21	329.34±27.25
4	Azoospermia	17.1±15	13.33±12.15	388.25±21.22
5	Oligoasthenoteratozoospermia	13.3±12	14.44 ±11.20	376.25±46.96
6	Oligoastenozoospermia	13.34±12.2	10.42±0.23	326.43±23.6
7	Oligozoospermia	12.6±13.18	11.50 ±5.20	332.65±21.1
8	Control	18.9±3.3	18.41±4.3	426.65±71.54

Table 6: Biochemical analysis (Carnitine) of control and Obese / Overweight men

Sl no conc	Condition	Carnitine 10-70µM
1	Normal	8.31± 0.75
2	High	87.86±5.78
3	Low	8.09±1.65

Table 7: Comparison of hormonal variations in obese /overweight individuals

Hormones	Normal	Elevated	Decline	Total abnormality
LH	61.25%	30.75%	8%	38.75%
FSH	70.51%	21.15%	8.9%	30.05%
Prolactin	67.08%	17.34%	15.46%	32.8%
Inhibib -B	62.15	8.25%	29.65%	37.85%

Table 8: The DNA fragmentation in semen samples of obese overweight cases compare to controls

No. Of Patients	Sperm Sample(ladder Assay)		Sperm sample(commet assay)	
	Positive%	Negative%	Positive%	Negative%
Obese/ overweight N=200	4.5	99.4	---	100
Control N=50	----	100	-----	100

Figure 1: Response of Sperms to Vitality test. Unstained (White) Sperms indicated normal viable sperms as positive response, while the stained sperms (Pink) indicated non-viable sperms as negative response.

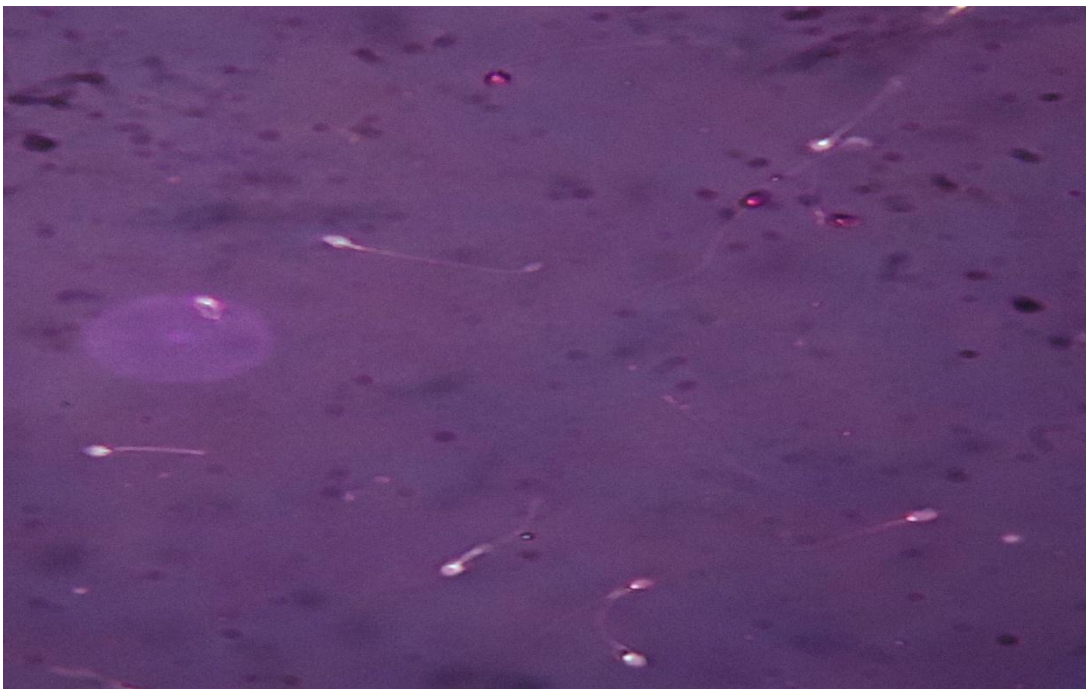


Figure 2: Sperms showing abnormal morphology

A: Normal sperm; B: pin head; C. Cytoplasmic droplets; D: arrow head

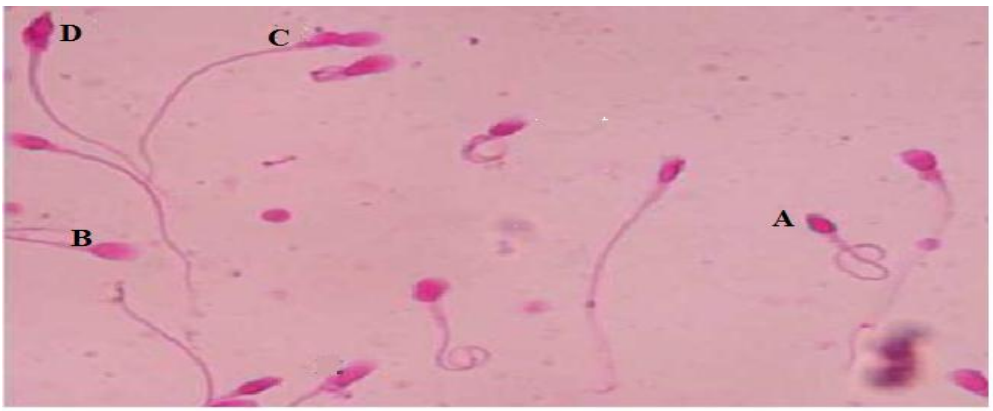


Figure 3: Acrosomal Intactness Test (AIT): Hallow formation in the acrosome region showing positive response(yellow arrow) and red arrow(negative response)

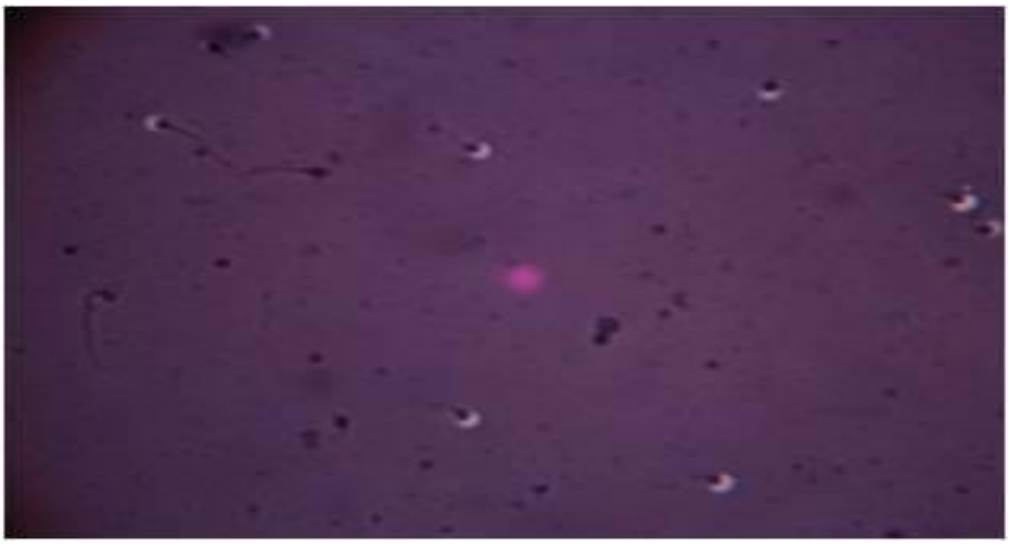


Figure4: Nuclear Chromatin Decondensation Test (NCD)

A: swollen head indicates positive response

B: Unswollen head indicates negative response

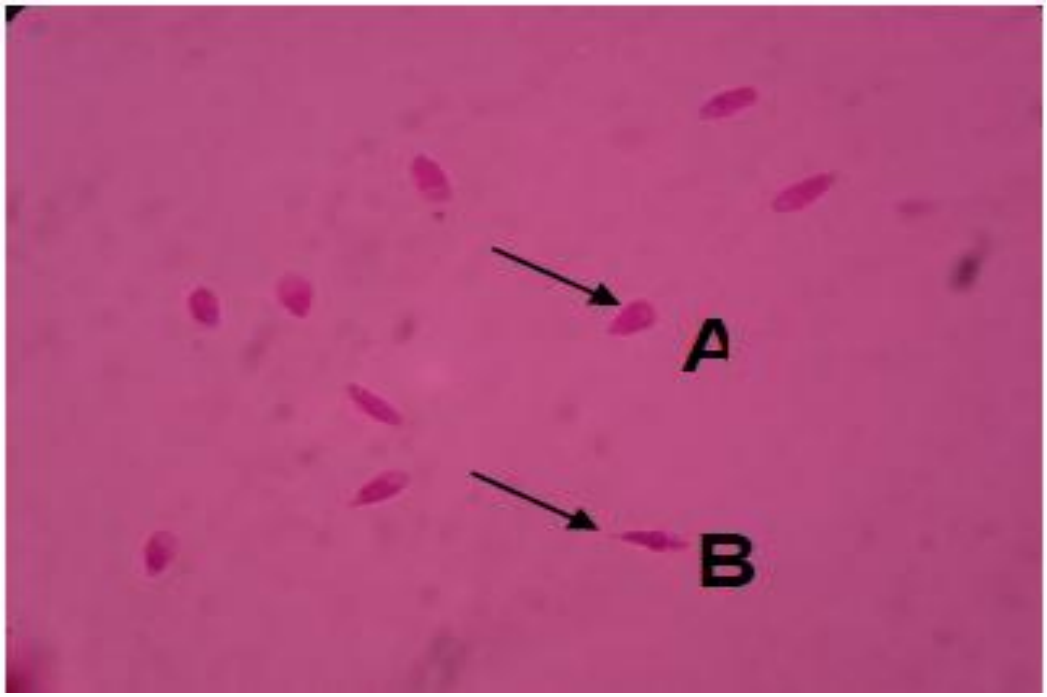


Figure5: Hypo-osmotic Swelling Test (HOS)

White Arrow: Coiled Tail (Positive Response), Yellow Arrow: Straight Tail (negative Response)

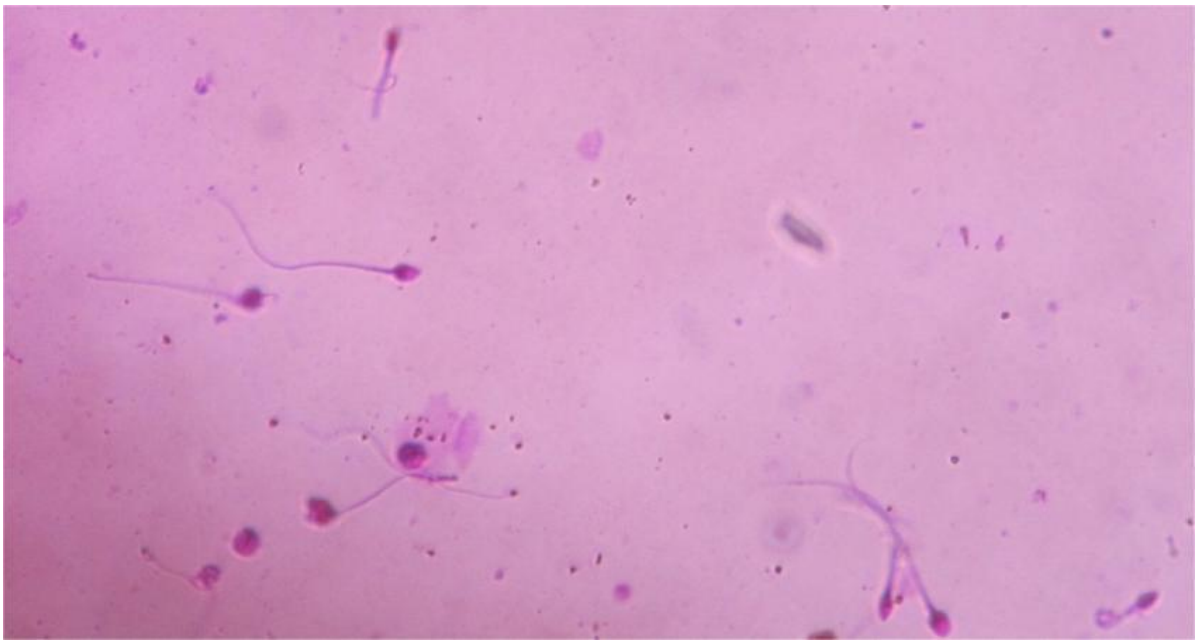


Figure 7: Different percentage Obese/overweight conditions when compared to control

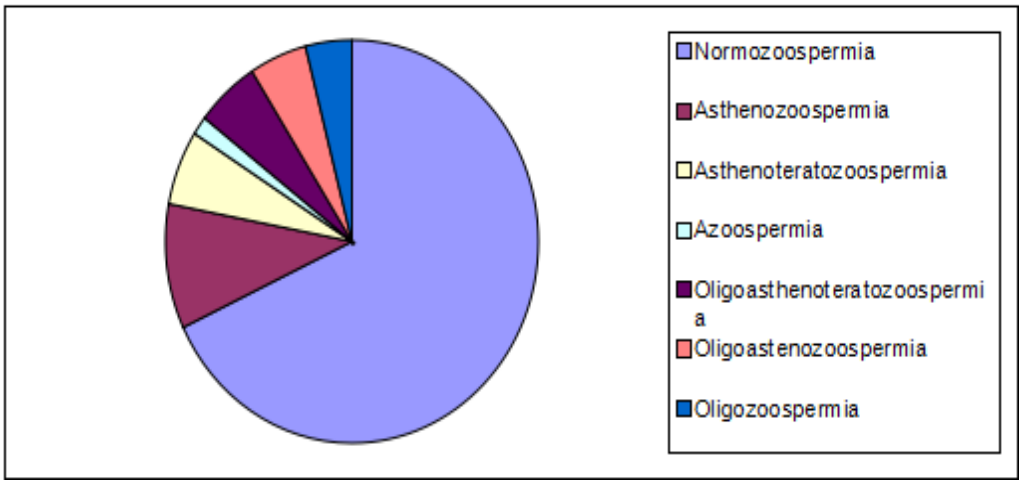


Figure 6: Semen volume among different Obese/overweight conditions when compared to control

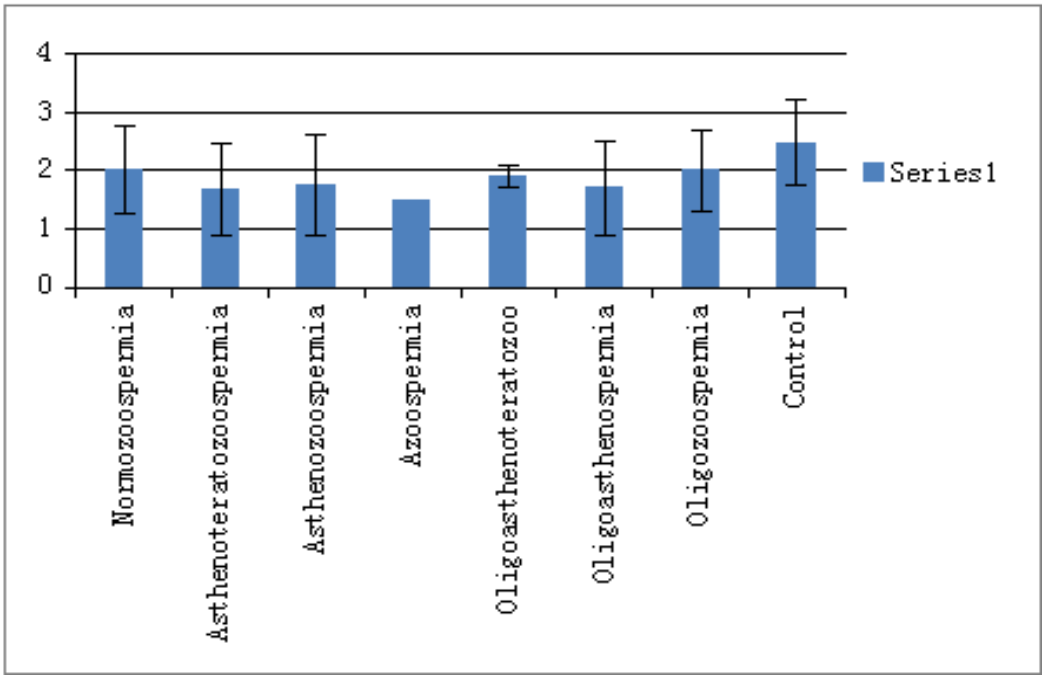


Figure 7: Sperm pH among different obese/overweight conditions in comparison with control individual

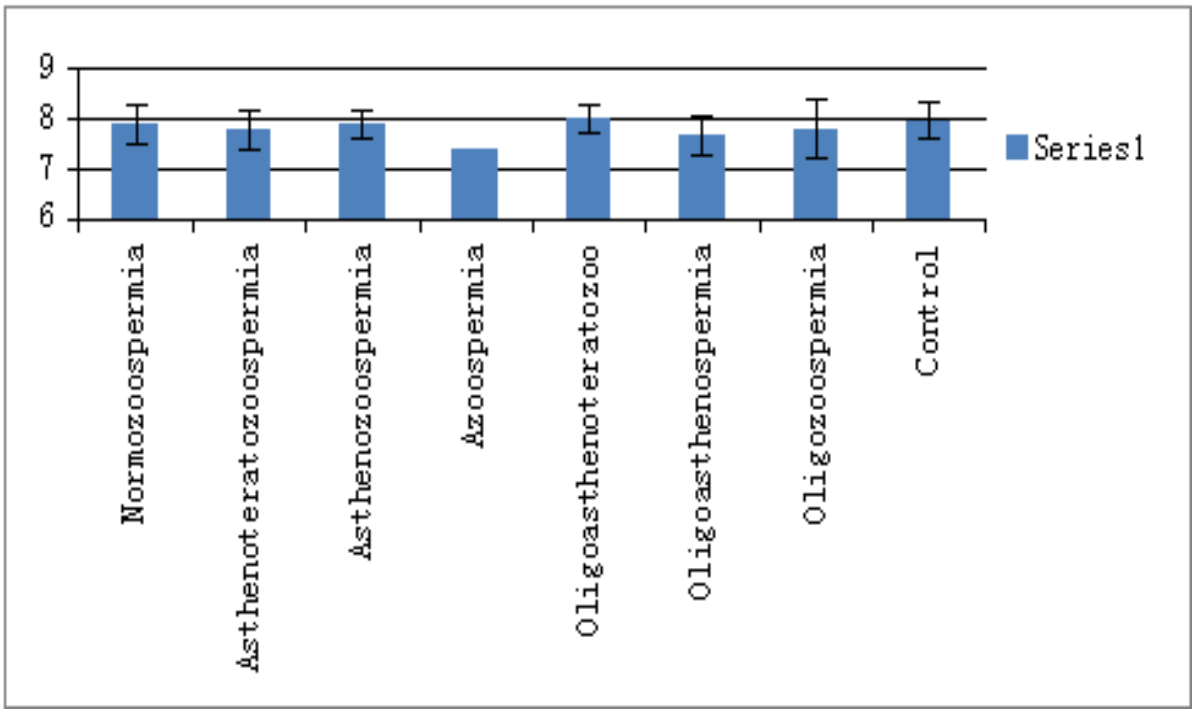


Figure 8: Sperm count among different obese/overweight conditions in comparison with control individual

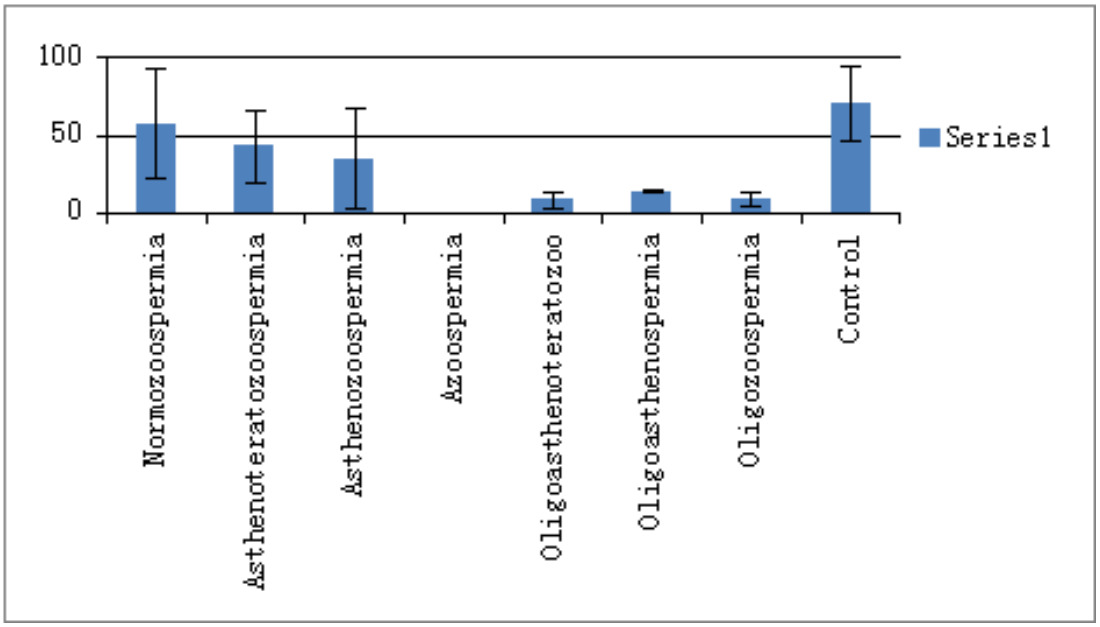


Figure 9: Sperm Vitality among different obese/overweight conditions in comparison with control individual

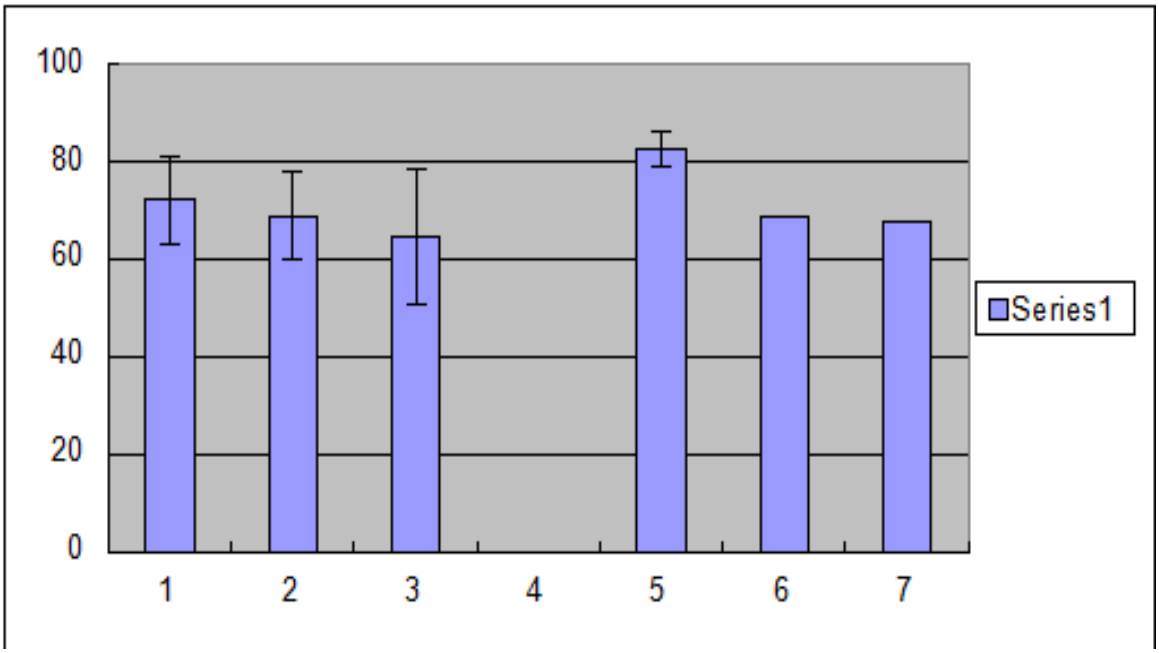
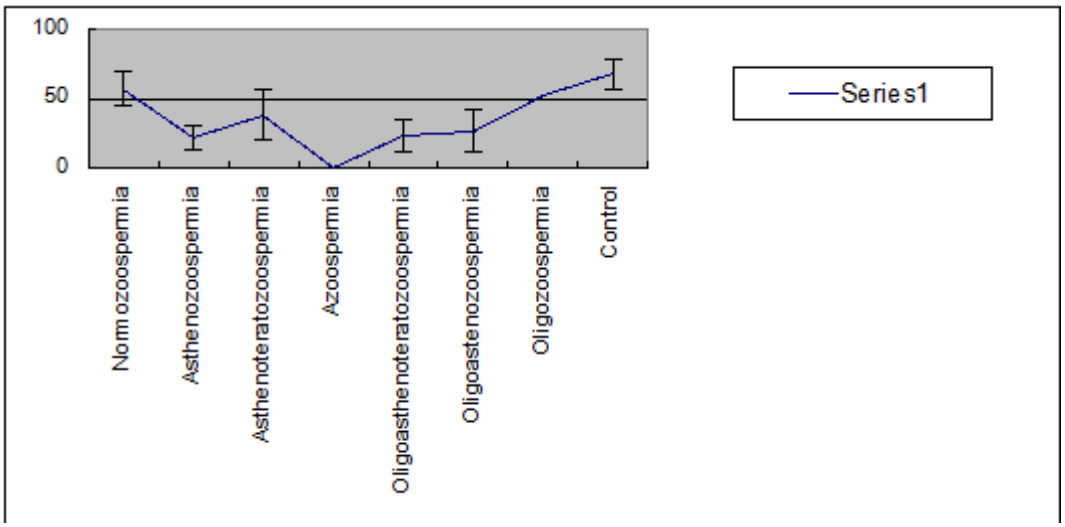


Figure 10: Sperm Mortility among different obese/overweight conditions in comparison with control individual



Sperm function test:

Figure 11: Sperm NCD among different obese/overweight conditions in comparison with control individual

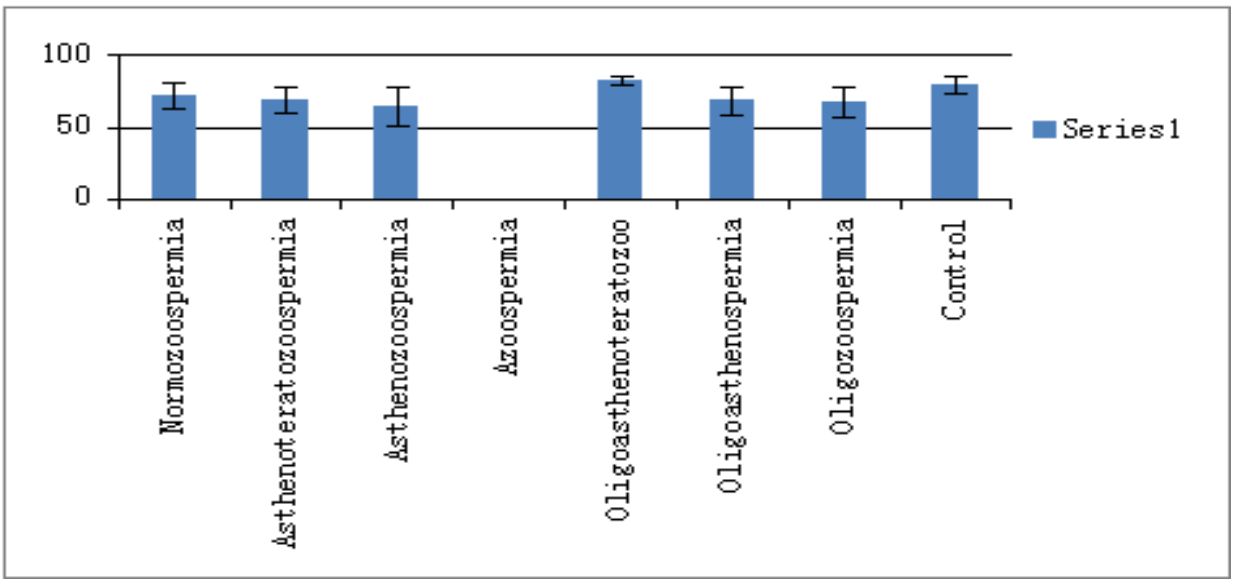


Figure 12: Sperm HOS among different obese/overweight conditions in comparison with control individual

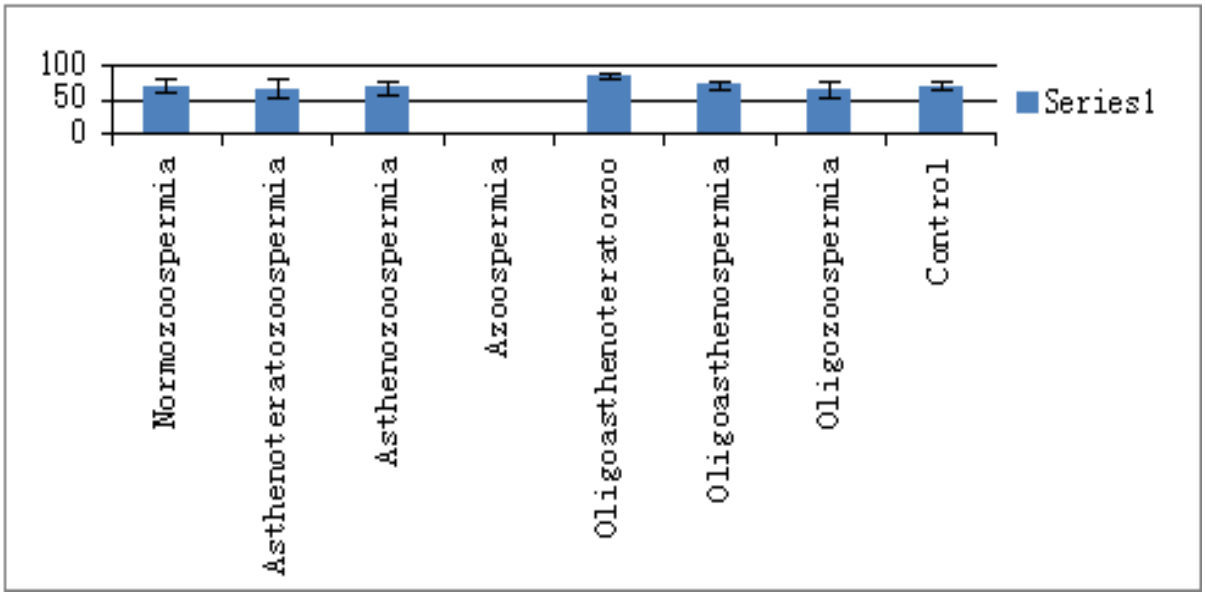


Figure 13: Sperm AIT among different obese/overweight conditions in comparison with control individual

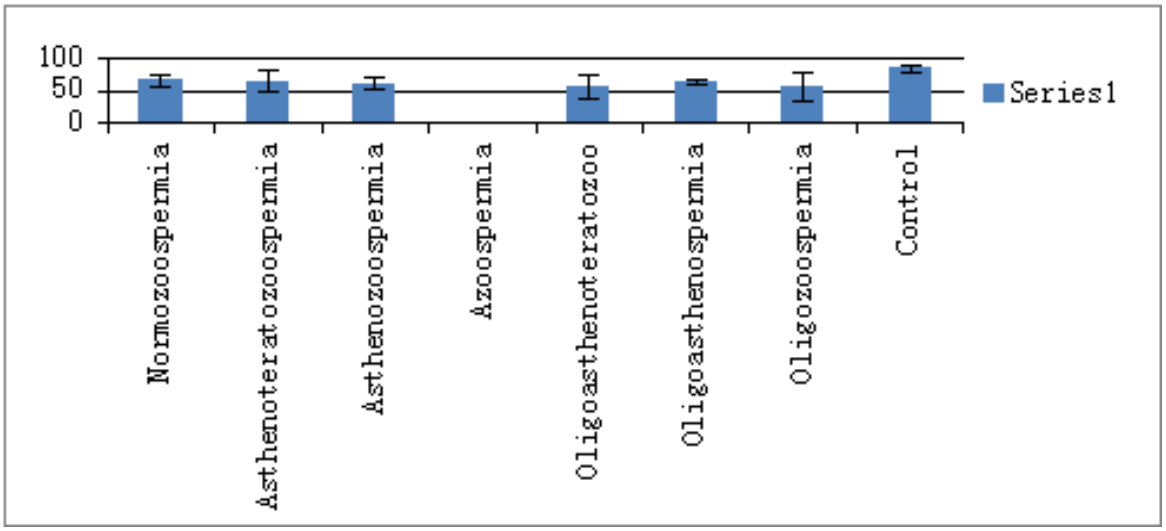


Figure 14: BMI among different obese/overweight conditions in comparison with control individual

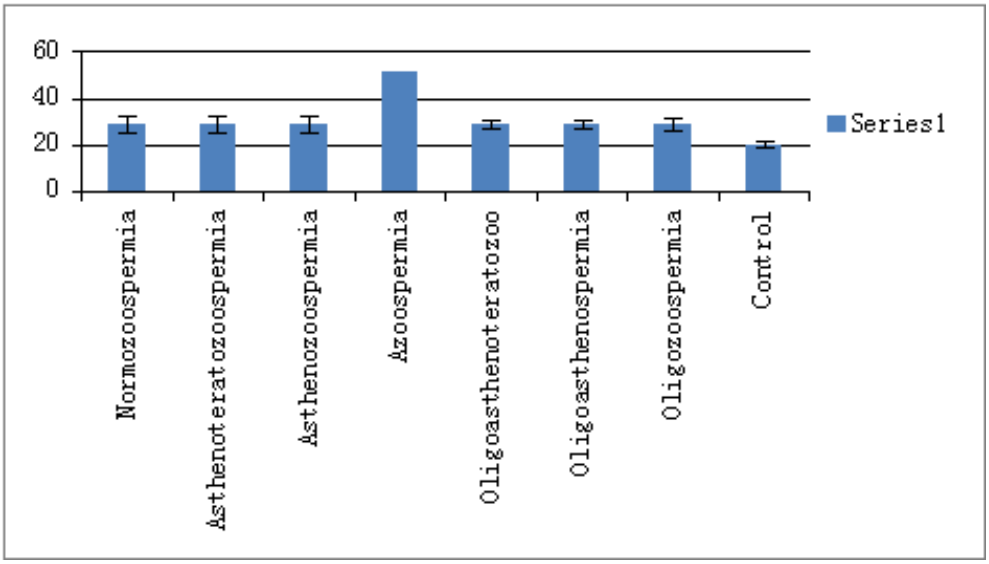


Figure 15: Figure showing biochemical test values (Fructose and Citric acid) Carnatine value)

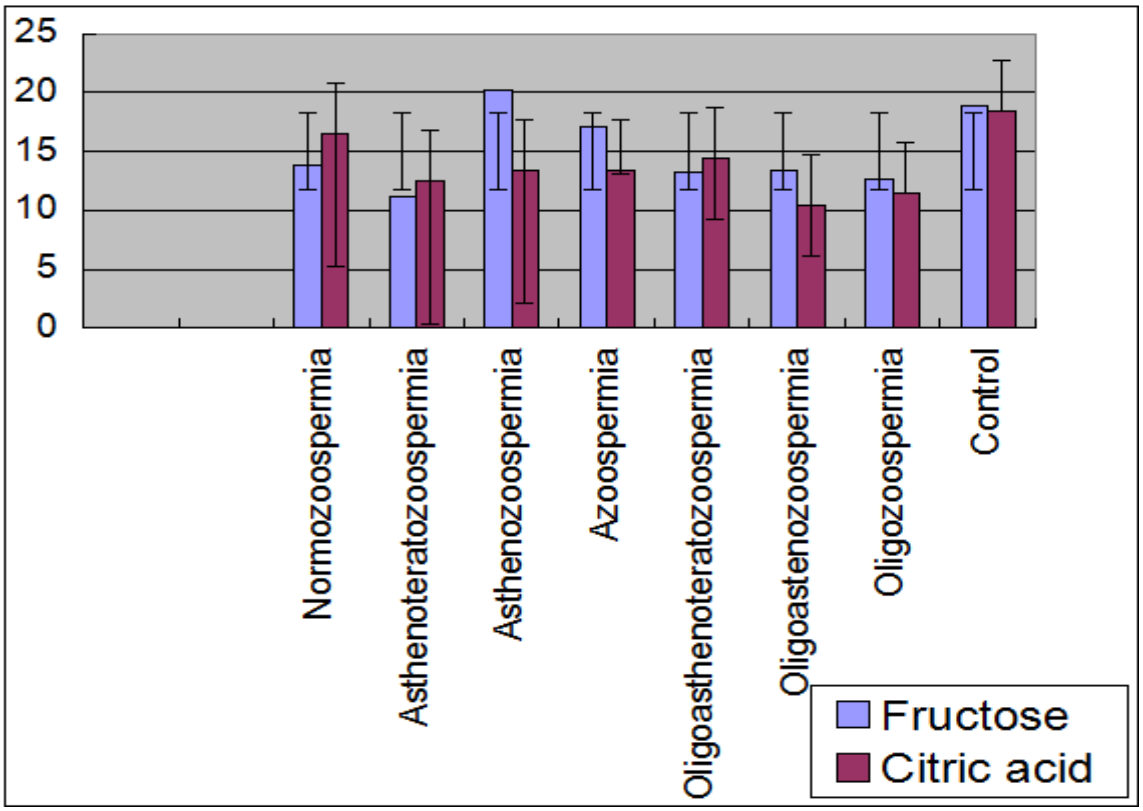


Figure 15: Figure showing biochemical test values (Acid Phosphase)

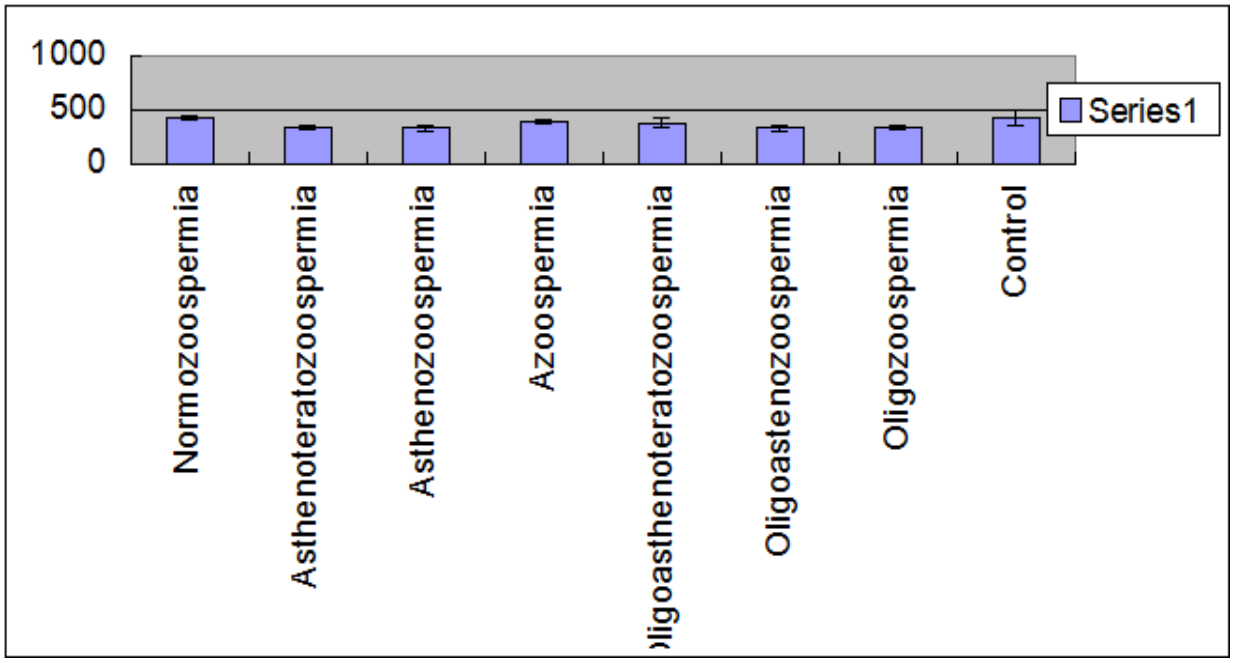


Figure 17: Figure showing biochemical test values (Fructose and Citric acid)

Carnatine value

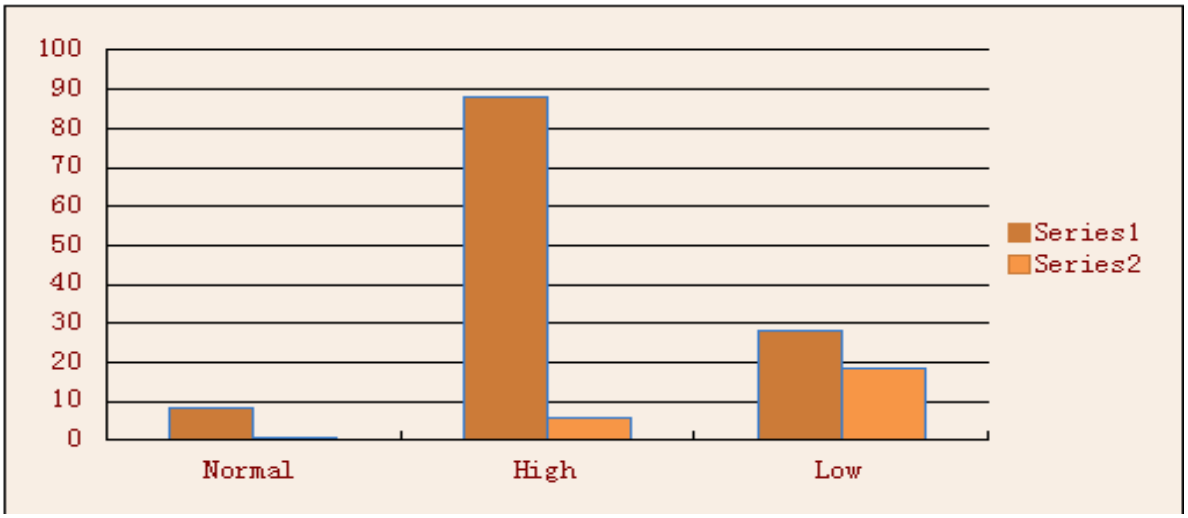
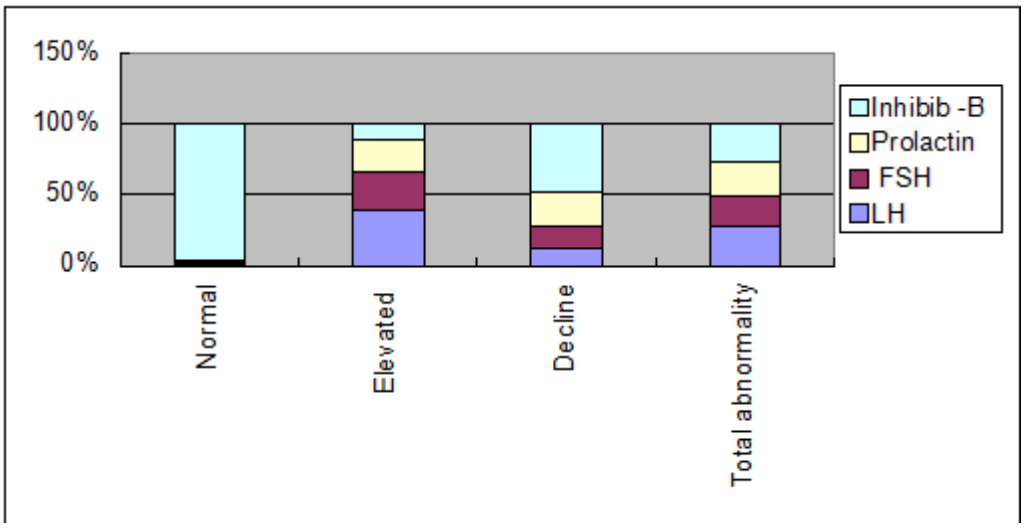


Figure 16: Hormone profile (FSH, LH, Prolactin, Inhibin-B) among different obese/overweight conditions in comparison with control individual



DISCUSSION

Semen analysis - For estimating the fertility rates in men the commonly advice test is semen analysis. In the present study, our data suggest that men with overweight and obesity are very prone to sub-fertility or infertility. Based on these available data we have found that the rate of infertility increased with BMI. Excess weight has been related directly or indirectly to biologic changes that could increase the risk of infertility. In this study, we found a significant decrease in semen volume, sperm count, sperm motility and sperm function tests in overweight and obese men that could be concluded that the increased BMI correlated negatively with above mentioned semen parameters. The prevalence of asthenozoospermia was high in our study. The possible reasons for variation in spermatogenesis in obese men could be due to fat deposition in supra-pubic area and thighs which alters the hormone profiles particularly sex hormones which can impair the expression of genes involving in spermatogenesis pathways leading to impaired semen parameters [decreased total sperm count, concentration and motility; increased DNA fragmentation index

In the present study the obese and overweight individuals were grouped into 7 different conditions based upon the microscopic observations like count, motility and morphology. Asthenozoospermia condition (10%) was found to be most reported followed by astenoteratozoospermia (6%) which was followed by oligoastenoteratozoospermia condition (5.5%) which was followed by oligoasthenozoospermia condition (5%) and oligozoospermia (4). Azoospermia condition (1.5%) was the least reported condition. The mean semen volume of all the infertile conditions was observed to be less than that of the control individuals. Asthenospermic and oligoasthenoteratozospermic conditions demonstrated decreased mean value forboth viability and motility when compared to other conditions and to the normal. Low sperm count was observed in oligoasthenoteratozospermic condition followed by oligozoospermia condition. All the conditions recorded low values for the sperm count when compared to normal

Sperm function tests

Sperm function tests enhance the spermiogram details. Apart from the routine physical and microscopic examination, analysis of sperm function tests plays a vital role to

evaluate the complete sperm profile. Further, it is cost effective and precise diagnostic toolto assess sperm-fertilizing potential at the initial stage to evaluate male fertility rates. Thus if abnormal levels of sperm dysfunction is diagnosed earlier, effective and personalized treatments can be recommended to improve the quality of sperms. Thus the chances of improving the fertility status of the individual are better instead of directly suggesting assisted reproductive technique. Hence sperm function tests must be carried out along with other routine analysis for precise diagnosis and correct treatment at the earliest. In this present study we attempt to unravel the functional status of spermatazoa's of obese and overweight men by Acrosome Intactness Test (AIT) it normally carried out to check the activity of the acrosomal enzymes which play a major role in fertilization of the oocyte. AIT assists in assessing sperm fertilizing ability and its reaction with zona pellucida. Exocytosis of sperm acrosomal contents is an important prerequisite for penetration of zona pellucida. The activity of the acrosomal enzymes is determined by the intact status of acrosome. Intact acrosome prevents loss of acrosomal enzymatic activity duringacrosomal reaction in female genital tract prior to fertilization (Tambe et al., 2001). Absence of acrosome reaction implies poor prognosis for fertilization. HOS test determines the plasma membrane intactness of the spermatozoa. Positive response of spermatozoa for HOS indicated good incidence of IVF (Toda et al., 1992). The normal spermatozoan membrane is the prerequisite for the specialized cell-to-cell communications and cell-to-cell binding. Membrane defects in the spermatozoa which could be the cause of defective membrane functions in the embryo leading to miscarriages. Intact membrane is essential for progressive motility which is very essential for the sperms to pass through the vaginal mucus (Patankar et al., 2001).The primary role of the sperm cell is to deliver paternal DNA and its associated factors to the oocyte for effective fertilization and embryodevelopment. The integrity of sperm DNA is a keystone of reproductive success. DNA-damaged sperm can form pronuclei at fertilization but may not allow the normal embryo development (Twigg et al., 1998). Nuclear chromatin of the spermatozoan is in a highly condensed state prior to fertilization. In vivo decondensation occurs in the ooplasm and is essential for successful fertilization and the formation of male pronucleus which fuses with the egg nucleus leading to the formation of the zygote (Levron et al., 1995). Sperm DNA integrity is poor in those couples whose natural pregnancy resulted in miscarriage as compared to that of the highly fertile couples (Virro et al., 2004). Sperm function test has not shown any

significant difference in the obese and overweight individuals. The values for the sperm function test were ranged in the normal conditions.

Biochemical Analysis:

Estimation of Fructose, Citric acid, Carnitine and Acid Phosphatase

The biochemical constituents of seminal plasma routinely studiedare fructose and citric acid carnitine and acid phosphatase. Fructose a readily glycolysable sugar is produced inhumans 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). **Fructose value is lower in Oligospermia, Asthenosteratozoospermia conditions when compared with normal range.** The results of Acid phosphatase assay in all the cases lie within the reference range **(Table 5)** where as described in **Table 6 and Figure 15.**

Citric acid is reliable indicator of prostatic function where the absence or low concentration suggests the dysfunction of prostatic glands. A decrease in its concentration is observed in cases of inflammation, cancer of the prostate gland and also acute or chronic prostatitis (Kammer et al., 1991; Costello and Franklin, 1998). **In the present study we observed decreased citric acid level in Oligoteratozoospermia. oligozoospermia and Asthenoteratoozoospermia cases** and subsequent increase in the alkaline nature of semen which indicates some extent of infection, inflammation and prostate gland malformation. Another biochemical marker carnitine is concentrated in high energy demanding tissues such as skeletal and cardiac muscles and in a specialized reproductive tract organ, the epididymis. It plays an important role in transferring long-chain fatty acids into the mitochondria for oxidation, producing energy. In epididymis, free carnitine is taken up from the blood plasma and is transported into the epididymal fluid. Our study revealed there is decreased level of carnitine normal value were 8.31± 0.75elevatedvalue were 87.86±5.78 and decreased value were 8.09±1.65could be due to passively diffused spermatozoa, which accumulates as both free and acetylated carnitine .The initiation of sperm motility occurs in parallel with increase in concentration of free carnitine in the epididymal lumen.

It has been known for a long time that human seminal plasma is a rich source of **Acid (prostatic) phosphatase** (Kutscher & Wolbergs, 1935). Gutman & Gutman (1941) were the first to report an alkaline phosphatase activity (at pH 9) in human seminal fluid, although with a very much lower activity than the acid phosphatase. Acid phosphatase is an enzyme (hydrolase), which catalyzes the hydrolysis of various phosphate esters with pH-optimum in the acid zone. It can be found in high concentrations in the prostate, bones, blood cells, the spleen and other organs. The detection of acid phosphatase in human semen has a diagnostic value in the treatment of prostate diseases, which can be used as a marker (Miteva1 et al., 2010). In our present study, the level of acid phosphatase is normal in both cases and control group indicate normal functional status of Prostate without inflammation.

Hormone analysis

The frequency of endocrine abnormalities observed in the present study is depicted in **Table 6 and Figure 16**. LH and FSH recorded 38.75%, 30.05% of abnormalities. Prolactin levels were found to be 32.5%. Inhibin-B level was found to be 37.85% with more variations. In 21 cases, 31 cases 39 cases and 21cases for LH. FSH, Prolactin and Inhibin-B was found to be elevated which indicates abnormal spermatogenesis. An elevated level of prolactin affects the pulsatile secretion of GnRH which in turn affects the pulsatility of FSH, LH and testosterone secretion. This can lead to spermatogenic arrest and low motility (de Rosa et al., 2003). The elevated levels of prolactin could have affected the fertility among these subjects by directly disrupting spermatogenesis and steroidogenesis.The absence or low concentration of these secretions suggests the dysfunction of the accessory glands like seminal vesicle and prostate. Spermatogenesis is initiated and regulated by the action of Follicle Stimulating Hormone (FSH) and Luteinizing hormone (LH), which are very much essential for normal spermatogenesis as well as accessory glandular function. Earlier studies have confirmed the direct role of prolactin in spermatrogenesis andsteroidogenesis as receptors for prolactin has been identified in Sertoli cells and Leydig cells (Arowojolu et al., 2004). Also the normal levels of FSH and LH along with elevated levels of prolactin indicates the role of prolactin inspermatogenesis independent of the gonadotropins (Colao et al., 2004).

SUMMARY

From our study it is obvious that various factors can be implicated as a cause for different conditions of male infertility observed among the individuals recruited for the present study. Seven different infertile conditions were observed among the obese and overweight individuals in the present study. Asthenozoospermia condition was found to be predominant while oligozoospermia was the least reported condition. About 32% of cases showing abnormality supporting that obesity and overweight may affect the semen parameters.

AN ANALYSIS OF THE EFFECT OF OBESITY ON MALE FERTILITY RATES IN KARNATAKA

GENETIC REGISTRY

NAME :
DATE :

AGE :
CASE NO :

ADDRESS :
HOSPITAL/CLINIC :

CONTACT NO :

NATIVE :

RELIGION/CASTE :

QUALIFICATION :

OCCUPATION :

MARRIED / UNMARRIED : WIFE'S NAME: AGE:

Sex/age of child (en) :

BLOOD GROUP : A/B/AB/O

Duration of marriage life:

MEDICAL HISTORY:

Type of health problem	Yes / No	Age	Drug name	D/W/M
Hypertension				
Diabetes(type 1 or type 2)				
Obesity(class 1/2/3)				
Renal problem				
Gall bladder disease				
Neurological problem				
Cardiac problems				
High cholesterol				
Asthma				
Urinary tract infection				
Erectile dysfunction/infertility				
Low volume ejaculation				

Bloody ejaculation				
Dry ejaculation				
Painful ejaculation				
Thyroid disorder				
Sleep apnea				
Varicocele				
Any Major surgery				

LIFE STYLE:

Physical exercise: Yes / No D/W/M/Y Time:

Yoga: Yes/No D/W/M/Y Time:

Meditation: Yes / No

Sleep Duration: > 7hrs / <6hrs

MOBILE PHONE HISTORY:

Since when:

Vibration / Ringing / Silent:

Where you keep: Shirt / Pant pocket

Computer/Laptop: Yes/No Duration:

OTHER HISTORY:

HABITS	DAILY	WEAKLY	MONTHLY	QUANTITY/ PACKS
Alcohol				
Smoking				
Tobacco				

FAMILY HISTORY

	Mother	Father	Brothers	Sisters	Children	Other
Diabetes						
Hypertension						
Heart Disease						
Stroke						
Cancer(type)						

Obesity						
Arthritis						
Infertility						
Other Disease						

DETAILS OF SUBJECT DIET:

FOOD	DAILY	WEEKLY	MONTHLY
Rice			
Ragi			
Fruits			
Vegetables			
Ghee			
Oil			
Non veg.			
Egg			
Junk Foods			

Meals per day (Including Tiffin):

Thrice / More than thrice

Eating between meals? Yes/No

Eating meals outside: Yes/No

Consumption of Tea/ Coffee:

No of times per day:

SYSTEMIC REVIEW:

Height				
Weight				
Chest circumference				
Waist circumference				
Hip circumference				
Skin fold thickness	Mid biceps	Mid triceps	Sub scapular	Supra iliac

BODY COMPOSITION FEATURES:

Character	Measurements
BMI	
Fat Mass	
Fat mass index	
Fat free mass	
Fat free mass index	

TYPE OF WORK : Easy / Moderate / Heavy

STRESS AT WORK : YES / NO

TYPE OF MARRIAGE :

	Consanguineous	Non-Consanguineous
Subject		
Subjects parents		
Spouse parents		

□ **Semen Analysis** When _____/_____/_____ Results ……..