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A Review on: Assessment of the Microbial Assay's Possible Aphrodisiac Effect on Male Testicular Hypogonadism

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ABSTRACT: Hypogonadism is a medical word for diminished gonad functional activity. Hypogonadotropic hypogonadism (HH) is an uncommon clinical disease caused by hypothalamic or pituitary dysfunction, which results in gonadal insufficiency. Male hypogonadism is defined by poor testicular function, which can interfere with spermatogenesis and/or testosterone synthesis. The term aphrodisiac is derived from Aphrodite, the Greek goddess of sexuality, love, and beauty. An aphrodisiac is a substance (food or chemical) that stimulates sexual desire. Spermatogenesis is the process or development of mature spermatozoa in the testis. Aphrodisiac cell culture conditions include: full culture medium, serum-free DMEM, freezing medium, thawing cells, subculturing, changing medium, and so on.Various ways of collecting sperm from animals such as rats and mice are described. The preparation of five sperm expanders—TL-HEPEST, ris-citratem,KRBL, EYP, and BS—was briefly seen. We are seeing the sterile collecting of animal sperm for study and microbiological investigation. Physical parameters of hypogonadism illness semen, such as viscosity, volume, pH, motility, hormonal assay, and ejaculation appearance, are observed.

KEYWORD: Aphrodisiac, hypogonadism, semen, TL-HEPES, sperm expanders, PBS, LEY, Tris – citrate, Hormonal assay, Ejaculation**.**

I. INTRODUCTION

Hypogonadism is a deficiency of testosterone in male patients that can be caused by a central (hypothalamic or pituitary) or testicular cause, or a mix of the two [1]. Male hypogonadism is a testicular malfunction characterized by reduced sperm and testosterone production [2]. Hypogonadism can be hypothalamic-pituitary or testicular in origin, or a combination of the two, which is becoming more frequent in the aging male population [1].

An aphrodisiac is a substance (food or chemical) that stimulates sexual desire [3].This word is taken from Aphrodite, the Greek goddess of love, and these substances are obtained from plants, animals, or minerals, and they have been man's passion since time immemorial. Aphrodisiacs are classified into two types: psychophysiological stimuli (visual, tactile, olfactory, and aural) preparations and internal preparations (food, alcoholic beverages, and love portion)[4].

Hypogonadotropic hypogonadism, central hypogonadism, or secondary hypogonadism refers to hypogonadism in male patients with gonadotropin insufficiency or dysfunction caused by disease or damage to the hypothalamicpituitary axis [3]. According to the World Health Organization (WHO), Hypogonadotropic hypogonadism is classed as a group 1 ovulation condition. Patients endure delayed or halted puberty, secondary amenorrhea, and infertility depending on the age of beginning.It is distinguished biochemically by low blood sex steroid hormone levels, low or normal luteinizing hormone (LH), and low follicle stimulating hormone (FSH) levels [5].

1.1. Classification of male hypogonadism

A) Primary hypogonadism
B) Secondary hypogonadism
C) Mixed Hypogonadism

1.2. Causes and symptoms of hypogonadism

A) Primary hypogonadism
I. Congenital anorchidism
ii. Cryptorchidism
iii. Mumps orchitis
-Genetic and developmental conditions:

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I. Klinefelter syndrome
ii.Autoimmune syndromes (anti-Leydig cell disorders)

B) Secondary hypogonadism
Genetic conditions:
i. Kallmann's syndrome
ii. Prader-Willisyndrome
iii. Pituitary tumours
iv. granulomas, abscesses
v. Hyperprolactinemia
vi. Cranial trauma

C) Mixed Hypogonadism
(Both primary and secondary)
i.Alcohol abuse
ii.Ageing [6]

1.4. Etiology

1. Hypergonadotropic hypogonadism (primary hypogonadism) in males:

- Klinefelter syndrome
- Cryptorchidism
- Previous chemotherapy or radiotherapy treatment
- Congenital bilateral anorchia
- Testicular trauma
- Gonadectomy
- Defects in testicular determination: gonadal dysgenesis
- Sertoli-cell-only syndrome
- Luteinizing hormone [LH] resistance
- Mumps virus
- Disorders of androgen synthesis:

- 17β-hydroxylase dehydrogenase deficiency
- 5α-reductase deficiency
- 17-hydroxylase deficiency

2. Hypogonadotropic hypogonadism (secondary hypogonadism): disruption of the hypothalamus or pituitary gland hormonal axis

- Sarcoidosis
- Hemochromatosis
- Tuberculosis
- Medications:

- Opioid analgesics
- Glucocorticoids
- Leuprolide

- Hypothalamic or pituitary tumors (pituitary adenoma)
- Congenital disorders:

- Kallmann syndrome
- Prader-Willi syndrome
- Angelman syndrome
- Gaucher disease

- Other disorders:

- Eating disorders
- Exercise-induced hypogonadism
- Hyperprolactinemia
- Cushing syndrome
- HIV/AIDS
- Morbid obesity
- Type 2 diabetes mellitus [7]

1.5. Epidemiology

- Hypogonadism may occur at any age.
- Often underreported
- Primary hypogonadism:

- Most common cause in women is Turner syndrome (incidence: 1 in 2000–2500 live births).
- Most common cause in men is Klinefelter syndrome (incidence: 1 in 600 live male births).
- Men > women (because incidence of Klinefelter syndrome > incidence of Turner syndrome)

- Secondary hypogonadism:

- Less common than primary hypogonadism
- Incidence: 1 in 10,000–86,000 people
- Men = women
- Kallmann syndrome causes approximately ⅔ of congenital cases [8]

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1.6. Mechanism of spermatogenesis

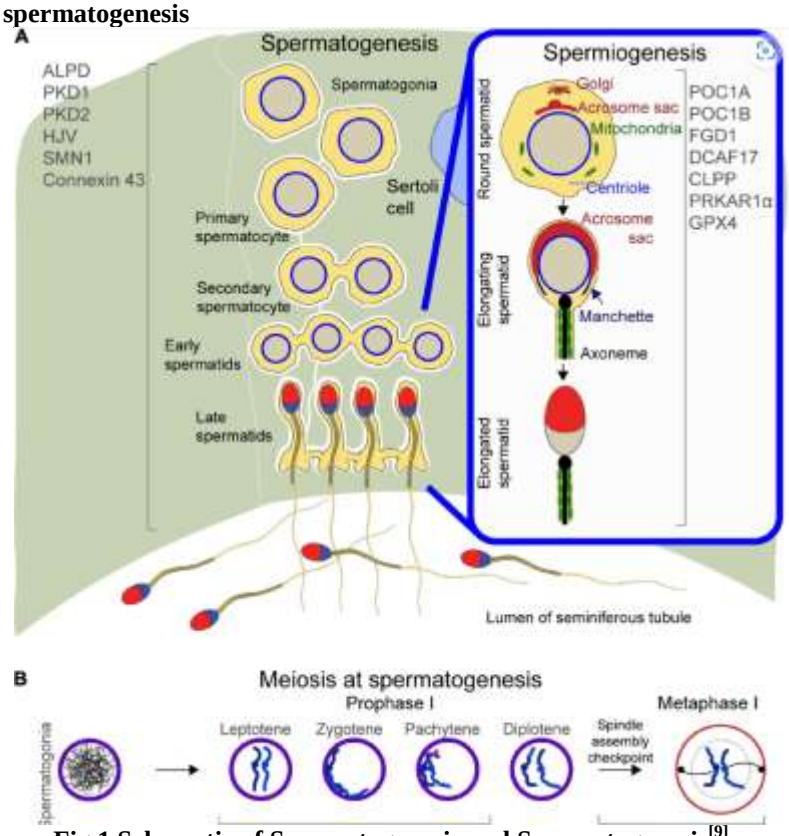

Fig.1.Schematic of Spermatogenesis and Spermatogenesis[9]

Spermatogenesis is the process by which male germ cells grow and produce sperm cells. It is found in the testicular seminiferous tubules.The key stages include:

Proliferation (Mitosis): Mitosis is the process by which spermatogonia (stem cells) divide to make additional spermatogonia. Some retain stem cells, while others develop into primary spermatocytes.

Meiosis I: The first meiotic division of primary spermatocytes results in the formation of two secondary spermatocytes.

Meiosis II: Secondary spermatocytes divide for the second time, producing four haploid spermatids.

Spermatogenesis: - Spermiogenesis is the process by which spermatids undergo morphological and functional changes before maturing into spermatozoa. This entails restructuring and excreting superfluous cytoplasm.

Spermiation: Mature spermatozoa are discharged into the lumen of the seminiferous tubules during spermiation.

Following this, sperm mature further as they move through the epididymis and vas deferens. The pituitary gland produces hormones such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which govern the entire process [10, 11, 12, and 13]

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1.6. Pharmacology of hypogonadism

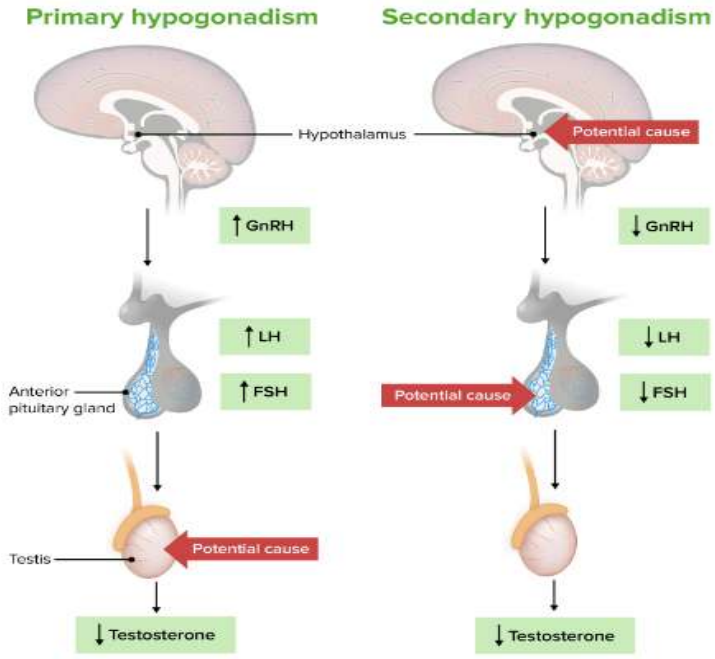

Fig.2.Pathogenesis of Primary verses Secondaryhypogonadism [14]

In primary hypogonadism, the hypothalamus GnRH level rises, sending a signal to the anterior pituitary gland, which raises LH levels while decreasing FSH. Then there's dysfunction, in which decreased testosterone production targets the testicle [15,16,17].

Secondary hypogonadism has probable causes for a decrease in GnRH level in the hypothelamus; as a result, there are potential causes for a decrease in LH and FSH in the anterior pituitary. They cause a reduction in testosterone production [15,16,18].

1.7. Mechanism of action of aphrodisiac potential

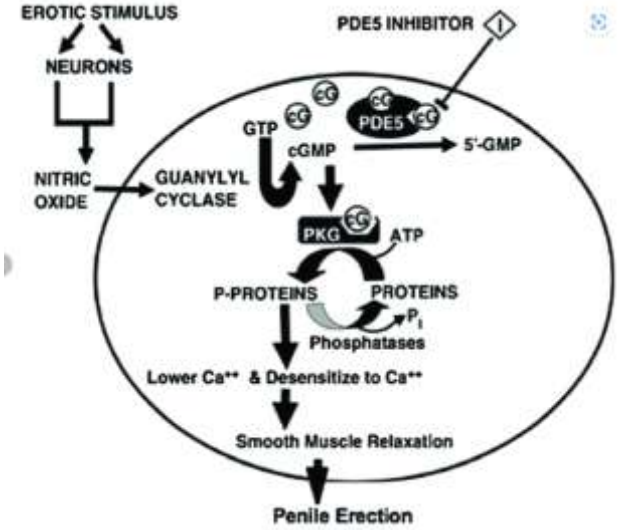

Fig.3. Mechanism of action of aphrodisiac agents [19]

- Aphrodisiacs are substances that can stimulate sexual desire or libido. These products cause the hypothalamus to release nitric oxide, dilates the blood vessels of the corpus

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carvenosum and activates the enzyme guanylate cyclase (GC), which converts nucleotide guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), resulting in penis erection. After a while, the erection stops because C.GMP is hydrolyzed by the phosphodiesterase type-5 enzyme (PDE-5) into inactive GMP. (The PDE-5 enzyme is found in penile tissues). PDE-5 hydrolysis is inhibited by aphrodisiac potentials, allowing active C.GMP to build. 'Uninterrupted' and extend the erection via increased blood flow [20,21,22,23].

II. METHODS AND MATERIALS

2.1. Cell culture conditions

A)Complete Culture Medium:

1. DMEM: 90%,
2. FBS: 10%,
3. L-glutamine 2.0 mM,
4. Amp 100 µg/ml,
5. Strep 100 µg/ml,
6. G418 700 µg/ml

B)Serum-free DMEM:

1. DMEM: 90%,
2. 0.1% BSA
3. L-glutamine 2.0 mM,
4. Amp 100 µg/ml,
5. Strep 100 µg/ml,
6. G418 700 µg/ml

C)Freezing Medium:

1. DMEM: 90%,
2. FBS: 10%,

F) Changing Medium:

Normally, this is done every other day.

a. Aspirate gently off medium.
b. Fill a flask with new warm complete DMEM media (37°C) (5 ml for T25 and 10 ml for T75)[24].

G) Freezing Cells:

a. Rep steps 1-3 from the subculturing segment.
b. For five minutes, centrifuge the cells at 1,000 rpm.
c. Remove the supernatant and resuspend the cells at a density of 2-3 106 cells/ml in fresh freezing media. Add 1 mL of cells to each cryogenic vial.
d. Place the cryogenic cell vial in the cryo freezing container. The container should then be moved to a -80°C environment and left there overnight.

3. L-glutamine 2.0 mM,
4. Amp 100 µg/ml,
5. Strep 100 µg/ml,
6. G418 700 µg/ml
7. 20% FBS
8. 10% DMSO

D) Thawing Cells:

a. Thaw frozen cells quickly in a 37°C water bath, stirring constantly.
b. Pipette the cells up and down five times with a 1 ml pipette and drop by drop into a 15 ml centrifuge tube containing 5 ml of fresh prewarmed full DMEM media. Then, for five minutes, centrifuge at 1,000 rpm.
c. Resuspend the cell pellet in 5 ml of new prewarmed full DMEM media after discarding the supernatant medium. Incubate the cells in a T25 flask at 37°C with 5% CO2 until they achieve >90% confluence. For frozen cells, the recovery rate is usually 90% or higher[24,25].

E) Subculturing:

When the cells attain confluence, they must be separated. This cell line is generally divided twice weekly at dilutions of 1:8 to 1:15.

a. Aspirate all media with care. Gently aspirate the cell layer after gently rinsing it with 0.2% trypsin-EDTA.
b. Wait for approximately 1-3 minutes. Tap the sides of the flask or dish gently to dislodge the cells.
c. Resuspend the cells in an adequate volume of full DMEM media and split them as needed[24,26] .
e. Fill the cryogenic vial with liquid nitrogen (-196°C)[24,27].

2.2. Collection of semen

A. Collection of semen for diagnosis or research purposes

- Masturbation and ejaculation into a clean, wide-mouthed glass or plastic container from a batch that has been proven to be spermatozoa-safe should be used to obtain the sample.
- To minimize substantial temperature variations that may alter the spermatozoa after they are ejaculated into the specimen container, keep it at room temperature, between 20°C and 37°C. It must bear the men's name and identifying number, as well as the date and time of collection.
- While the semen liquefies, the specimen container is placed on the bench or in an incubator (37°C).

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- If the sample is incomplete, especially if the initial, sperm-rich fraction is absent, make a note of it in the report. If the sample is incomplete, a second sample should be collected after a 2- to 7-day abstinence interval [16].

B. Sterile collection of semen for assisted reproduction

The procedure is the same as for diagnostic collection, except that the specimen container and pipette tips for mixing must be sterile [16].

C. Sterile collection of semen for microbiological analysis

Microbiological contamination from non-semen sources (e.g., commensal organisms from the skin) must be avoided in this case. Sterile specimen containers, pipette tips, and pipettes for mixing are required.

The animal should:-

- Urine passes.
- Wash hands and penis with soap to prevent the danger of commensal organisms from the skin contaminating the samples.
- Rinse the soap away.
- Use a fresh disposable towel to dry hads and penis.
- Empty your ejaculate into a sterilized container.[28]

2.3. Animal [Rat]

1. Animal and housing conditions

The National Laboratory Animal Center at Mahidol University in Thailand provided 24 male and 20 female Wistar rats (Rattus norvegicus) 6 weeks old and weighed roughly 180-200 g. The animals were kept in normal polypropylene cages with sawdust bedding, with relative humidity kept between 60% and 70% and room temperature kept between 25 and 2°C. 12-hour light/dark cycles were used to govern lighting periods. Throughout the experiment, rats were given unlimited access to food and water. The animals were given a week to acclimate to the laboratory surroundings before the trial began [29].

2. Sperm collection of Animal

As sperm donors, outbred Sprague-Dawley male rats (aged 16 to 20 weeks) were employed. The rats were housed in compliance with the Animal Care and Use Committee's rules and the Guide for the Care and Use of Laboratory Animals. Male rats were euthanized with CO2 inhalation for sperm collection, and cauda epididymides were removed and deposited in a 35-mm culture dish with TL-HEPES solution supplemented with 3 mg/mL BSA (fraction V). The cauda epididymides were dissected with fine scissors to allow sperm to float out at room temperature for 10 to 15 minutes. The sperm suspension was gently drawn into a plastic transfer pipette (inner diameter, 2 mm; Samco, San Fernando, CA) and deposited in a 5-mL tube for further analysis. Each sperm sample had a final concentration of 12 to 15 106 sperm/mL. The motility study was done with a phase-contrast microscope, and the sperm samples were kept at 22 °C for subsequent testing. Each experiment was carried out six times with sperm from a single donor [30,31].

3. Preparation of sperm extenders.

1.TL-HEPES
2. Tris–citrate
3.mKRB
4. LEY
5.PBS

1. TL-HEPES :- The TL-HEPES solution contains 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO3, and 0.4 mM NaH2PO4.•H2O, 10 mM lactic acid, 2 mM CaCl2•2H2O, 0.5 mM MgCl2•6H2O, 10 mL/L penicillin-streptomycin (10 mg streptomycin and 10,000 IU penicillin sulfate per 1 mL). To create a workable solution, BSA (fraction V, 3 mg/mL) was added. Because of its impact on fluorescence staining, phenol red was not used. The TL-HEPES extender had a pH of 7.2 and an osmolality of 290 5 mOsm [24,30,32].

2. Tris–citrate:- Salamon modified Tris-citrate was used to create this extender, which comprised 27.0 g/L Tris, 14.0 g/L citric acid, 10.0 g/L fructose, 50 g/mL streptomycin, and 75 g/L penicillin sulfate. This extender's osmolality was 416 5 mOsm, and the pH was set to 7.0[24,30,33].

3.mKRB :-mKRB buffer included 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl2•0.2H2O, 1.19 mM MgSO4•0.7H2O, 1.19 mM KH2PO4, 25.07 mM NaHCO3, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 5.56 mM glucose, 50 µg/mL streptomycin, and 75 µg/mL penicillin sulfate. The mKRB extender's pH and osmolality were 7.0 and 300 5 mOsm, respectively [24,30,34,35].

4. LEY :- Lactose solution was made by dissolving 8% lactose monohydrate (w/v) in filtered, deionized water; 23 mL egg yolk was combined with 77 mL lactose solution and centrifuged at

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15,000 g for 1 hour in sterile tubes. The supernatant was transferred to a fresh tube, and the solution was spiked with 50 g/mL streptomycin and 75 g/mL penicillin sulfate. The pH and osmolality were both 7.0 and 330 5 mOsm [24,30].

5. PBS: - Diluting the 10 stock and adding 50 g/mL streptomycin and 75 g/mL penicillin sulfate resulted in PBS extender. PBS's pH and osmolality were 7.3 and 280 5 mOsm, respectively [24,30].

4. Sperm collection analysis

The sperm was collected in a sterile container, and all individuals abstained for 2-5 days. The world health organization (WHO) laboratory manual for the testing and processing of human sperm (Organization, 2010) was used for the sperm analysis. Masturbation induced ejaculation, which was liquefied at 37 °C for 30 minutes. Mackler chamber was used to evaluate sperm analysis, including count and motility parameters (e.g., progressive, non-progressive, and immotile). For sperm morphology and viability evaluation, Papanicolaou and Eosin-Nigrosin staining tests were performed [36,37].

5. Microorganism identification

Homogenized sperm samples were grown on sheep blood agar and eosin methylene blue agar (EMB) (Condapronadisa, Spain), respectively, to isolate gram positive and negative bacteria. Differential tests for phenotypic identification of the species were performed, including Bile Esculin Agar (BEA), growth in 6.5% NaCl, catalase, and hemolysis assays. Staphylococcus species were identified using mannitol salt agar (MSA), DNase agar, coagulase, and catalase assays. Catalase, oxidase, Simmons citrate agar, methyl red-voges-proskauer (MR-VP), lysine decarboxylase, triple sugar iron (TSI), and sulfide-indol-motility (SIM) tests were used to characterize Gram-negative bacteria [19].

PCR reactions were used to detect various growing colonies. Qiagen extraction kit (QIAamp DNA micro kit, USA) was used to extract chromosomal DNA from pure colonies. A Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, UK) and 1% gel electrophoresis were used to determine DNA content and purity. Templet DNA was stored at -20 °C until further usage.PCR was carried out in an Eppendorf Master Cycle Gradient thermocycler (Eppendorf, Hamburg, Germany) in a final volume of 25 l. PCR products were electrophoresed in a 1% agarose/0.5TBE gel (45 mM-Tris-borate, 1 mM-EDTA) and stained with 0.1 l/ml Gel RedTM (Biotium, USA) before being photographed with a UV trans-illuminator (Tanon, China)[36].

6. Physical parameters

1] semen viscosity

The sample's viscosity can be evaluated by gently aspirating it into a wide-bore (about 1.5 mm diameter) plastic disposable pipette, allowing the semen to drop by gravity, and measuring the length of any thread. A typical sample emerges from the pipette in small, distinct drops. If the viscosity is abnormal, the drop will create a thread that is longer than 2 cm.

Alternatively, the viscosity can be determined by inserting a glass rod into the sample and measuring the length of the thread that forms when the rod is withdrawn. When the thread's viscosity surpasses 2 cm, it should be reported as abnormal.

A viscous semen specimen, in contrast to a partially unliquefied sample, exhibits homogenous stickiness and consistency that does not alter with time. The elastic characteristics of the sample, which adheres, indicate high viscosity. When pipette attempts are made, it sticks hard to itself[28,37].

2] Appearance of the ejaculate

A normal liquefied sperm sample appears uniform and grey-opalescent. If the sperm concentration is very low, it may appear less opaque; the color may also differ, for example, red-brown when red blood cells are present (haemo-spermia), or yellow in a guy suffering from jaundice or who is taking specific vitamins or medicines [28].

3] Semen Volume:-

Semen volume was determined by aspirating semen ejaculated at the glass dish's corner into a pre-warmed tuberculin syringe graded to 0.01 mL precision.

Weighing the sample in the vessel in which it is collected yields the most accurate volume measurement.

• Collect the sample in a clean, disposable, pre-weighed container.
• Weigh the vial containing the sperm.
• Subtract the container's weight.
• Calculate the volume based on the sample weight, assuming a density of 1 g/ml for the sperm (Auger et al., 1995). (The density of sperm ranges from 1.043 to 1.102 g/ml (Huggins et al., 1942; Brazil et al., 2004a; Cooper et al., 2007).

Alternately, the volume can measure directly.

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- Collect the sample immediately into a graduated glass measuring cylinder with a wide mouth that has been modified. These are commercially available.
- Directly read the volume from the graduations (0.1 ml precision).

Lower reference limit: -The lower limit for sperm volume is 1.5 ml (5th centile, 95% confidence interval (Cl) 1.4-1.7)[28,38].

4] Semen pH

The pH of sperm reflects the equilibrium of the pH values of the several accessory gland secretions, most notably the alkaline seminal vesicular secretion and the acidic prostatic secretion. The pH should be determined after liquefaction at a consistent time, preferably after 30 minutes, but in any event within 1 hour of ejaculation because it is affected by CO2 loss after production.

For normal sample, pH paper in the range 6.0 to 10.0 should be use.

• Mix the semen sample well

• Distribute a drop of sperm equally onto the pH paper.

• Wait for the colour of the impregnated zone to become uniform (<30 seconds).

• To determine the pH, compare the color to the calibration strip [28,38].

5] Semen motility

Sperm motility was measured by diluting a half drop of semen on a pre-warmed clean glass slide with a drop of warm 2.9% Sodium citrate, covering with a slip, and inspecting at X100 magnification under an Olympus CX21 (Japan) light microscope. Under a light microscope with an X40 magnification, mass activity was measured on a scale of five. Individual sperm motility was measured using a modified approach reported by Wheeler and Andrews (Cyriac et al., 2013), and the results were expressed as a percentage [38,39] .

6] Hormonal assay

A capillary tube was used to collect 4-5 mL of blood from the inner canthus of each rat's eye and place it into heparinized and plain sample vials. After centrifuging the blood at 2500 rpm for 10 minutes, serum was collected, and appropriate serum samples (50 l) were pipetted into allotted wells. A 100 l solution of hormone-enzyme reagent was applied to each well. The microplate was gently spun for 20-30 seconds before being covered and incubated at room temperature for 60 minutes. The contents of the microplate were then removed, and 350 l of wash buffer was added and aspirated twice more for a total of three washes. Working substrate solution (100 l) was applied to all wells and incubated for 15 minutes at room temperature. Each well received 50 l of stop solution, which was mixed for 15 - 20 seconds. Within 30 minutes of adding the stop solution, the absorbance of each well was measured at 450 nm using a micrometer reader. The test was carried out in duplicate[38].

III. CONCLUSION:

Male patients with hypogonadism have low testosterone levels, which can be brought on by either a central (pituitary or hypothalamic) or testicular source, or by a combination of the two. An aphrodisiac is a food or chemical that piques one's desire for sex. The process by which male germ cells develop into sperm cells is known as spermatogenesis. The testicular seminiferous tubules contain it. Among the cell culture techniques included in the review above are the following: circumstances for cell culture.

Complete culture medium , serum-free DMEM , freezing medium, thawing cells, subculturing, changing medium , and so on Cells in Freeze: Gathering of seed Semen collection for study or diagnosis, Sterile semen collection for assisted reproduction , Sterile semen collection for microbiological analysis. A liquid diluent called semen extender is added to semen in order to maintain its fertility-promoting properties. It serves as a barrier to keep sperm cells safe from the harmful byproducts they produce. The aforementioned reviews: TL-HEPES semen extender preparation techniques: TL-HEPES, Trisocyanate, mKRB, LEY. The physical characteristics of semen and sperm include Semen volume, pH, viscosity, and volume are the first four factors to consider. Hormone test, the way the ejaculation looks.

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