Bani-Suef University
Faculty of Veterinary Medicine
Department of Theriogenology

Seminar on

Advanced studies on infertility in male dogs with special reference to diagnosis and treatment

As a requirement for PhD degree
(Theriogenology Department)

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Introduction

In recent years, there has been a significant surge in interest in commercial breeding dogs **(Jang, G., Kim, M. K., and Lee, B. C. 2010),** especially in terms of genetic improvement and reproduction of dogs with high zootechnical and economic importance **(Thomassen, R., and Farstad, W. 2009).** Domestic dogs, too, are not only excellent companions but also ideal experimental models due to their reproductive physiology's resemblance to that of wild species and humans **(Kirchhoff, C. 2002)**. Owners often require a semen evaluation of their dogs at the beginning of their reproductive career, when showing a decreased fertility, or if intended for semen cryopreservation. A semen evaluation is advisable on samples collected for fresh semen artificial insemination (AI) **Freshman JL.(2002)**.

The ultimate goal of semen evaluation is to predict the fertilizing capacity of a semen sample. Generally, males with optimal fertility produce semen with high number of progressively motile, viable and morphologically normal spermatozoa. Whereas infertile or sub-fertile individuals contain low percentage of normal viable sperm. Till date, light microscopy is routinely used to evaluate the principal parameters of semen, concentration, motility and morphology. Concentration is usually determined using a Neubauer-counting chamber. Motility is assessed subjectively on a pre-warmed glass slide **(Johnston, 1992; Iguer-ouada and Verstegen, 2001)**, whereas morphology defects are assessed with various staining techniques **(Oettle, 1993; Johnston, 1992)**. Recently, several techniques have been described which may enable more accurate prediction of the fertilizing capacity of canine spermatozoa.

This allows assessment of many functional characteristics of spermatozoa which are related to the binding, penetrating and fertilizing capacity of an oocyte **(Van Soom*et al*., 2001; Hewitt and England 2001).**

Review of Literature

The reproductive organs of the male dog consist of the testicles with epididymides and the vas deferens, the prostate gland, the urethra and the penis. The testicle contains the seminiferous tubules, producing spermatozoa, and the interstitium with Leydig cells which produce testosterone. The epididymis consists of a single long duct in which the spermatozoa undergo maturational changes and capacity for motility. The cauda, is the storage site for the matured spermatozoa. Prostatic fluid constitutes the major portion of the ejaculate, and contains several enzymes, cholesterol and lactate. The penis consists of a pelvic part, and the glans penis, varies from 5-15 cm long. The glans penis has two cavernous parts, the bulbus glandis and the pars longa glandis, which fill with blood during sexual arousal creating an erection. The dog also has a dorsal penile bone, which enables coital intromission of the non-erect penis, **C. Linde Forsberg, (2007)**.

The ejaculates from young dogs contain high percentages of abnormal spermatozoa **(Taha *et al.*, 1981)**. **(Andersen and Wooten, 1959)** found that male dogs usually become sexually mature 2 to 3 months after they have reached adult body weight. **(Takeishi *et al*. 1980a)** reported that Beagles reached puberty at 6 months, but optimal sperm production was first seen at 15-16 months of age.

The ejaculate contains between 100 and 5000 x 10^6 spermatozoa, depending on the size of the dog. The percentage of abnormal spermatozoa should not exceed 20-40% and motility should be at least 70% **(Feldman and Nelson, 1987; Oettlé, 1993)**. It has been suggested that a higher number of spermatozoa to some extent may compensate for a higher percentage of abnormal spermatozoa **(Linde-Forsberg and Forsberg, 1989)**. Also found that in dogs the total number of normal, motile spermatozoa was more important than the percentage, **(Mickelsen *et al*. 1993)**.

The acrosome reaction is necessary for a spermatozoon to acquire its fertilizing capacity. It is believed to be triggered by an intracellular rise of Ca^{2+}. During the acrosome reaction the apical and pre-equatorial domains of the sperm plasma membrane fuse with the outer acrosomal membrane **(e.g. Wassarman, 1990)** leading to a release of the acrosomal contents including hydrolytic enzymes, which are necessary for the spermatozoon to be able to penetrate the zona pellucida of the oocyte and accomplish fertilization.

The daily sperm production has been found to be 12 to 17 x 10^6 spermatozoa per gram testis parenchyma **(Davies, 1982; Olar *et al*., 1983)**.
The volume of the testicular parenchyma, the total number of spermatozoa and the ejaculate volume show a distinct correlation with body weight **(Günzel-Apel**

***et al*., 1994)** and daily sperm production, therefore, normally varies with the size of the dog. It is generally considered that mature, healthy dogs can accomplish matings every second day without a decrease in ejaculate volume or number of spermatozoa **(Boucher *et al*., 1958)**.

Till date, light microscopy is routinely used to evaluate the principal parameters of semen, concentration, motility and morphology. Concentration is usually determined using a Neubauer counting chamber. Motility is assessed subjectively on a pre-warmed glass slide **(Johnston, 1992; Iguer-ouada and Verstegen, 2001),** whereas morphology defects are assessed with various staining techniques **(Oettle, 1993; Johnston, 1992)**. Recently, several techniques have been described which may enable more accurate prediction of the fertilizing capacity of canine spermatozoa. This allows assessment of many functional characteristics of spermatozoa which are related to the binding, penetrating and fertilizing capacity of an oocyte **(Van Soom*et al*., 2001; Hewitt and England 2001)**.

The most commonly used method of semen collection in dogs is manual. **(Axnér and Linde-Forsberg, 2002)**. A teaser bitch is used for semen collection. Preferably in proestrus or oestrus. Vaginal secretion swab of proestrus/oestrus bitch, which is applied on the vulvar area and tail head of an anoestrus bitch **(Seager, 1986)**. Sexual rest for 4 to 5 days is ideal as compared to sexual rest of more than 10 days, that can cause increased morphological abnormalities, decreased motility due to spermatozoal aging and increased debris **(Purswell*et al*., 1992; Johnston *et al*., 2001)**. An inexperienced or nervous dog can be allowed to sniff and play with the bitch before collection **(Schubert and Seager, 1991)**. Lubricating agent is not recommended during semen collection **(England and Allen, 1992; Freshman, 2001)**. The collector's left hand holds the collection cone with attached collection tube at the tip of the prepuce. As the dog's penis reaches 40 to 50% erection, the prepuce is pushed behind the bulbus-glandis with the collection cone. A circular, firm pressure should be maintained with the left hand to simulate the copulatory lock or tie. **(Freshman, 2001; Johnston *et al*., 2001; Seager, 1986)**. Continue to apply pressure until crystal clear prostatic fluid begin to flow into the collection tube **(Freshman, 2001)**.

Dogs ejaculate in three fractions. The first fraction is termed as pre-sperm fraction which originates from the prostate gland. Normally it is clear or slightly cloudy and volume ranging from 0.5 to 20 ml or more **(Feldman and Nelson, 1996; Freshman, 2001)**. The second fraction is called as sperm-rich fraction which is normally opaque, milky-white in color and ranging from 0.5 to 2.0 ml. This fraction comes from storage in the tail of the epididymis, as well as from daily sperm output **(Johnston, 1989)**.
Contact of spermatozoa with first or third fraction fluid may decrease motility after two hours of collection **(England and Allen, 1992)**. The third or prostatic fraction is normally clear and may consist of more volume, depending on how

long pressure is maintained proximal to the bulbusglandis **(Johnston, 1989)**. This prostatic fraction is also useful for evaluating the prostate diseases.

A clear semen sample contains no spermatozoa. Cloudy or milky samples probably contain spermatozoa. Yellow color can indicates the urine contamination. Green colour denotes presence of pus. Red or brown colour indicates fresh or haemolysed blood in semen **(Johnston *et al*., 2001)**.

The volume of semen is not an indicator of semen quality in dogs. However, the volume should be measured as the part of the calculation of total number of spermatozoa in the sample **(1589 M. Arokia Robert, G. Jayaprakash, Mayur Pawshe, T. Tamilmani, and M. Sathiyabarathi)**.

Motility is a manifestation of structural and functional competence of spermatozoa; **(Kumi-Diaka, 1993; Rodriguez-Gil *et al*., 1994)** and of normal morphology **(Ellington *et al*., 1993)**. Motility should be evaluated immediately after collection. If the sample is too concentrated to evaluate motility a drop of semen can be diluted with a drop of buffered saline solution at the appropriate pH **(Johnston, 1989**). Normal motility is described as rapid, progressive, forward motion. **(Feldman and Nelson, 1996)**. The percentage of total motile spermatozoa in normal canine ejaculates is between 70 to 90% **(Johnston *et al*., 2001; Iguer-Ouada and Verstegen, 2001)**.

Abnormality is evaluated by counting about 100 to 200 spermatozoa in a stained semen slide under 100× (oil immersion). Count only free heads and not free tails **(Feldman and Nelson, 1996**). Abnormalities can be classified as primary (occurring during spermatogenesis) or secondary (occurring during maturation or sample preparation) **(Oettle and Soley, 1988)**. Normal semen samples should have <10% primary abnormalities and <20% secondary abnormalities. Total abnormalities should be <10-20% **(Feldman and Nelson, 1996; Freshman, 2001; Purswell*et al*., 1992)**.

The hemacytometer technique has been reported to be equally accurate or more accurate than CASA systems and its considered the gold standard. **(Root Kustritz*et al*., 2006)**.

Eosin–nigrosin is one of the best method to determine the percentage of live-dead sperm in given sample. Live spermatozoa with intact acrosomes appears to be white against the dark background of nigrosin exhibiting a regular and well defined apical ridge. Good canine semen sample should contain at least 80% morphologically normal and viable spermatozoa **(Johnston *et al*., 2001)**. When the proportion of normal spermatozoa was below 60 percent, fertility was found to be adversely affected **(Oettlé, 1993)**. Problems with this test include inability

to classify spermatozoa with partial staining and interference with staining if glycerol or fat globules are present in the seminal fluid **(Rijsselaere et al., 2005)**.

The HOS test involves submersion of spermatozoa into a hypo-osmotic medium. Those spermatozoa that have intact plasma membranes will swell as fluid moves into the sperm cell; this will cause swelling and coiling of the tail **(Martinez, 2004)**. Remember to evaluate the percentage of spermatozoa with coiled tails before performing the HOS test; the initial value must be subtracted from the percentage of spermatozoa with coiled tails after incubation to get the true percentage of spermatozoa with presumed intact plasma membranes as determined by this **test (England and Plummer, 1993; Kurni-Diaka and Badtram, 1994; Inamassu *et al*., 1999)**.

Cytology of the sperm-rich and prostatic fractions should be evaluated separately. Normal cytology of the sperm-rich fraction contains spermatozoa, white blood cells (WBC), red blood cells (RBC), epithelial cells and bacteria. Prostatic fluid also contains epithelial cells, WBC and bacteria **(Johnston*et al*., 2001)**.

Semen is not sterile. A wide variety of normal flora is present in semen. **(Feldman and Nelson, 1996)**. Culture of the distal urethra to compare flora may also be useful. Greater than 10,000 CFU of aerobic bacteria per mL of semen indicates infection.
ALP is produced from the epididymis. This makes it an excellent marker for patency of the ductal system. In normal semen samples, the ALP has a range of 5,000 to 40,000 U/L **(Johnston, 1989)**.

Sperm DNA integrity is a fundamental prerequisite in fertilization and embryo development. Among DNA integrity tests, the Comet assay is an accurate and sensitive test for the detection of sperm oxidative damage the Comet assay has ample potential for clinical and research purposes in dogs. **(Pereira AF, *et al.* 2017).**

The Comet assay is an accurate and specific test for detection of DNA damage, adaptable to sperm **(Sipinen et al., 2010)**. The basic principle of the assay is the migration of fragmented DNA of lysed cells under electrophoretic conditions in an agarose matrix. When viewed under a microscope, a cell has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating in the direction of the anode **(Hartmann et al., 2003)**. The standard alkaline Comet assay detects DNA strand breaks and alkali-labile sites. The Comet assay also gives information about the extent of several kinds of damage (basal breaks, oxidative damage and others), depending the enzyme used on the incubation **(Collins, Duthie, & Dobson, 1993; Dušinská & Collins, 1996)**. Considering the importance of DNA integrity for the success of natural or assisted fertilization and embryonic development **(Fraser & Strzezek, 2004)**.

(Helena Lemos , Jesus Dorado , Manuel Hidalgo , Isabel Gaiv˜ ao , Ana Martins-Bessa, 2020). Shows the role of testicular artery blood flow assessment via Doppler ultrasonography in diagnosing subfertility in dogs. Significant correlations were found between blood flow parameters and semen characteristics, particularly progressive motility and sperm concentration. Additionally, sperm morphology and integrity were associated with vascular parameters, underscoring the impact of testicular perfusion on reproductive health. These findings suggest that higher testicular blood supply is linked to better sperm quality, reinforcing Doppler ultrasonography as a valuable diagnostic tool."

Testicular doppler ultrasound is a non-invasive, highly repeatable method to detect and measure blood flow in testicular artery. the Resistive Index and Pulsatility Index seem to be potential markers of seminal quality in dogs. **(R.Zelli, A. Troisi, A. Elad Ngonput, L. Cardinali, A. Polisca . 2013)**

Antimicrobial resistance (AMR) poses a global threat to humans and animals due to the widespread use of antibiotics in veterinary and human healthcare. With the increase in pet ownership, there has been a surge in veterinary care expenditure, leading to elevated antibiotic usage. Consequently, AMR has spread among animals, posing a risk to human health through close contact and potential transmission of resistant pathogens (**Satish Kumar, Ayana Krishnan, Bilal Ur Rehman, Brijesh Yadav and Vijay Pal Singh; 2023)**. This has made treatment of infectious diseases less effective. Beside spontaneous emergence of mutant microorganisms **(Blate ME; 2023).**

Olive oil coproducts and their phenolic extracts have shown beneficial effects when added to the diets of food-producing animals, whereas data on their effects on pets are scarce **(Sara Minieri, Iolanda Altomonte, Virginia Bellini , Lucia Casini and Angelo Gazzano, 2024).**

The many mechanisms associated with the beneficial effects of polyphenols from olive coproducts include the modulation of gene expression, immune functions, antimicrobial actions, and antioxidant activities **(Scicutella, F.; Mannelli, F.; Daghio, M.; Viti, C.; Buccioni, A., 2021 ; Sant' Anna Monteiro, C.; Adedara, I.A.; Farombi, E.O.; Emanuelli, T., 2024)**

Antioxidant supplements in the diet play a role in maintaining oxidative balance in dogs and could help protect blood lipids from oxidative stress, thus counteracting the oxidative action of free radicals and the cellular damage they generate and therefore potentially protecting animal health. **(Sara Minieri et al., 2024)**

Oleuropein has been shown to have strong antimicrobial activity against both Gram-negative and Gram-positive bacteria. **(Bisignano G, Tomaino A, Lo Cascio R, Crisafi G, Uccella N, Saija A., 1999).** as well as mycoplasma **(Furneri PM, Marino A, Saija A, Ucella N, Bisignano G., 2002)**. Phenolic structures similar to oleuropein seem to produce its antibacterial effect by damaging the bacterial membrane and/or disrupting cell peptidoglycans. Different authors have used biophysical assays to study the interaction between oleuropein and membrane lipids **(Caturla N, Perez Fons L, Estepa A, Micol V., 2005)**

(Ayoob Rostamzadeh, Hossein Amini-khoei, Mohammad Javad Mardani Korani, Mohammad Rahimi-madiseh, 2020) showed that simultaneous administration of the OLE with oleuropein significantly increased the count of viable sperm in mice, moreover increase count of progressive motility. Concluded that administration of OLE and oleuropein exerted protective effects against CP- induced negative alterations in the male reproductive system in mice.

The 30 day administration of OLE significantly improved the sperm qualitative parameters, sperm count, motility and viability. Also improve antioxidant status of the testis in rat. **(Maryam Sarbishegi, Enam Alhagh Charkhat Gorgich, Ozra Khajavi; 2017)**

Oleuropein at 0.1% of the diet for 28 days in male rats was able to increase testicular concentrations of testosterone in a linear relation with overall dietary protein intake (with rats consuming 40% dietary protein experiencing a larger increase than 25% or 10%, the latter experiencing no increase) and the highest protein group experienced a decrease in urinary nitrogen excretion by 19.7% with oleuropein. **(Oi-Kano Y, Kawada T, Watanabe T, Koyama F, Watanabe K, Senbongi R, Iwai K. 2012).**

Objectives

The present study aims to:

1. Surveying some infertility cases of stud-dog that originated from semen-related issues, along with treatment methods using different tools for fertility assessment.
2. Trying to establish the most reliable and field applicable fertility assessment forum to used clinically .
3. Studying effect of Oleuropein (olive leaves extract “OLE”) on semen fertility status of stud-dog using different analytical and examination technique.
4. Represent a comparative study on the effect of incorporating a Oleuropein into the main infertility treatment versus using it as a standalone treatment .

Material and Methods

1. Animals

Animals will be examined and sampled during this study will include Imported and native Stud breeds. Stud dogs belonging to local breeder farm and Private stud dogs as clinical cases come to VHC clinic in Helwan will be subjected to this study. Also, dog studs of variable age (2 – 10 years) will be reviewed.

2. History

The Studs age, breed, work history, breeding records (of the last two previous breeding seasons), the fate of former breeding nor insemination and potential reproductive problems will be noted. General health, parasite control, vaccination history and the condition of the Studs will be also noted.

3. Type of sample :

3.1. Blood : samples will be collected during preliminary exam from saphenous vein in both plain and EDTA 3 ml vaccutainer tubes.

3.2. Semen : Samples will be collected from Studs using a masturbation methods after sufficient and proper disinfection to prepuce and area surrounded. All procedure are performed using sterile and non-spermicidal material.

- Ensuring the following items in sampling :
  - ✓ The period of rest don't less than 48 hour or exceed 2 weeks.
  - ✓ Double sampling technique for representative sample.
  - ✓ Only 2nd sperm rich fraction will be collected .
  - ✓ Under presence of estrous bitch or vaginal swab from estrous bitch .
- Semen sample will be fractioned immediately in sterile vials into 3 equal proportion (semen analysis – culture & sensitivity – COMET)

3.3. Fine-needle biopsy : from testis in case of azoospermic semen. The tissue pieces were collected and fixed in 10% Neutral buffered formalin (NBF). The fixed tissue pieces were processed for paraffin sectioning. Paraffin sections of 3-5 µm thickness were made using Leica Rotary Microtome (RM 2145) and stained with routine haematoxylin and eosin for studying the histoarchitecture **(J Bhagyalakshmi, K Balasundaram, P Selvaraj, S Kathirvel, P Balachandran and GSS Chandana . 2020)**.

5. **General Health Assessment :**

   5.1. **Clinical Examination** : Studs external genitalia (testicle, prepuce and penis) will be evaluated for position, painfulness and size.

   5.2. **Ultrasonographic examination:** assessment of the Stud's testis and prostate will be mostly performed with a transabdominal approach. using either real time B-mode and P/W Doppler scanners equipped with 8 and 8.5 MHz real time B-mode transducer **(Paddy Mannion, 2006).**

   5.3. **Blood sample :** Blood samples will be examined **(Lilliehöök and Tvedten, 2009)** as following :

      5.3.1. **EDTA tube :** examined immediately for infertile dogs for complete blood count CBC

      5.3.2. **Plan tube :** will be centrifugated at 15000 RPM for serum. testosterone, Biochemical marker Liver (ALT and AST), Kidney (BUN and creatinine) and Brucella titer immediately after collection. Also, the biochemical marker will be repeated every 1 week for monitoring issue.

6. **Semen assessment :** by the following different analytical methods

   6.1. **Semen analysis :** will be subjected for complete semen analysis using CASA "Computer Assisted Sperm Analysis" , CASA systems use software to calculate output measures describing and defining motion traits in successive frames, viz. total motility (TMOT, %): [percentage of total motile spermatozoa]; progressive motility (PMOT, %): [percentage of spermatozoa with a progressive motility]; velocity average path (VAP, μm/s): [average velocity of the smoothed cell path]; velocity straight line (VSL, μm/s): [average velocity measured in a straight line from the beginning to the end of the track]; velocity curvilinear (VCL, μm/s): [average velocity measured over the actual point-to-point track followed by the cell]; amplitude of lateral head displacement (ALH, μm): [mean width of the head oscillation as the sperm cells swim]; beat cross frequency (BCF, Hz): [frequency of sperm head crossing the average path in either direction]; straightness (STR, %): [estimates the proximity of the cell's pathway to a straight line and is a measure of forward progression, also expressed as the ratio of VSL/VAP]; linearity (LIN, %): [estimates the proximity of the cell's track to a straight line, also expressed as the ratio of VSL/VCL]; sperm size (SS, μ): [elongation ratio of minor to major axis of each sperm]; and sperm nucleus (SN, μ): [size of each sperm nucleus] **(A.K.SINGH, A.KUMAR and A.BISLA, 2021).**

6.2. Sperm viability : The viability of sperm was determined using the Eosin-Nigrosin staining technique **(Campbell, R.C., Dott, H.M and Glover, T.D. 1956).** The stain was made up of Eosin-y (1.67 g) and Nigrosin (10 g) in 100 ml of 2.90% sodium citrate buffer. Two hundred spermatozoa per slide of live sperm (non-stained head) and dead sperm (red head) were evaluated by a light microscope with a 40x objective.

6.3. Plasma membrane integrity : The plasma membrane integrity of canine spermatozoa was assessed by the hypo-osmotic swelling (HOS) test Pinto, **(C. R. F., and Kozink, D. M. 2008)**. The solution of HOS consisted of sodium citrate (0.73 g) and fructose (1.35 g), dissolved in 100 ml of distilled water. The assay was carried out by mixing 50 µl of frozen-thawed canine sperm sample with 500 µl of HOS solution and incubating it for 40 minutes at 37 ºC. After incubation, a drop of the sample was examined under a light microscope with a 40x objective. Two hundred spermatozoa were counted for their swelling. Spermatozoa with coiled or swollen tails had functional intact membranes.

6.4. Sperm acrosome integrity : The acrosome integrity of canine spermatozoa was assessed by Trypan Blue-Giemsa (TBG) stain: Staining was performed as described by **(Kovacs, A., and Foote, R. H. 1992).** One drop of trypan blue and one drop of the sample were mixed on a glass slide and distributed over the entire surface with another slide. Slides were air-dried vertically, then placed in a jar of fixative for two minutes before being rinsed with tap and distilled water. Slides were left in jars containing Giemsa stain at room temperature overnight (16 to 20 hours). Slides were washed again, air-dried vertically. Sperm cells were examined under a microscope at 100x oil immersion magnification. Spermatozoa were evaluated as acrosome intact (light purple) and damaged/lost acrosome (unstained or blue acrosome).

6.5. DNA fragmentation testing : cell gel electrophoresis (alkaline comet test) was used to examine the DNA integrity of frozen-thawed canine spermatozoa, according **to (Codrington, A. M., Hales, B. F., and Robaire, B. 2004)**. Sperm samples were washed with phosphate buffer saline (PBS) mixed with 50 µl of 1% low melting point agarose and placed onto frosted microscope slides pre-coated with 50 µl of 1% normal melting point agarose. The slides were lysed for 1 hour in lysis buffer. Electrophoresis was performed on the lysed cells. Following neutralization in Tris solution, the slides were stained with ethidium bromide (pH 7). A fluorescent microscope (Olympus, Japan) was used to study 200 sperm cells at a magnification of 400x. The amount of DNA present was expected to be proportional to the intensity of the stain in the comet tail area. Based on measurements of the DNA % in the tail, tail length, and olive tail moment, image analysis software was

utilized to evaluate DNA damage (Comet-Score program). Spermatozoa with fragmented DNA had a higher rate of DNA migration from the nucleus to the anode, but spermatozoa with intact DNA did not produce a "comet" **(Kovacs, A., and Foote, R. H. 1992)**.

6.6. Alkaline Phosphatase "ALP" : used as marker for patency of the ductal system. Sample examined using commercial kits and spectrophotometer. In normal semen samples, the ALP has a range of 5,000 to 40,000 U/L

7. Medical treatment :

7.1. Medical treatment :

7.1.1. Antimicrobials : Concomitant broad-spectrum antimicrobial therapy should be administered during any treatment protocol. Ideally, identification and sensitivity were done from semen sample before initiating any antimicrobial treatment **(Verstegen *et al*., 2008)**.

7.1.2. Enrofloxacin : Stud-dogs that confirmed Brucella spp. Positive are given 5 mg/kg of enrofloxacin *"MUV-Enrofloxacin 20% ®"* orally every 12 h for 30 days **(Wanke, Delpino and Baldi, 2006)**.

7.2. Fertility Enhancer : Oleuropein

7.2.1. Group (I) : healthy animal that receive a dose of 50 mg per Kg of body weight for a period of 60 days

7.2.2. Group (II) : healthy animal that receive a dose of 100 mg per Kg of body weight for a period of 60 days

7.2.3. Group (III) : infertile animal due to genital infection that receive a dose of 50 mg per Kg incorporation with antimicrobials declared in culture and sensitivity results

7.2.4. Group (IV) : Infertile animal due to genital infection that receive a dose of 100 mg per Kg standalone.

8. Statical analysis

Data of hormone assaying, Doppler ultrasound, Biochemical marker, semen analysis and COMET assay will be subjected to statistical analysis using IBM SPSS 20 according to **(Snedecor, G. W., and Cochran, W. G. 1989)**.

Re-testing of semen analysis, biochemical and DNA fragmentation testing after and during treatment of infertility status

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