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Physiological and Biochemical Evaluation of the Immature Male Reproductive System in Rats Treated with Biosynthesized Starch-Selenium Nanoparticles

A Thesis

**Submitted to the Council of the College of Veterinary Medicine,
University of Shatrah, in Partial Fulfillment of the Requirements
for the Master's Degree in Veterinary Sciences (Physiology)**

By

Abdulsattar Mohammed Afrawee

B.V.M.S. (1990)

Veterinary Medicine College /Baghdad University

Supervised by :

Assist. Prof. Dr Aamir M. A. Al Ghareebawi

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1448 A.H.

(بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ)

وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ
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Declaration

I declare that this thesis, entitled "**Physiological and Biochemical Evaluation of the Immature Male Reproductive System in Rats Treated with Biosynthesized Starch-Selenium Nanoparticles,**" is my original work except for quotations and citations, which have been fully acknowledged. I also declare that this thesis has not previously been submitted for any other degree at the University of Shatrah or any other institution.

Signature:

Name: Abdulsattar Mohammed Afrawee

Date: / /

Certification

We certify that the preparation of this thesis, "**Physiological and Biochemical Evaluation of the Immature Male Reproductive System in Rats Treated with Biosynthesized Starch-Selenium Nanoparticles,**"

was carried out under my supervision at the Department of Physiology, Pharmacology and Biochemistry, College of Veterinary Medicine, University of Shatrah, in partial fulfillment of the requirements for a Master's degree in Veterinary Medicine (Physiology).

Supervisor

Name: **Dr Aamir M. A. Al Ghareebawi**

Title: - **Assist. Prof.**

Date: -

In view of the available recommendations, I forward this thesis for debate by the examining committee.

Signature

Name: **Professor Dr. Rabab Shakir Salman**

Title: Department Head of Physiology, Pharmacology, and Biochemistry

Date: -

Examining Committee Decision

We are members of the examining committee for this thesis (**Physiological and Biochemical Evaluation of the Immature Male Reproductive System in Rats Treated with Biosynthesized Starch-Selenium Nanoparticles**), and after examining the student (Abdulsattar M. Afrawee) and his content, we found it adequate for the award of the Master's degree in Veterinary Medicine / Physiology

Professor

Dr. Baida Rihan Ali

Chairman

Professor

Dr. Jawad Kadhim Taher

Member

Professor

Mohammed Abdulabbas Hasan

Member

Assistant Professor

Aamir M. A. Al Ghareewi

Member and Supervisor

Approval of the college committee on postgraduate studies

Professor

Dr. Rabab Shaker Salman

Head of Physiology Department

Assistant Professor

Dr. Nabeel Mahdi Abd

Dean of the Veterinary Medicine College

Date of examination 3/9/2026

Dedication

To the light of the nation and guide of humanity, the Prophet of Mercy, Muhammad (peace and blessings be upon him and his family).

To those purified from all defilement, the family of the Chosen One (peace be upon them all). To the awaited Imam, the promised one.

To the soul that planted in me the seed of love for knowledge and passion for learning in my early years, and departed too soon, my dearest father (may God have mercy on him).

To the great heart and the striving, sacrificing, and patient soul, my mother (may God have mercy on her).

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Summary

The acceleration of the male reproductive system is an important issue in animal husbandry. The research aimed to evaluate the effects of biosynthesized selenium nanoparticles (SeNPs) on the maturation of the immature male reproductive system (testis) in male rats. The experiment was divided into two parts: I concerned with the biosynthesis of SeNPs using starch as a stabilizing agent with ascorbic acid as a reducing agent. After synthesis, the produced nanomaterial was characterized by fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), zeta potential, and field-emission scanning electron microscopy (FESEM), demonstrating that it exhibits nanoparticle properties and size. The part II of the experiment involves utilizing 40 immature male rats divided into 5 groups as follows: the G1 group, which received Distilled Water (D.W) only, the G2 group received 50µg/kg B.W of biosynthesized SeNPs orally, the G3 group received 100 µg/kg B.W of biosynthesized SeNPs orally, the G4 group received 200 µg/kg B.W of biosynthesized SeNPs orally, and the G5 group received 400 µg/kg B.W of biosynthesized SeNPs.

The second part of the experiment continued for 30 days. After that, the experimental animals were sacrificed under inhalation anesthesia to obtain blood for hormonal (testosterone, FSH, and LH) concentrations, functional sperm protein (protamine and acrosin) concentrations, and antioxidant (Glutathione reductase, Glutathione peroxidase, and Selenoprotein P) concentrations. Also, testes from experimental male rats were obtained to investigate testicular weight, sperm count, live/dead ratio, histologic changes, and histometry of the study testis, including seminiferous tubule maturation score, seminiferous tubule diameter, and germinal layer thickness. The results of the current experiment revealed a significant ($p \leq 0.01$) - ($p \leq 0.05$) increase in LH, FSH, selenoprotein p, acrosin, sperm count, live/dead ratio, testicular weight, seminiferous tubule maturation score, seminiferous tubule diameter, and germinal layer thickness. In addition, there is a significant ($p \leq 0.01$) - ($p \leq 0.05$) decrease in the concentrations of

testosterone, glutathione reductase, glutathione peroxidase, and protamine across different experimental groups. The histological changes revealed many alterations, indicating increased maturation of the seminiferous tubules.

The experiment concludes that the use of biosynthesized SeNPs accelerates testis maturation in male rats, with caution regarding some studied doses.

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List of Abbreviations

Abbreviations	Meaning
%	percent
40x	Magnification power
BTB	Blood -testis-barrier
C	Celsius
DIOs	Iodothyronine deiodinases
D.W	Distal water
ELISA	Enzyme-linked immunosorbent assay
FESEM	Field emission scanning electron microscopy
FTIR	Fourier transform infrared spectroscopy
FSH	Follicular-stimulating hormone
FSHr	Follicle-stimulating hormone receptor
G	Group
GnRH	Gonadotropin-releasing hormone
GPXs	Glutathione peroxidase
GRs	Glutathione reductases
GSE	Grape seed extract
H&E	Hematoxylin &Eosin stain
HPG	Hypothalamic-Pituitary-Gonadal
LH	Luteinizing hormone
mm	Millimeter
µg	Microgram
MPOA	Medial preoptic area
mIU	Milli-international unit
µl	Microliter
MUMP	Male urogenital mating protuberance
Na ₂ SeO ₃	Sodium selenite
ng	Nanogram
p≤	Probability value
pg	picogram
PH	Potential of hydrogen
PLAL	Pulsed-Laser liquid ablation in liquid
ROS	Reactive oxygen species
rpm	Revolution per minute
Se	Selenium
SE	Standard Error
Se ^o	Elemental Selenium
SER	Smooth endoplasmic reticulum
SeNPs	Selenium nanoparticles
SELENOP	Selenoprotein p

SSCs	Spermatogonial stem cells
TFF	Total fertilization failure
TXNRDs	Thioredoxin reductases
UV	Ultra violet
XRD	X-ray Diffraction
ZP	Zona pellucida

Chapter one

Introduction

1. Introduction

1.1 The male reproductive system

The male reproductive system is composed of the scrotum and penis on the outside and the testes, epididymis, prostate gland, and vas deferens on the inside. Many ducts and glands play a major role in the production, storage, and ejaculation of semen as well as the synthesis of hormones required for the development of male organs (Tiwana and Leslie, 2017).

The cranium contains another structure that governs the growth and operation of the male reproductive system. The brain regulates sexual and parental behavior and determines when puberty will occur. The hypothalamus gland is essential for regulating sexual behavior and reproduction. The primary source of sex hormone release is gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary to produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The hypothalamus generates GnRH and stimulates the pituitary gland. After that, the two hormones (LH, FSH) enter the bloodstream and travel to the testes (Müller and Graem, 1993).

According to Carreau *et al.* (2010), the testis' primary tasks are the production of semen and the synthesis of the steroid hormone testosterone. While the interstitial tissue is a thin web of connective tissue that contains Leydig cells, the seminiferous tubules account for approximately 90% of the weight of the adult testis (Cordeiro *et al.*, 202).

Ohmura *et al.* (2003) explain that spermatogenesis is a complex and regulated process that typically continues throughout a person's life. Spermatogonial stem cells, which reside in the seminiferous tubules at the basement membrane and proliferate to generate differentiated spermatogenic cells

such as primary and secondary spermatocytes, spermatids, and spermatozoa, are the initial stage of this process (Nakamoto *et al.*, 2004).

Apart from selenium, other chemicals play a significant role in male fertility, with copper being the primary one. Copper affects the bull's testicular tissue, preventing him from having sex and perhaps making him sterile. It also affects libido. Sperm are protected from oxidative stress by copper, which is necessary for energy synthesis (Kreplin and Yaremicio, 1992). Sperm motility, hormone synthesis, and male fertility all depend on zinc (Zn). Male animals lacking zinc exhibit decreased levels of FSH and LH (Kumar *et al.*, 2011). This may lead to reduced libido, smaller testicles, and severe disruption of spermatogenesis (Omu *et al.*, 2015; Kumar, 2023).

Follicular maturation and luteinizing hormone release are both significantly impacted by chromium (Cr). Low sperm counts and reduced fertility result from insufficient (Cr) concentration (Tuormaa, 2000). A molybdenum deficiency (Mo) may result in decreased libido, impaired sperm production, and male infertility; in females, it may cause delayed puberty, low conception rate, and absence of estrus (Kumar *et al.*, 2011). Molybdenum contributes to sperm quality by increasing sperm count and motility and lowering oxidative stress.

Selenium (Se), an essential trace element, is integrated as selenocysteine by key selenoproteins such as glutathione peroxidases (GPXs), thioredoxin reductases (TXNRDs), iodothyronine deiodinases (DIOs), and selenoprotein P (SELENOP) (Alexander and Olsen, 2023). Male fertility depends on both redox homeostasis and endocrine balance, which these proteins preserve. While SELENOP protects Leydig cells during hormone production by transporting Se to the testes, glutathione peroxidase (GPX4) is essential for sperm maturation, providing structural integrity to the mitochondrial sheath and facilitating chromatin condensation (Barchielli *et al.*, 2022).

Spermatogenesis and semen quality are affected by selenium deficiency, while adequate levels improve semen quality. Despite its advantages, there is an urgent need to develop new techniques to ensure safety and effectiveness, as the optimal dosage for the animal is close to the toxic threshold (Bai *et al.*, 2024). The bioavailability and toxicity concerns of the chemical forms of selenium (selenomethionine, selenite) vary. By improving bioavailability and stability and reducing toxicity, selenium nanoparticles (SeNPs) biosynthesized with natural stabilizers such as starch address this issue (Sampath *et al.*, 2024; Geng *et al.*, 2025).

Nanoparticles (NPs) are defined as particles that are smaller than 100 nm and have special characteristics that distinguish them from bulk materials. Nanoparticles have a superior surface area-to-volume ratio due to their small size. In this way, the bloodstream can transport many types of medications (Huang *et al.*, 2018). For several years, the medical community has used nanotechnology to treat some bacterial infections and wounds (Alexander and Jotterand, 2014; Matharu *et al.*, 2018). Also, it is used to improve the immune system's response to immunizations and to promote the healing of bone and tissue damage (Asgary *et al.*, 2016). Reduce cellular damage, which may prevent diabetes (Saratale *et al.*, 2017). Because of their antioxidant activity, starch-SeNPs are particularly beneficial for reproductive system applications.

In immature male rats, biosynthesized selenium nanoparticles (SeNPs) derived from starch (Starch-SeNPs) can accelerate the development of the reproductive system and exhibit lower toxicity than bulk selenium. The current study revealed the role of nano-selenium in the development and maturation of the reproductive system in immature male rats. Also, it determined safe dosages by investigating the effects of SeNPs on the male reproductive system and growing testes in immature male rats. Understanding how biosynthesized starch-selenium nanoparticles (Starch-SeNPs) affect the development of male

reproductive systems in rats would be the primary goal if new fertility therapies and restrictions for nano-selenium in food and medicine were to be put into place.

The creation and study of Starch-SeNPs are the main objectives of this work, which is based on physicochemical methods, including (1) Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), zeta potential, and field-emission scanning electron microscopy (FESEM). Other goals include: (2) how the drug affects the rats' testicular histology and structure; (3) testicular weight and sperm quantity (viability and concentration); (4) reproductive hormones (testosterone, FSH, and LH) and sperm proteins (protamine and acrosin); (5) antioxidants (glutathione peroxidase, glutathione reductase, selenoprotein p).

1.2 Aims of the study:

- 1-To evaluate the effects of selenium nanoparticles (SeNPs) on the maturation and development of the male reproductive system in rats.
- 2- To study the effect of selenium nanoparticles on sex hormone (Testosterone, LH, FSH) levels in immature male rats.
- 3- To study the effect of selenium nanoparticles on antioxidant enzymes (GR, GPX, SELENOP).
- 4- To study the effect of selenium nanoparticles on sperm proteins (Acrosin, protamin).
- 5- To study histological changes in the testes after treatment with selenium nanoparticles.

Chapter Two

Literature Review

2. Literature Review

2.1 Male reproductive system

The male reproductive system consists of the testes, reproductive ducts (including the epididymis and vas deferens), accessory sex glands (Seminal Vesical, Prostate Gland, Bulbourethral or Cowper's glands), and the external genitalia (penis and scrotum)(Hall and Hall, 2020); The androgen hormone play a central role in the development and differentiation of both the internal reproductive tract and the external genitalia (Amato *et al.*, 2022). These structures function in an integrated manner to support spermatogenesis, sperm maturation, and semen transport, while accessory glands contribute important biochemical components to seminal plasma (Bhattacharya and Dey, 2023; Rochdi *et al.*, 2025 B). In addition, the physiological regulation and development of the male reproductive system are controlled by the hypothalamic–pituitary–gonadal (HPG) axis, in which hypothalamic signaling regulates pituitary gonadotropin hormones (LH and FSH), thereby modulating Leydig-cell and Sertoli-cell function, hormone secretion, and male fertility (Amato *et al.*, 2022; Bhattacharya and Dey, 2023).

The brain, particularly the hypothalamus, plays a central role in determining the timing of puberty and in regulating male reproductive neuroendocrine function (Koysombat *et al.*, 2023). In addition to its well-established role in regulating reproductive (including sexual) behavior via hypothalamic and broader neuroendocrine circuits, recent evidence also highlights the importance of hypothalamic regions—especially the medial preoptic area (MPOA) in the hormonal regulation of parental behavior (Pal *et al.*, 2024; Mills *et al.*, 2025). The initiation and regulation of male reproductive function occur through the hypothalamic-pituitary-gonadal (HPG) axis, in which gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile manner into the hypophyseal portal circulation, stimulating gonadotroph cells in the anterior

pituitary to form and release FSH and LH (Koysombat *et al.*, 2023; Esteves and Humaidan, 2025). These gonadotropins then act on the testes: LH primarily stimulates Leydig cells to produce testosterone, while FSH acts on Sertoli cells; together, they support and maintain spermatogenesis (Esteves and Humaidan, 2025).

2.1.1 Parts of the male reproductive system

2.1.1.1 Testis

The testes are paired, oval male gonads located within the scrotum, and their position supports thermoregulation, keeping testicular temperature approximately 2–4°C below body temperature, which is a requirement for normal spermatogenesis (De Toni *et al.*, 2023). In male rats, the measures of each testis are about 1.3-1.6 grams in weight, 18- 22 millimeters in length, and 10-14 millimeters in width (Parker and Picut, 2016; Li W. *et al.*, 2024; Lin *et al.*, 2025). The growth of germ cells within the seminiferous tubules is supported structurally and metabolically by Sertoli cells, which also aid in forming the blood-testis barrier, which divides the seminiferous epithelium into basal and adluminal compartments (Li *et al.*, 2024). Accordingly, the testis serves as an endocrine organ for the production of androgens, particularly testosterone, as well as a sperm generation organ (Li *et al.*, 2024).

2.1.1.1.1 Sertoli cells

Sertoli cells are specialized somatic cells of the seminiferous epithelium that remain in contact with developing germ cells and are essential for normal spermatogenesis. Adjacent Sertoli cells form specialized junction complexes, including tight junctions, that form the blood–testis barrier (BTB), a selective barrier that preserves an immune-privileged microenvironment while allowing regulated germ-cell movement (Thumfart and Mansuy, 2023; Alesawi and Ergun, 2023). Sertoli cells provide metabolic and structural support to the germ cells,

help maintain the seminiferous tubular microenvironment, and coordinate multiple stages of germ-cell development from early spermatogonial support through maturation and release into the lumen (O'Donnell *et al.*, 2022; Li *et al.*, 2025; Xiao *et al.*, 2025).

In addition to directly supporting the germ cells, Sertoli cells play a central role in regulating testicular development and function, the organization and functional coordination of other testicular cell types (including Leydig cells, peritubular myoid cells, and testicular macrophages), and contributing to immune homeostasis within the testis (O'Donnell *et al.*, 2022; Feng *et al.*, 2025). The endocrine regulation of Sertoli-cell function is also important, in which FSH acts primarily through FSH receptors (FSHR) on Sertoli cells. Sertoli cells secrete inhibin B, which participates in negative feedback regulation of pituitary FSH secretion within the hypothalamic–pituitary–gonadal axis (Cannarella *et al.*, 2024; Li *et al.*, 2025).

2.1.1.1.2 Leydig cells

Leydig cells are the main steroid-producing cells of the testicular interstitium (intertubular compartment), where they are present as single cells or in clusters in close association with blood vessels and adjacent to seminiferous tubules. The interstitial compartment also contains other cell populations, including macrophages and additional immune mesenchymal endothelial cells. Male testosterone is produced by Leydig cells, which also express luteinizing hormone-choriogonadotropin receptors (LHCGR). LH primarily increases the production of androgens through cAMP-dependent signaling (Walczak-Jedrzejowska *et al.*, 2022; de Mattos *et al.*, 2023; Wu *et al.*, 2025). A well-developed smooth endoplasmic reticulum (sER), tubulovesicular mitochondria, and lipid droplets consistent with active steroidogenesis are highlighted in recent descriptions of the morphology of adult Leydig cells, which display an abundance of cytoplasm (Walczak-Jedrzejowska *et al.*, 2022; Bhattacharya and Dey, 2023).

Importantly, FSH does not act directly on Leydig cells as LH does, because FSH signaling is centered on Sertoli cells (FSHR is predominantly expressed in Sertoli cells). Therefore, any effect of FSH on Leydig cells is considered indirect, mediated through Sertoli cell paracrine factors and Sertoli-Leydig interactions (Wang *et al.*, 2022; Bhattacharya and Dey, 2023; Feng *et al.*, 2025). In addition, androgen signaling supports Leydig cell maturation and function. Thus, estrogen effects on Leydig cells can be described as regulatory rather than as simple suppression (Walczak-Jedrzejska *et al.*, 2022; Adamczewska *et al.*, 2022).

2.1.1.2 Epididymis

The epididymis is a single, highly convoluted duct connected to the efferent ductules. The epididymis is composed of three parts, which are the caput, corpus, and cauda regions. The extended length of the epididymis in rats can reach approximately 6 meters, while the visible length is about 1.5-2 cm (James *et al.*, 2020). It is derived embryologically from the mesonephric (Wolffian) duct. Furthermore, its development and maintenance are androgen-dependent processes (Wu and De Cicco, 2020). The major functions of the epididymis are sperm maturation, concentration, storage, and transport of the semen. Spermatozoa leaving the testis are still functionally immature and acquire more progressive motility and fertilizing capacity during transport through the epididymis (James *et al.*, 2020). However, the final maturation step required for fertilization capacity occurs later in the female reproductive tract, not in the epididymis (James *et al.*, 2020).

The transport of sperm occurs mainly by the rhythmic contractions of the surrounding smooth muscle. Then, the sperm reach the cauda epididymidis, which serves as the principal storage site for mature spermatozoa (James *et al.*, 2020). In current histological descriptions, motile cilia characterize the efferent ductules, while the epididymal duct epithelium is characterized mainly by stereocilia (Gocht *et al.*, 2025).

2.1.1.3 Vas deferens

The vas deferens (ductus deferens) is a muscular duct that continues from the cauda epididymis, and its function is to transport sperm toward the ejaculatory ducts. It also secretes alkaline mucus to protect sperm from the acidity of the urethra. It ascends within the spermatic cord, passes through the inguinal canal, and joins the duct of the seminal vesicle to form the ejaculatory duct (Wu and De Cicco, 2020). Like the epididymis and seminal vesicles, it is derived from the mesonephric duct during embryogenesis (Wu and De Cicco, 2020; Yu and Wang, 2023).

Histologically, the vas deferens wall consists of an inner mucosa, a thick smooth muscle coat (with a prominent circular layer between the inner and outer longitudinal layers), and an outer adventitia (Koslov and Andersson, 2013). Its nervous supply is autonomic and sensory, with adrenergic fibers predominating in the muscle coat and additional cholinergic fibers present, contributing to the coordinated contractions required for sperm propulsion during transportation (Koslov and Andersson, 2013).

2.1.1.4 Seminal vesicle

The seminal vesicles are a pair of accessory male sex glands located after the urinary bladder, superior to the prostate gland, and anterior to the rectum. Each gland is a highly coiled tubular structure. Moreover, its excretory duct joins the distal vas deferens (ampulla) to form the ejaculatory duct, which crosses the prostate and opens into the urethra (Gaspar *et al.*, 2024). The seminal vesicles contribute the major proportion of seminal plasma (forming ~40–80% of semen volume) and secrete various components such as fructose, prostaglandins, coagulating factors, and bicarbonate, which support sperm nutrition, semen coagulation, and buffering of the vaginal acidic environment (Gaspar *et al.*, 2024; Rochdi *et al.*, 2025 A).

The major seminal vesicle-derived energy substrate for sperm function is fructose. In contrast, citrate and zinc are primarily associated with prostate gland secretion, an important distinction in the biochemical evaluation of male reproductive system function (Rochdi *et al.*, 2025 A). The secretory activity of the seminal vesicle is androgen-dependent, and recent evidence indicates that testosterone-driven metabolic remodeling in the seminal vesicle epithelium can modify seminal plasma composition, thereby improving sperm motility parameters (Yamanaka *et al.*, 2025). More broadly, seminal plasma plays essential roles in sperm protection, maturation, and fertilization-related processes and is recognized as a biomarker for diagnosing male infertility (Vashisht and Gahlay, 2024). Furthermore, prostaglandins, cytokines, and chemokines are seminal plasma components that support endometrial receptivity, implantation, and successful reproduction while also modulating immunological responses in the female reproductive tract. However, experimental data also suggest that seminal vesicle secretions play a role in improving *in vivo* fertilization and in shaping the female reproductive environment during the early stages of development (Ahmadi *et al.*, 2022).

2.1.1.5 Prostate gland

The major accessory gland of the male reproductive system is the prostate gland, which is located inferior to the urinary bladder and surrounds the prostatic urethra. In contemporary anatomy, the human prostate is described using zonal anatomy, divided into three zones (peripheral, central, and transition zones), with an anterior fibromuscular stroma, rather than relying solely on the traditional lobe-based description (Ittmann, 2018). Histologically, the prostate gland consists of branching acini and ducts embedded in a fibromuscular stroma, and its epithelium is mainly composed of luminal and basal cells, with scattered neuroendocrine cells (Ittmann, 2018).

Prostatic secretions contribute an important portion of the seminal plasma (commonly around 20–30%) and contain biologically relevant components such as citrate, zinc, spermine, acid phosphatase, and proteolytic enzymes, which support semen quality and function. It also plays a role in male reproductive physiology (Anamthakul and Winuthayanon, 2020). The prostate gland secretes about 75% of the seminal fluid and is somewhat acidic (pH 6.6) (Kumar and Majumder, 1995). It has a high quantity of citric acid and hydrolytic enzymes, especially fibrinolytic enzymes. In rodents, the prostate gland is composed of distinct lobes (e.g., anterior, ventral, dorsal, and lateral), which differ from the zonal organization of the human prostate (Ittmann, 2018).

2.1.1.6 Cowper's glands

Also known as the bulbourethral glands, these small, round structures are located behind the urethra. (Hall, 2021). Cowper's gland secretion is an alkaline, mucus-like fluid that lubricates the urethra, helps balance the acidity of urine, and neutralizes vaginal acidity. The Cowper's gland fluid does not contain sperm (Chughtai *et al.*, 2005).

2.1.1.7 Penis

The penis is the male external copulatory organ and also serves as the terminal duct for urine and semen transport. Anatomically, it is divided into three parts: the root, body (shaft), and glans (Filipoiu *et al.*, 2024). The root anchors the penis to the perineum and includes the bulb and paired crura, while the shaft contains the principal tissues for erectile function (Filipoiu *et al.*, 2024).

Structurally, the penis is composed of three erectile bodies: two are located dorsally, called the corpora cavernosa. Moreover, one ventral structure, the corpus spongiosum, surrounds the penile urethra and enlarges distally to form the glans (Filipoiu *et al.*, 2024; Kodali *et al.*, 2025). The corpora cavernosa is enclosed by the tunica albuginea, which is a dense fibrous layer that contributes to penile

rigidity during erection (Kodali *et al.*, 2025). The distal portion of the corpus spongiosum, the glans penis, is characterized by a large number of sensory nerve endings beneath the epithelium, which play an important role in reproductive function and sexual sensation (Hall, 2021). The penis in male rats features unique structures absent in humans, including the baculum (os penis), a cartilaginous bone located in the shaft providing constant stiffness for rapid intromission, and the MUMP (male urogenital mating protuberance), which is a pointed cartilaginous tip protruding beyond the urethral opening (Pinheiro *et al.*, 2003).

2.2 Spermatogenesis

Spermatogenesis is the highly coordinated process by which male germ cells develop into mature spermatozoa. The process occurs within the seminiferous tubules of the testes. It begins at puberty and continues throughout the male reproductive system's lifespan, although age-related changes in the testicular microenvironment can affect spermatogenic efficiency (Maroto *et al.*, 2025). The process is supported by somatic cells, particularly Sertoli, Leydig, and peritubular myoid cells. It is tightly regulated by the hypothalamic–pituitary–testicular axis, in which GnRH stimulates pituitary secretion of FSH and LH, while testosterone produced by Leydig cells and FSH signaling in Sertoli cells coordinate germ-cell development (Bhattacharya and Dey, 2023; Maroto *et al.*, 2025).

Spermatogenesis is commonly divided into three major phases: (1) mitotic proliferation of spermatogonial stem cells (SSCs) and spermatogonia along the basement membrane, (2) meiosis, during which primary spermatocytes undergo meiosis I to form haploid secondary spermatocytes and then meiosis II to form haploid spermatids, and (3) spermiogenesis, in which round spermatids undergo morphological and biochemical remodeling to become elongated spermatozoa (Maroto *et al.*, 2025; Cui *et al.*, 2025). During spermiogenesis, major changes

include chromatin condensation (with histone-to-protamine replacement), acrosome formation, and flagellum development (Miyata *et al.*, 2024).

Mature spermatozoa consist of the first part, the head, which contains the nucleus and acrosome, and the second part, the midpiece and the flagellum (tail), which is structurally organized into the principal piece and end piece and is essential for motility (Miyata *et al.*, 2024).

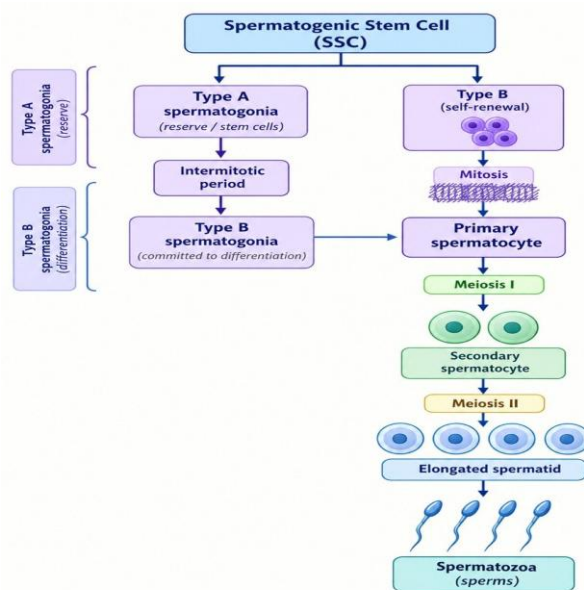


Figure 2-1 stages of spermatogenesis in rats

2.3 Testicular functional protein

2.3.1 Acrosin

Acrosin is a trypsin-like serine protease associated with the sperm acrosome and is stored predominantly as the inactive zymogen proacrosin, which becomes activated during acrosomal exocytosis. Acrosomal exocytosis is a regulated event linked to sperm capacitation and can be stimulated by signals from the female reproductive tract and zona pellucida (e.g., progesterone and zona-derived signals) (Suhaiman and Belmonte, 2024). In humans, the zona pellucida (ZP) is composed of four types of glycoproteins (ZP1, ZP2, ZP3, and ZP4).

Current evidence suggests that fertilization-related sperm–ZP interactions are not mediated solely by ZP3; rather, multiple ZP glycoproteins contribute to sperm binding and/or the induction of the acrosome reaction (Gupta, 2021).

Functionally, acrosin appears to facilitate sperm passage through the ZP by limited proteolysis and remodeling of the zona matrix rather than simple bulk degradation. In a mouse study, acrosin-mediated cleavage sites were identified in ZP1, ZP2, and ZP3, and this limited proteolysis altered zona mechanical properties and sperm-binding behavior (Kuske *et al.*, 2021). Recent human evidence showed that Acrosin deficiency can cause total fertilization failure (TFF) by preventing sperm penetration of the zona pellucida. Consequently, acrosin should be characterized as a significant protease associated with fertilization that facilitates zona penetration and matrix remodeling, with effects that vary across species and experimental settings. (Kuske *et al.*, 2021; Hua *et al.*, 2023)

2.3.2 Protamine

Protamines are small, highly basic (arginine-rich) nuclear proteins that play a central role in sperm chromatin remodeling during spermiogenesis. In mammals, the paternal genome undergoes extensive chromatin reorganization in post-meiotic spermatids, where the majority of histones are replaced by protamines (primarily protamine 1 and protamine 2). The result is a highly compacted sperm nucleus (Moritz and Hammoud, 2022). This extreme condensation helps stabilize and protect paternal DNA, supporting proper sperm head construction and function (Arévalo *et al.*, 2022).

Protamine expression and incorporation are tightly regulated in time and space during spermiogenesis. Recent data highlight precise post-transcriptional regulation during the histone-to-protamine transition, and experimental research demonstrates that appropriate protamine transport and nuclear localization are necessary for effective chromatin condensation (Agudo-Rios *et al.*, 2023).

Disruption of protamine processing or incorporation leads to defective histone-to-protamine exchange, abnormal protamine ratios, impaired sperm chromatin packaging, increased DNA damage, and male infertility (Arevalo *et al.*, 2022).

2.4 Maturation stages of the male reproductive system in rats

The male reproductive system in mammals, such as rats, undergoes many developmental stages from birth to sexual maturity; these stages are under the control of the hypothalamus and pituitary glands. In immature rats, the testes are unable to produce sperm, and their hormonal levels remain stable (Plant & Zeleznik, 2014). In rats, there is no period of testicular quiescence or interruption in reproductive gland secretion. The maturation and development of the male reproductive system occur early and in four stages. (Ojeda *et al.*, 1980).

The neonatal period, which lasts from birth to seven days of age, the infantile period, which lasts from eight to twenty-one days; the juvenile period, which lasts for thirty-five days; and the peripubertal period, which lasts from fifty-five to sixty days. These phases end when the vas deferens contains the first mature spermatozoa. The spermatocytes deteriorate on days 3 and 7 after birth, after ceasing to divide on day 18 of pregnancy. After being around 25% at birth, the remaining cells begin to split into spermatogonia (Setchell 1978; Griswold, 2016).

Testicular testosterone concentration and testicular luteinizing hormone receptors (LHR) in the testes and interstitial cells rise throughout pregnancy and peak after birth. This is associated with a rise in testosterone levels in the latter stages of pregnancy that rapidly declines following birth (Warren *et al.*, 1984). Sertoli cells in the seminiferous tubules will start to proliferate on day 16 of pregnancy, reach their peak division on day 19, and cease to do so between days 14 and 16 of postnatal life (Steinberger and Steinberger, 1971). The follicle-stimulating hormone receptor (FSHR) on Sertoli cells also dramatically rises before birth (Wang *et al.* 2022).

2.5 Selenium

One of the crucial trace elements is selenium (Se), which is incorporated as selenocysteine into a family of human selenoproteins, many of which are central to redox regulation and endocrine homeostasis. In humans, major selenoprotein families include glutathione peroxidases (GPXs), glutathione reductases (GRs), and iodothyronine deiodinases (DIOs). These proteins collectively support antioxidant defense and thyroid hormone metabolism, both of which are closely linked to male reproductive function (Barchielli *et al.*, 2022).

In the male reproductive system, selenium is increasingly recognized as a biologically important determinant of testicular function, spermatogenesis, semen quality, and steroidogenic balance. Mechanically, two selenoproteins are particularly important in male fertility: glutathione peroxidase 4 (GPX4) and selenoprotein P (SELENOP). Selenium is a component of glutathione peroxidase, an enzyme that protects germ cells, Sertoli cells, and Leydig cells from oxidative stress. It is also essential for the normal stages of germ cell development and differentiation, and one of its most important proteins is selenoprotein P, which contributes to providing selenium to reproductive tissues and also regulates the structure of the sperm midpiece, thus improving motility and normal function. (Qazi *et al.* 2019). By preserving chromatin stability and mitochondrial sheath integrity, glutathione peroxidase 4 (GPX4), another antioxidant enzyme, contributes to sperm maturation. Another important selenium transport protein, selenoprotein P (SELENOP), is produced in Leydig cells and may play a role in antioxidant defense during steroidogenesis. It is involved in selenium transport to the testis. However, high-quality evidence is still lacking. Dietary supplements containing selenium have not been demonstrated to enhance pregnancy or live births, according to a recent systematic review and meta-analysis (2025). However, in pooled studies, selenium enhanced a few sperm motility-related

outcomes. The same study noted that heterogeneity, small sample sizes, and bias were the primary causes of the evidence's typically low to extremely low certainty.

Therefore, rather than being a general treatment for male infertility, selenium should be considered a potential adjuvant, particularly depending on the clinical situation and baseline nutritional status (Michaelsen *et al.*, 2025). Although selenium (Se) is a vital trace element for both humans and animals, its physiological requirement and toxicity are so close together that careful dose control is required in both therapeutic and nutritional applications (Alexander & Olsen, 2023). Selenium is mostly found in nutritional contexts as inorganic forms (like selenite and selenate salts) and organic forms (such as selenomethionine and selenocysteine). On the other hand, selenoproteins containing selenocysteine, such as glutathione peroxidases (GPXs), thioredoxin reductases (TXNRDs), iodothyronine deiodinases (DIOs), and selenoprotein P (SELENOP), are primarily responsible for their biological functions in mammals (Alexander and Olsen, 2023; Bai *et al.*, 2024). Dietary sources and soil selenium levels significantly affect selenium status, as selenium's toxicity and bioavailability are dose-dependent. As a potentially safer and more bioactive element form, selenium nanoparticles (SeNPs) have drawn growing interest (Sampath *et al.*, 2024).

2.5.1 Effects of selenium on male reproductive hormones

2.5.1.1 Testosterone

Testosterone is produced primarily by adult Leydig cells, which are located in the testicular interstitium, and is the principal androgen in the male reproductive system. It is essential for the maintenance of spermatogenesis and for the development and preservation of male reproductive and somatic characteristics under the regulation of the hypothalamic–pituitary–gonadal (HPG) axis (Li *et al.*, 2024).

Selenium contributes to male reproductive physiology mainly through selenoproteins that act as regulators of redox balance, antioxidant defense, and inflammatory control in testicular tissue. Recent reviews indicate that both selenium deficiency and excess may adversely affect testicular development and male reproductive function; therefore, selenium is better described as a regulator of reproductive homeostasis rather than a uniformly useful factor across all exposure levels (Yuan *et al.*, 2024).

Regarding steroidogenesis, current evidence suggests that selenium may indirectly help preserve testosterone production by protecting Leydig cell function from oxidative and inflammatory damage. However, the effect of selenium supplementation on testosterone is not uniform across all species, doses, or experimental conditions, so it should be presented as a context-dependent effect rather than a universal response (Yang *et al.*, 2025; Zečević *et al.*, 2025).

2.5.1.2 Luteinizing hormone

In males, LH primarily acts on Leydig cells to stimulate testosterone synthesis, which is crucial for maintaining spermatogenesis and male reproductive function (Lei *et al.*, 2025). Immature mammals have low circulating LH levels that increase significantly during puberty, thereby causing immature Leydig cells to differentiate into adult cells (Nalbant, 2002). When LH attaches to its receptor on the Leydig cell membrane, Leydig cell steroidogenesis begins. (De Mattos *et al.*, 2023; Lei *et al.*, 2025). There are four distinct stages in the postnatal development of adult Leydig cells in rats: stem cells, progenitor cells, immature cells, and adult Leydig cells (Ye *et al.*, 2017).

. According to Stanley (2012), several studies indicate that luteinizing hormone is a key regulator of Leydig cell activity. By binding to the LH receptor on Leydig cell membranes, luteinizing hormone increases intracellular cAMP levels by activating adenyl cyclase, which in turn activates protein kinase A,

transcription factors, and signals that modify Leydig cell function (Casarini *et al.*, 2020).

The evidence for a direct and consistent effect of selenium on LH is weaker than the evidence for its broader antioxidant role in male reproduction. Recent literature supports the view that selenium may help stabilize the hypothalamic pituitary testicular axis under specific pathological or oxidative stress conditions, but this should not be generalized to imply that selenium invariably increases LH in healthy males (El-Shaer *et al.*, 2024).

2.5.1.3 Follicle-stimulating hormone (FSH)

Follicle-stimulating hormone (FSH) is a heterodimeric glycoprotein synthesized and secreted by pituitary gonadotrophs under hypothalamic control through gonadotropin-releasing hormone (GnRH). In males, FSH is a major endocrine regulator of testicular development, pubertal maturation, and spermatogenesis. Its biological actions in the testis are primarily mediated by the follicle-stimulating hormone receptor (FSHR), which is expressed exclusively on Sertoli cells. Follicle-stimulating hormone (FSH) signaling promotes Sertoli cell proliferation and differentiation. It supports germ-cell development by stimulating Sertoli cells to produce essential paracrine factors, nutrients, and structural proteins that create a specialized microenvironment for germ-cell survival and development, particularly during the early phases of spermatogenic progression (Oduwole *et al.*, 2021; Wang *et al.*, 2022).

Follicle-stimulating hormone acts exclusively through Sertoli cells to maintain the microenvironment required for germ cell survival, differentiation, and maturation. In adults, FSH is essential for maintaining the quantitative efficiency of spermatogenesis, particularly by supporting the late stages of germ cell maturation (spermiogenesis) and preventing Sertoli cell and germ cell apoptosis. Thus, normal spermatogenesis depends on the coordinated action of

FSH and intratesticular testosterone rather than on a direct FSH effect on Leydig cells (Oduwole *et al.*, 2021; Wang *et al.*, 2022). Studies have shown no direct relationship between selenium and follicle-stimulating hormone (FSH), but there is an indirect effect via its effects on pituitary secretion and its antioxidant properties (Razavi *et al.*, 2016).

2.5.2 Selenium dietary requirements and health implications

The chemical form of selenium strongly influences its bioavailability, metabolism, and toxicity. Current nutritional evidence indicates that adult human requirements for selenium are generally 55–60 micrograms/day, whereas excessive intake may lead to adverse effects due to a relatively narrow safety margin (Ren *et al.*, 2024). Recent evaluations also support a more conservative view of upper safe intake, with newer assessments proposing that the tolerable upper intake level for adults may be closer to 255 µg/day rather than the historically cited 400 µg/day (Han *et al.*, 2025).

A lack of selenium can reduce antioxidant defenses and increase vulnerability to oxidative stress by impeding the manufacture and function of various selenoenzymes, including glutathione peroxidases, selenoproteins, and thioredoxin reductases. Low selenium status may be linked to negative health effects, such as thyroid dysfunction, compromised immune function, and reproductive problems, in severe or prolonged selenium-deficient conditions (Ren *et al.*, 2024). However, the relationship between selenium intake levels and health outcomes is typically nonlinear or U-shaped in a variety of studies, and depending on the baseline level of selenium, population characteristics, and the studied endpoint, the physiological effects of either too much or too little selenium may be detrimental (Wang *et al.*, 2023).

Selenium supplementation should be used with caution and should not be advised due to this limited therapeutic window. Recently, selenium nanoparticles

(SeNPs) have attracted interest as a novel method for selenium delivery. In certain preclinical models, SeNPs have been shown to exhibit favorable physical, chemical, and biological properties, including strong surface reactivity and possibly lower toxicity than other conventional selenium compounds. However, several issues remain regarding dose standardization, long-term safety, formulation stability, and translational applicability before they can be considered therapeutic agents or supplements. Research on its use in human nutrition and medicine is still ongoing (Geng *et al.*, 2025). Studies show that rats need 0.15 mg of selenium per kilogram of dry feed weight per day (Jia *et al.*, 2025).

2.6 Nanotechnology and Nanoparticles

The creation, development, and use of materials and systems at the nanoscale—typically with at least one exterior dimension in the 1–100 nanometer range—is referred to as nanotechnology. The physical, chemical, and biological characteristics of materials at this scale, which differ significantly from their bulk properties, underpin many of their biomedical applications (Bayda *et al.* 2019). The name "nanotechnology" was first used in 1974 by Norio Taniguchi, although Richard Feynman's 1959 lecture "There is Plenty of Room at the Bottom" has been credited with laying the groundwork for the field (Szczyglewska *et al.*, 2023). Because of their high surface area-to-volume ratio and their ability to alter surface chemistry, nanoparticles are especially useful in medicine. They can be utilized to enhance tissue targeting, boost drug loading, modify release kinetics, and protect cargo. When properly designed, these characteristics may reduce off-target toxicity and help develop more effective drug delivery systems (Mitchell 2021). For medications that harm the kidneys, such as gentamicin, nanoparticles can improve drug stability, reduce toxicity, shield tissues, and offer novel treatment approaches.

Nanoparticle-based platforms are being used across a variety of medical specialties, including oncology, infectious diseases, and vaccinology, according

to recent clinical and translational data. Notably, albumin-bound paclitaxel (such as Abraxane®; nab-paclitaxel) and liposomal doxorubicin (such as Doxil®) are clinically proven instances. They have shown benefits in improving therapeutic delivery and safety profiles for specific indications. (Kumarasamy *et al.* 2024).

2.6.1 Nano selenium

Selenium nanoparticles (SeNPs) constitute a promising family of nanomaterials due to their adaptable physical and chemical properties, biocompatibility, and very low toxicity compared to some other nanomaterials. Their potential in antioxidant (Liu *et al.*, 2021), anti-inflammatory (Ansari *et al.*, 2024), antimicrobial (Chen *et al.*, 2023), and anticancer (Wadhwani *et al.*, 2017) applications is highlighted by recent reviews, and there is increasing interest in biological and translational usage (Zhang *et al.*, 2025).

2.6.2 Synthesis of selenium nanoparticles (SeNPs)

The most recent reviews categorize SeNPs synthesis into three broad categories: chemical, physical, and biological (green or biosynthetic) pathways. They emphasize that the type of synthesis plays a crucial role in determining the size, shape, stability, and surface chemistry of nanoparticles, which, in turn, dictate their performance in biological systems (Zhang *et al.*, 2023). While biosynthetic processes are often preferred because they are less hazardous and better suited to mild environments, chemical reduction remains the most popular method. It is typically carried out by taking selenium precursors like Na_2SeO_3 along with reducing and stabilizing agents (Sampath *et al.*, 2024). The most recent literature also highlights the importance of standardizing the physicochemical characterization (particle size distribution, zeta potential, morphology, and surface chemistry) and the production of SeNPs to ensure their use in veterinary and biomedical applications (Zhang *et al.*, 2023; Geng *et al.*, 2025).

2.6.3 Chemical reduction method for selenium nanoparticle synthesis

Chemical reduction, one of the three primary fabrication routes—physical, chemical, and biological in recent work, is one of the most widely used techniques for producing selenium nanoparticles (SeNPs). It starts by reducing selenium precursors to Se_0 in the presence of reducing agents and stabilizers (Sampath *et al.*, 2024). Although alternative selenium precursors, such as selenate, selenium salts, and acids, have also been documented in certain procedures, sodium selenite (Na_2SeO_3) is the most prevalent selenium precursor. The most widely used technique for creating SeNPs is chemical synthesis, which involves reducing selenium ions in a sodium selenite solution using stabilizers and reducing agents (Qin *et al.*, 2025). Common reducing agents reported in recent literature include ascorbic acid (Al-Bassam *et al.*, 2025) and glutathione (El-Batal *et al.*, 2021), while tabulated examples in the same review also document glucose-based (Nie *et al.*, 2016) and cysteine-based reduction systems for SeNPs preparation (Li *et al.*, 2010).

In parallel, stabilizing or capping materials such as polysaccharides (Shi *et al.*, 2021), glycoproteins (Zhang *et al.*, 2025), and polyphenols (Indiarto *et al.*, 2022) are commonly used to improve colloidal stability and reduce aggregation. Shar *et al.* (2019) used a straightforward, inexpensive, and environmentally benign hydrothermal technique to produce nano-sized selenium particles. Sodium selenite was used as a precursor, and L-ascorbic acid was used as a stabilizing and reducing agent (Chellapa *et al.*, 2020). Theobroma cacao L. bean shell extract was used by Mellinas *et al.* (2019) to produce SeNPs, and they successfully regulated their size and antioxidant activity over an extended period of storage. (Mellina *et al.* 2019).

2.6.4 Biological synthesis of selenium nanoparticles (SeNPs)

2.6.4.1 Microbial synthesis

Because biological systems are employed as reducing and stabilizing systems, frequently under mild reaction conditions, and have less reliance on chemicals, microbial biosynthesis of selenium nanoparticles (SeNPs) is a more popular and environmentally friendly method for this study (Sampath *et al.*, 2024; Qin *et al.*, 2025; Geng *et al.*, 2025). Microbial redox and detoxification systems are typically used to convert microorganisms cultivated in media containing selenite precursor materials (sodium selenite or sodium hydrogen selenite) to elemental selenium (Se^0) (Ao *et al.*, 2023; Fadel *et al.*, 2025).

The size, surface chemistry, and colloidal stability of SeNPs can be affected by biomolecules produced by microorganisms, such as proteins, polysaccharides, and lipids, which can act as reducing, capping, and stabilizing agents (Ao *et al.*, 2023). According to recent research, certain bacterial species, such as *E. coli*, are utilized in the production of SeNPs (Kora and Rastogi, 2017). *Bacillus subtilis* (Wang *et al.*, 2024) and *Penicillium tardochrysogenum* (El-Sayed *et al.*, 2024). *Pseudomonas aeruginosa* (Elshikiby *et al.*, 2025), *Lactobacillus casei* (Spyridopoulou *et al.*, 2021), *Aspergillus* (Asghari-Paskiabi *et al.*, 2023) and others.

2.6.4.2 Plant extract-assisted synthesis of selenium nanoparticles (SeNPs)

Using phytochemicals and other plant-derived biomolecules as reducing and capping agents, plant extract-assisted synthesis of selenium nanoparticles (SeNPs) is a green biosynthesis method that transforms selenium precursors (like sodium selenite, Na_2SeO_3) into elemental selenium nanoparticles (Se^0). Because plant-mediated synthesis can be carried out under moderate, cost-effective conditions and does not require the time-consuming maintenance of microbial cell cultures, it is often more practicable in the lab (Pyrzynska, 2024).

Plant-produced phytochemicals and other plant-derived biomolecules are employed as reducing and capping agents in the green biosynthesis process known as "plant extract-assisted synthesis of selenium nanoparticles" (SeNPs), which can transform selenium precursors (sodium selenite, Na_2SeO_3) into Se^0 (Pyrzynska, 2024). Because microbial cell cultures can be maintained at very low cost and under very mild conditions, plant-mediated synthesis is often more feasible at the laboratory level than microbial-mediated synthesis (Qin *et al.*, 2025).

Recently, selenium ions can be reduced and nanoparticles stabilized by hydrogen bonding, electrostatic interactions, and surface capping with plant extracts rich in polyphenols, flavonoids, and other functional biomolecules containing hydroxyl, carboxyl, amino, or aldehyde groups (Cheng *et al.*, 2025). This dual role is significant because it decreases nanoparticle aggregation during synthesis and improves colloidal stability. The physicochemical properties of plant-derived SeNPs—especially particle size, polydispersity, and stability—are strongly influenced by the composition of the extract and by synthesis parameters such as precursor concentration, extract concentration, pH, temperature, and reaction time (Qin *et al.*, 2025). Therefore, optimization and comprehensive characterization are essential for reproducibility and for comparing results across studies (Pyrzynska, 2024).

For example, Cheng *et al.* (2025) reported a green synthesis strategy using grape seed extract (GSE) synergized with ascorbic acid for the reduction of sodium selenite, followed by response surface optimization of the synthesis conditions. Their optimized GSE-SeNPs exhibited a favorable particle size distribution and zeta potential values consistent with good colloidal stability, and the authors demonstrated that GSE contributed to both the reduction and stabilization of SeNPs (Cheng *et al.*, 2025). Similarly, Mekky *et al.* (2025) synthesized SeNPs using *Dahlia pinnata* tuber extract and sodium selenite and documented a visible color change during nanoparticle formation, while UV-Vis

and FTIR analyses confirmed SeNP formation and the involvement of plant biomolecules in capping and stabilization (Mekky *et al.*, 2025).

Overall, plant extract-assisted synthesis represents a promising green route for producing functional SeNPs with a reduced chemical burden; however, standardization of plant extract composition and synthesis conditions remains critical to improve reproducibility and inter-study comparability (Pyrzynska, 2024; Qin *et al.*, 2025). There are many examples of using various plant species in the green manufacturing process for different nanoparticles, including *Camellia sinensis* (Rolim *et al.*, 2019) and *Tarragon* (Yilmaz *et al.*, 2021).

2.6.4.3 Physical method for SeNPs synthesis

The most popular physical techniques for producing SeNPs include electrochemical, laser ablation, hydrothermal, and microwave radiation methods. Pulsed-laser liquid ablation in liquids (PLAL) offers several advantages over conventional methods for synthesizing selenium, including the wet chemical route, physical vapor deposition, and chemical vapor deposition. These advantages include a simple structure, a high surface purity of the SeNPs (with no needless adducts or byproducts), and the ability to collect or store the resultant nanoparticles as colloidal solutions with ease. Moreover, it is a simple, eco-friendly technique that does not require a clean room or a vacuum (Zhang *et al.*, 2023).

However, nanoparticles produced by liquid-phase pulsed laser ablation (LP-PLA) may not be contaminated by chemical reagents. Due to the lack of a vacuum chamber, the equipment is cheap, collecting nanoparticles after synthesis is simple, and nanoparticles may be maintained stably in colloidal solution (Zhang *et al.*, 2023). A novel PLAL method for SeNPs, twice-pulsed laser irradiation, produces pure SeNPs with numerous biological functions, small sizes, and exceptional stability (Geoffrion *et al.*, 2020).

2.7 The role of selenium nanoparticles as antioxidant defense

Oxidative stress results from an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defense systems. In the male reproductive system, ROS exert a dual effect: at physiological levels, they participate in essential events such as sperm capacitation, motility, and fertilization. However, excessive ROS production disrupts redox homeostasis and promotes lipid peroxidation, protein oxidation, and DNA fragmentation (Mohammadi and Jafarpour, 2024).

Male infertility is a result of these alterations, which also harm sperm membrane integrity, motility, fertilization capacity, and genomic stability (Wang *et al.*, 2025). Selenium helps maintain the balance between antioxidant defense and cellular redox. Because of the high concentration of polyunsaturated fatty acids in their plasma membranes and their low capacity for repair, spermatozoa are particularly vulnerable to oxidative damage. However, since both excess and deficiency can have detrimental effects on male reproductive function, selenium homeostasis must be controlled (Zhang *et al.*, 2023; Yuan *et al.*, 2024).

Selenium nanoparticles (SeNPs) have attracted increasing interest as antioxidants in recent years. Because SeNPs release bioactive selenium ions gradually and under control, preventing abrupt concentration spikes that could be cytotoxic, their antioxidant activity may be less harmful than that of typical selenium compounds (such as selenite or selenate). In oxidative conditions, they affect redox-related enzyme activity. The biological effects of SeNPs, however, are inconsistent and largely depend on their size, surface chemistry, production method, dose, and the experimental model used (Shi *et al.* 2023).

For instance, one study found that oral SeNP supplementation improved several antioxidant indices in boars without appreciably improving semen quality. However, a different study discovered that using a semen extender containing

SeNPs improved the quality of short-term stored boar semen and the results of conception. According to Horky *et al.* (2023), it would be more accurate to characterize nano-selenium as a promising but context-dependent antioxidant method that has not yet attained the status of a superior and widely recognized therapeutic antioxidant.

2.8 Nano-selenium and its role in spermatogenesis

Selenium is linked to spermatogenesis, sperm maturation, and sperm motility in the male reproductive system, as well as to the regulation of inflammatory and oxidative responses. Current research indicates that there is a dose-dependent, U-shaped relationship between selenium status and testicular function and semen quality; therefore, selenium homeostasis, rather than opportunistic supplementation, is necessary for normal male reproductive function (Yuan *et al.*, 2024; Yang *et al.*, 2025). Selenium's interaction with reactive oxygen species (ROS) is a key factor in its reproductive role. While modest ROS levels are physiologically necessary for optimal sperm activity, excessive ROS causes DNA damage, lipid peroxidation, and poor motility. A crucial structural element of the mitochondrial sheath of mammalian spermatozoa is glutathione peroxidase 4 (GPX4), a selenium-dependent enzyme that is involved in antioxidant defense throughout germ cell development (Ghafarizadeh *et al.* 2018).

The primary plasma selenium transport protein, selenoprotein P (SeP), produced in the liver, is responsible for transporting selenium to peripheral tissues like the testis, as well as for testicular selenium homeostasis and selenoprotein biosynthesis (Saito, 2021). According to recent research, SeNPs may have biological advantages over some traditional forms of selenium (inorganic selenite or organic selenomethionine) in terms of toxicity (lower toxicity in some experiments) and bioactivity (better cellular uptake or more effective incorporation into selenoproteins). The regulated release of selenium ions, which

can reduce abrupt concentration swings associated with the toxicity of some small-molecule forms, may be the cause of this toxicity (Zhang *et al.* 2023).

Their positive effects on male fertility are mostly associated with inflammatory processes, apoptosis, and oxidative stress, all of which can help maintain sperm quality. However, the present data point to a cautious interpretation rather than a universal therapeutic generalization, as these effects are highly dependent on dose, particle properties, formulation, and biological context. Therefore, SeNPs are a promising yet still developing approach to male reproductive health, and rigorous, standardized, and translational research is needed to fully characterize their therapeutic potential (Yuan *et al.*, 2024).

Chapter Three

Materials and Methods

3. Materials and Methods

Table (3-1): The equipment and materials used in this study

No.	Material	Company	Origin
1	Acrosin ELISA Kit	ELK Biotechnology	China
2	Automated tissue processor- ATP700	Histo-Line	Italy
3	Centrifuge	Generic	China
4	Conical flask, Cylinder	Kern	China
5	Digital Thermometer	LC Tech	China
6	Disposable Syringe 5 ml	Sterile EO	China
7	ELISA-Reader	Human reader HS	Germany
8	Embedding system-TEC2800 -C	HESTION	China
9	Eppendorf tube 2. 0 mL	Eppendorf	Germany
10	Field emission scanning electron microscopy (FESEM)	TESCAN Mira3	Czechia
11	Filter paper	Bright Sign	China
12	Fourier transform infrared spectroscopy (FTIR)	Perkin Elmer TWO	USA
13	FSH ELISA Kit	ELK Biotechnology	China
14	Gel tube 5 ml	Biozek	The Netherlands
15	Glutathione Peroxidase ELISA Kit	Reed Biotech	China
16	Glutathione Reductase ELISA Kit	Reed Biotech	China
17	Hemocytometer	Hirschmann Laborgeräte Gmb	Germany

18	Hot plate – HYSH11	K&K	Korea
19	Incubator	Memmert	Germany
20	LH ELISA Kit	ELK Biotechnology	China
21	Magnetic stirrer	ALFA	Pakistan
22	Micropipette	VWR	USA
23	Microscope	Euromex	The Netherlands
24	Protamine ELISA Kit	ELK Biotechnology	China
25	Semi-automatic microtome- MRS3500	Histo-Line	Italy
26	Sensitive balance	Sartorius	Germany
27	Slenoprotein ELISA Kit	Reed Biotech	China
28	Testosterone Hormone ELISA Kit	ELK Biotechnology	China
29	Ultrasonic homogenizer	Hwasheen	Korea
30	Water bath - BI	FALC	Italy
31	X -ray diffraction	Philips OW1730	The Netherlands
32	Zeta potential analyzer	HORIBA SZ100	Japan

Table (3-2): The chemicals and their remarks used in this study.

No.	Chemicals	Company	Origin
1	Alcohol (ethanol 96%)	Aljoud	Iraq
2	Ammonia water solution	Scharlau	Spain
3	Ascorbic acid	Thomas Baker	India
4	Chloroform	Thomas Baker	India
5	Eosin stain	Himedia	India
6	Ethanol	BDH	UK
7	Formalin	Chem-Lab NV	Belgium

8	Harris's Hematoxylin	Sigma	USA
9	Mounting Medium	Sigma	USA
10	Nigrosine stain	Sigma	USA
11	Paraffin Wax	Leica Biosystems	Germany
12	Sodium selenite	Avonche limited	United Kingdom
13	Starch	Thomas Baker	India
14	Xylene	BDH	UK

3.1 Part one of experiment: Synthesis and characterization of biosynthesized SeNPs.

3.1.1 Preparation of solutions

3.1.1.1 Preparation of starch solution

A 0.2% starch solution was prepared by dissolving 2 g of starch powder in 1 L of distilled water. The mixture was heated gently while being stirred continuously to produce a clear, uniform solution. The starch solution was allowed to cool to room temperature before being used in the synthesis procedure (Kazemi, 2021).

3.1.1.2 Preparation of sodium selenite solution

An aqueous sodium selenite (Na_2SeO_3) solution with the required molar concentration of 20 millimolar was created by dissolving 3.46 grams of Na_2SeO_3 in 800 milliliters of distilled water. To prepare a 0.346% solution of Na_2SeO_3 . The volume was then completed to 1000 milliliters. Then, stir the mixture until it dissolves completely. The solution should be stored in a dry, cold area in a dark bottle. The concentration and preparation date were written on the bottle's label (Tabibi *et al.*, 2023).

3.1.1.3 Preparation of ascorbic acid solution

A fresh ascorbic acid solution was made by dissolving 3.5 g of ascorbic acid in 25 ml of distilled water. The solution was transferred to a 100 mL volumetric flask to prepare a 3.5% ascorbic acid solution, and the final volume was completed to 100 mL with distilled water (Malhotra, 2014).

3.2. Synthesis of selenium nanoparticles

Selenium nanoparticles were manufactured via a green chemical reduction approach utilizing prepared 0.2% starch for stabilization and 3.5% ascorbic acid for reduction (Siddique *et al.*, 2024). First, a magnetic stirrer was used to mix the produced starch solution at room temperature continuously. The starch solution was then gradually mixed with the prepared 0.346% sodium selenite solution and continuously stirred to ensure the selenium ions were evenly distributed throughout the starch matrix. Following thorough mixing, the prepared 3.5% ascorbic acid solution was gradually added to the reaction mixture while stirring constantly until the PH of the solution dropped from 12 to (7-6,8).Figure 4-1

The solution was visually confirmed by a gradual change from colorless to orange-red (Vishakha *et al.*, 2020). indicating the formation of selenium nanoparticles. To promote nanoparticle production and increase the reduction rate, the reaction temperature was slightly raised to 50–60 °C. For 4.5 hours, the reaction mixture was continuously stirred to guarantee that the nanoparticles were completely reduced and stabilized (Tabibi *et al.* 2023).

3.3 Purification of selenium nanoparticles

Centrifugation was used to purify the produced selenium nanoparticles for 15 minutes at 6000 rpm. To remove unreacted materials and excess stabilizing agents, the sediment was washed three times with distilled water, then with alcohol(ethanol 96%). After that, the product was dried at 40 °C for 24 hrs in an incubator.

3.4 Characterization of green-synthesized selenium nanoparticles (SeNPs)

Green-synthesized selenium nanoparticles (SeNPs) can be characterized using complementary techniques, as no single method can fully characterize their physical and chemical properties. (Pyrzynska, 2024).

3.4.1 Fourier transform infrared (FTIR) spectroscopy

It is used to identify functional groups on the surface of nanoparticles contributed by biological reducing/capping agents. In green synthesis, FTIR bands commonly reflect carbonyl, hydroxyl, and C–O-related groups, supporting the particle's surface function and defining the elemental selenium core alone (Mekky *et al.*, 2025). PerkinElmer Spectrum TWO FT-IR Spectrometer (USA) is used to identify the functional groups of biosynthesized SeNPs.

3.4.2 X-ray diffraction (XRD) analysis

Determines the structural and crystalline nature of selenium nanoparticles (SeNPs) and can also provide an approximation of the size of the crystallite. Green synthesis of selenium nanoparticles may show crystalline selenium reflections in some systems, while other preparations appear weakly crystalline or predominantly amorphous; thus, XRD supports identification of phase and structure, not morphology (Mekky *et al.*, 2025; Hosseinpour *et al.*, 2025). The (PHILIPS, PW1730, The Netherlands) is used to determine the crystalline nature of(SeNPs).

3.4.3 Zeta potential

Zeta potential is a commonly used measure of colloidal stability and aggregation tendency in solution, as it reflects surface charge (Bhiri *et al.*, 2025; Hosseinpour *et al.*, 2025). The (HORIBA, SZ100, Japan) is used to measure the stability and aggregation tendency.

3.4.4 Field emission scanning electron microscopy (Fe-SEM)

The size and morphology of the synthesized Selenium nanoparticles (SeNPs) were examined using field-emission scanning electron microscopy (FESEM), which showed spherical nanoparticles (Mohammed *et al.*, 2026). TESCAN MIRA3 (Czechia) was used to show the images.

3.5 Selenium nanoparticles storage

To reduce oxidation and aggregation until further experimental use, the produced selenium nanoparticles were kept at low temperatures (4°C) in dark, tightly sealed containers (Xu *et al.*, 2023).

3.6 Preparation of dosage solution

To prepare the dosing solution, 0.4 g of nano-selenium was dissolved in 100 mL of D.W. and placed in an ultrasonic bath to disperse the nano-selenium particles. Then, the volume was completed to 1000 mL. The solution was then stored in an opaque bottle at -4 °C. In this way, the concentration will be 400 µg/mL. Then, to prepare the second concentration (200 µg/mL), take 50 mL of the stock solution and add 50 mL of D.W. to obtain a concentration of 100 µg/mL; take 25 mL of the stock solution and add 75 mL of D.W. for the last concentration of 50 µg/mL; take 12.5 mL of stock solution and add 87.5 mL of D.W.

3.7 Part two of the experiment

3.7.1 Experimental animals

Forty immature white male rats (*Rattus norvegicus*) weighing 100 ± 10 grams at 21 days of age were employed in the experiment, which was carried out in the animal house of the Veterinary Medicine College - University of Shatrah. For roughly 7 days of acclimatization, the animals were housed in special cages, with eight animals per cage, to maintain identical laboratory conditions. All animals were subjected to the same experimental conditions, including an air conditioner that maintained a room temperature of 25 ± 2 °C, 12 hours of light per day from four lid lamps, a humidity level of 40-50%, and daily access to food and water *Ad libitum*.

The animals were divided into 5 groups, with 8 male rats per group. The five groups were given a daily oral single dose for 30 treatment days as follows:

G1 group: Administered orally 0.2 mL of D.W.

G2 group: Administered orally 50 µg/kg BW of SeNPs daily.

G3 group: Administered orally 100 µg/kg of SeNPs daily.

G4 group: Administered orally 200 µg/kg BW of SeNPs daily.

G5 group: Administered orally 400 µg/kg BW of SeNPs daily.

Note: The weight of experimental animals at the time of the experiment was 120 ± 10 gm. The volume of the administered solution was changed weekly according to the weight change of experimental animals to obtain the same dose administration

3.7.2 Collection of blood samples

After 30 days of oral administration of SeNPs, the 8 male rats of each group were sacrificed. Following chloroform anesthesia by inhalation, the animal was secured, its abdomen was opened, and a 5 mL disposable syringe was used to collect blood from its heart Figure 3-1. To separate blood serum and allow it to clot at room temperature, blood was transferred into gel anticoagulant-free test

tubes. The tubes were then centrifuged at 3000 rpm for 10-15 minutes, and the serum samples were kept at -20 °C until they were needed to measure the levels of the studied hormones (testosterone, luteinizing hormone, and follicle-stimulating hormone). sperm proteins (protamine and acrosin) and antioxidant parameters (selenoproteins, glutathione reductase, and glutathione peroxidase)

3.7.3. Measurements of the testis weight

After the animals were sacrificed at the end of the first part of the experiment, the one free epididymis testis was weighed, and the specimens were preserved in 10% formalin. The formalin solution was changed after 24 hrs to remove fats, blood, and impurities before the samples were sent for histological examination.

3.8 Semen examination

3.8.1 Epididymal sperm count

To assess sperm viability, the epididymis was dissected at the time of sacrifice. The number of sperm was counted using a hemocytometer (Improved Neubauer chamber) following the separation of the cauda epididymis. The epididymis was dissected in a Petri dish with two milliliters of normal saline to create a diluted sperm suspension, which was then added to a counting chamber. The amount of sperm per epididymis was calculated by multiplying the total sperm count in five squares by 10000 (Figure 3-1; Al-Ghareebawi & Alkalby, 2012).

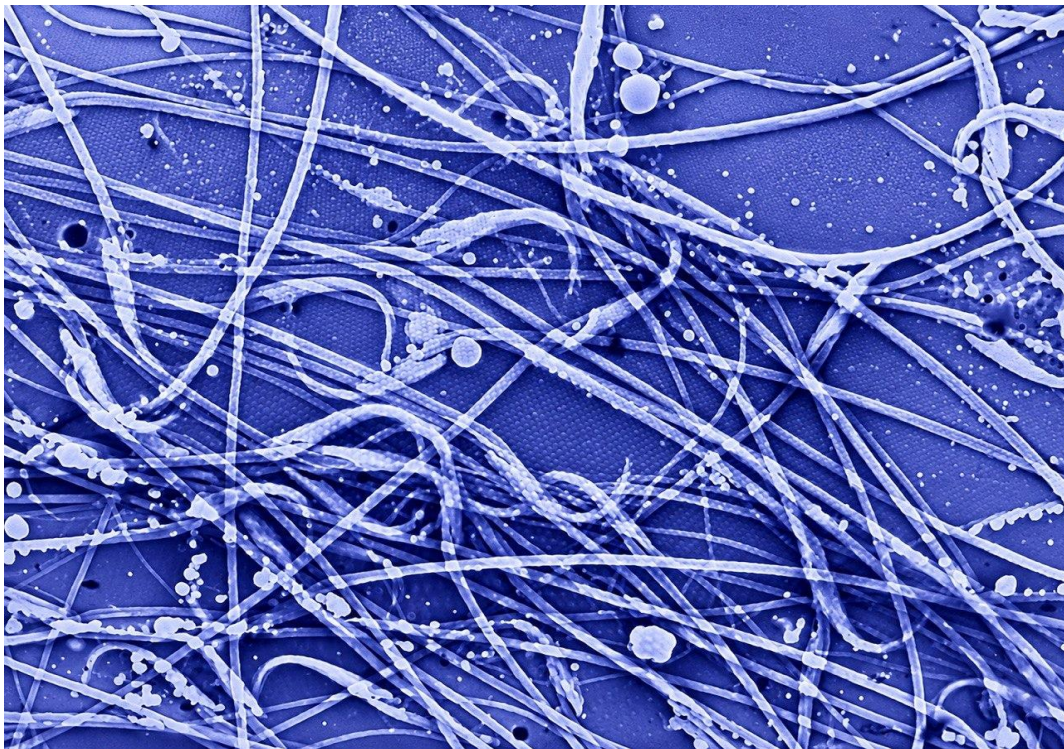


Figure 3-1 Epididymal sperm count

3.8.2. Live/dead spermatozoa percentage

According to Maxwell *et al.* (1996), dead sperm were counted.

- 1) A clean slide was covered with diluted semen.
- 2) Using a glass rod, eosin-nigrosin stain was applied to the semen and thoroughly mixed. A clean slide was placed at an angle on the semen slide and pulled horizontally to perform a smear.
- 3) The slide was left to air dry.
4. The slide was examined under the light microscope using oil-immersion 100× power lenses. Live sperm appeared in white, and the dead sperms appeared in red.

Figure 3-2.

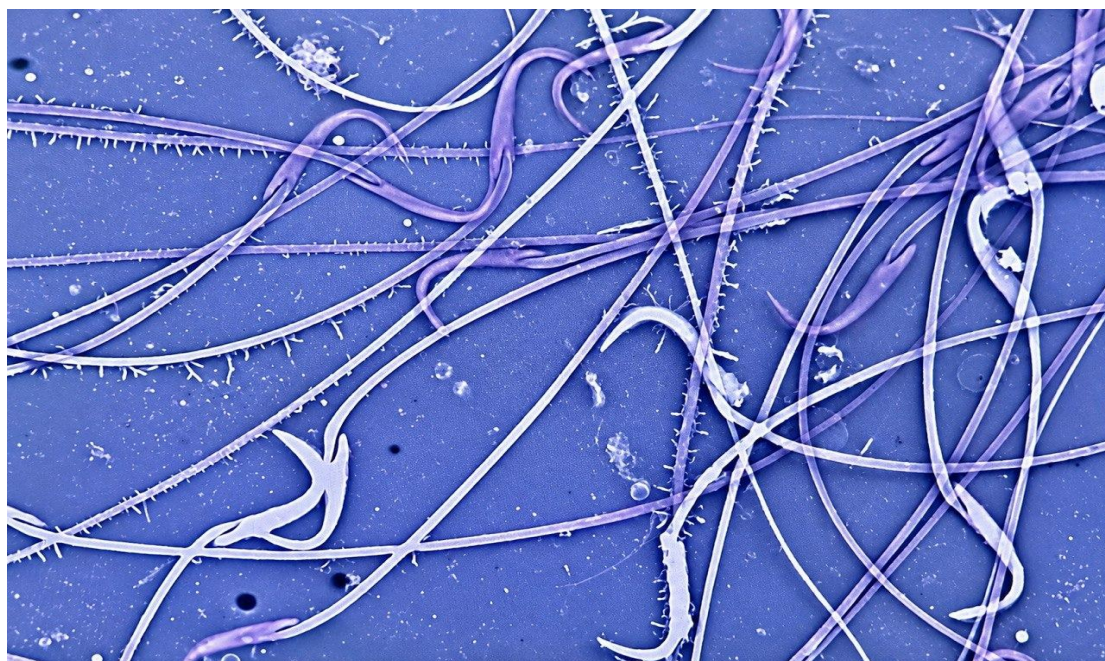


Figure 3-2 live /percentage sperm count

3.9 Hormonal assay

Testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) concentrations in the serum were quantified by commercially available ELISA Kits (ELK Biotechnology /China)

3.9.1. Determination of testosterone concentration

3.9.1.1 Test principle

This assay is based on a competitive enzyme immunoassay (EIA) principle. The microtiter plate included in the kit is pre-coated with rat testosterone antigen. Standard preparations and sample solutions are added to the assigned wells, and a biotinylated antibody specific to rat testosterone is then introduced. An avidin-HRP conjugate is then added, and the plate is incubated to facilitate the binding reaction. Following incubation, tetramethylbenzidine (TMB) substrate is applied to generate color, and the reaction is stopped with sulfuric acid, resulting in a detectable color change. The absorbance is then measured at $450 \text{ nm} \pm 10$

nm using a spectrophotometer. The optical density (OD) readings of the samples are subsequently compared with the standard curve to determine the concentration of rat testosterone in each sample.

3.6.1.2 Assay procedure

1) Testosterone was determined through competitive ELISA. The wells of the microplate were then pre-coated with rat testosterone antigen. Standard preparations and sample solutions were added to the wells, followed by biotinylated antibody specific for rat testosterone. An avidin-HRP conjugate was applied, and the plate was incubated. After that, TMB substrate was used to develop color, and the reaction was stopped with sulfuric acid. The absorbance was read with a spectrophotometer at $450\text{ nm} \pm 10\text{ nm}$. The OD values of each sample were then compared to the standard calibration curve to determine the concentration of rat testosterone.

2) All the contents are pulled out of every well. Aspiration is done; 200 μL of 1 $\times$ Wash Solution is added to each well and left in place for 1–2 minutes. The wash buffer is disposed of, then the plate is firmly pressed onto absorbent paper to remove any remaining liquid. This washing process is repeated three times. The liquid should be completely removed for reliable assay results. After the last wash, the plate is flipped over and blotted on clean paper towels to remove any remaining traces of buffer.

While washing, the pipette tip should not touch the inner surface of the wells to avoid contamination.

When the liquid is discarded, it should be done carefully to prevent the wash solution from being splashed into adjacent wells.

3) One hundred μL of streptavidin-HRP working solution is added to each well. The plate is sealed with the provided cover and incubated at 37°C for 60 minutes.

- 4) Aspiration and washing steps described in Step 2 are repeated until all wells are washed 5 times.
- 5) Ninety μL of TMB substrate solution is added to every well, and the plate is covered with a fresh sealer. The plate is incubated at 37°C in the dark for 20 minutes, with a maximum incubation time of 30 minutes. A blue color will develop in the wells after the substrate is applied. Before determining the optical density (OD), the microplate reader is warmed up for about 15 minutes. The plate should also be protected from direct sunlight before the reading is taken.
- 6) Fifty μL of Stop Reagent is added to each well. After its addition, the solution will turn yellow. The sides of the plate are gently tapped to ensure the contents are fully mixed. The Stop Reagent should be added in the same sequence as the TMB Substrate Solution.
- 7) The surface of the liquid is kept free of air bubbles and any moisture or fingerprints are wiped away from the underside of the plate. Then the microplate reader is switched on, and the absorbance is immediately recorded at 450 nm.

3.9.2 Determination of luteinizing hormone concentration

3.9.2.1 Test principle

This assay kit uses the sandwich ELISA method. The wells of the supplied microtiter plate are pre-coated with an antibody specific for rat LH. After standard solutions or test samples are dispensed into the appropriate wells, a biotinylated antibody against rat LH is added. An avidin-HRP conjugate is then added to each well, followed by incubation.

A color reaction develops only in wells containing rat LH, the biotinylated antibody, and HRP-conjugated avidin. Following the addition of the TMB substrate, the enzymatic reaction produces a visible color change. The reaction is then terminated with sulfuric acid, and the resulting absorbance is measured at

450 nm $\pm$ 10 nm using a spectrophotometer. The concentration of rat LH in the samples is then determined from the measured signal.

3.9.2.2 Assay procedure

1. Individual wells are allocated for the blank, standards, and sample solutions. One blank well and seven wells for the serially diluted standards are prepared. 100 μ L of Standard Working Solution is added to each standard well, and 100 μ L of each sample is added to the sample wells. The plate is covered and incubated at 37°C for 80 minutes. While the liquids are being dispensed, contact between the pipette tip and the inner surface of the wells is avoided, and the solutions are loaded carefully to avoid unnecessary disturbance.
2. All liquid is completely removed from the wells. Each well is washed with 200 μ L of 1 $\times$ Wash Solution and allowed to stand for 1–2 minutes. The wash buffer is discarded, then the plate is inverted and tapped firmly on absorbent paper to remove any residual liquid. The washing process was repeated three times. After the final wash, any remaining buffer is eliminated by aspiration or decanting, and the plate is blotted dry on absorbent paper. Contact between the pipette tip and the well wall when the wash solution is dispensed, as contamination can be introduced. The wash buffer is added carefully to prevent splashing or overflow into neighboring wells.
3. One hundred μ L of Biotinylated Antibody Working Solution is poured into each well. The plate is covered and incubated at 37°C for 50 minutes.
4. Aspiration and washing steps are repeated as described in Step 2, and the wash cycle is repeated until three washes have been done.
5. One hundred μ L of Streptavidin-HRP Working Solution is added to every well. The plate is sealed and incubated at 37°C for 50 minutes. 6. The wells are washed again according to the procedure described in Step 2. In this round, the washing step is done five times.

6. The wells are washed again according to Step 2, but five times in this round.
7. Ninety μL of TMB Substrate Solution is added to each well, and the plate is covered with a fresh sealer. Next, the plate is incubated at 37°C in the dark for 20 minutes, ensuring the total incubation time is no more than 30 minutes. Once the substrate is added, the liquid will gradually turn blue. Before the optical density is measured, the microplate reader is warmed up for approximately 15 minutes. 50 μL of Stop Reagent is added to each well. Once it is added, the color will go from blue to yellow. The edge of the plate is lightly tapped to mix the contents thoroughly.

3.9.3 Determination of Follicle-stimulating hormone concentration

3.9.3.1 Test principle

This assay kit uses a sandwich ELISA method. The microtiter plate we received has wells pre-coated with an FSH-specific antibody. The standard preparations or sample solutions are added to the wells, a biotinylated anti-FSH antibody is added, and the plate is incubated. Then, avidin-HRP conjugate is added to each well and allowed to bind. After TMB substrate is added, the color is visible only in wells where the full immune complex has formed, containing FSH, a biotinylated antibody, and avidin-HRP. The enzyme reaction is then stopped with sulfuric acid, and the absorbance is measured at $450\text{ nm} \pm 10\text{ nm}$ using a spectrophotometer. The FSH levels in the samples are then calculated by comparing their absorbance to that of the standard solutions.

3.9.3.2 Assay procedure

1. Separate wells are used for the blank, the standards, and test samples. One blank well and seven wells for the serially diluted standards are set aside. 100 μL of Standard Working Solution is dispensed into each standard well, and 100 μL of each sample is dispensed into sample wells. The micro-ELISA plate is covered and incubated at 37°C for 80 minutes. When the samples are loaded, the inner

surface of the wells should not be touched by the pipette tip, and the liquid is dispensed gently to minimize foam formation.

2. The contents of all wells are completely removed. Following aspiration, 200 μL of $1\times$ Wash Solution is dispensed into each well and allowed to stand for 1–2 minutes. The wash buffer is discarded, then the plate is inverted and tapped firmly on absorbent paper to eliminate any residual liquid. The washing step is repeated until three complete wash cycles have been carried out. To avoid contamination, it is ensured that the pipette tip does not contact the well walls when the wash solution is dispensed.

- The wash buffer is added carefully and directly into each well to avoid spillover or carryover into adjacent wells.

3. One hundred μL of Biotinylated Antibody Working Solution is added to each well. The plate is sealed with the cover and incubated for 50 minutes at 37°C .

4. The aspiration and washing procedure described earlier is repeated until 3 wash cycles have been completed.

5. One hundred μL of Streptavidin-HRP Working Solution is added to all wells, the plate is resealed, and incubated again at 37°C for 50 minutes.

6. The aspiration and washing procedure is performed again, but this time with five washes.

7. Ninety μL TMB Substrate Solution is added to each well, and the plate is sealed with a new sealer. If the plate is incubated at 37°C in the dark for 20 minutes, the total reaction time should not exceed 30 minutes. After the substrate is added to the wells, the surfaces will turn blue. If optical density is measured with the microplate reader, allow it to warm up for about 15 minutes.

8. Fifty μL of Stop Reagent is added to every well. After adding it, the solution will change to yellow. The plate is tapped to mix all the contents. If the color is

uneven, then it is lightly tapped again to achieve uniform mixing. The Stop Reagent should be added in the same order as the TMB Substrate Solution.

9. It is ensured that there are no air bubbles on the liquid surface, and the underside of the plate is wiped to remove any moisture or fingerprints. Then the plate is placed into the microplate reader, and the absorbance is immediately recorded at 450 nm.

3.10 Determination of antioxidant enzymes

3.10.1 Determination of glutathione peroxidase (GPX) concentrations

3.10.1.1 Test principle

This assay employs a sandwich ELISA technique. The wells of the supplied micro-ELISA plate are pre-coated with an antibody specific for rat GPX1. When standards or sample solutions are loaded into the wells, the GPX1 antigen binds to the immobilized capture antibody. A biotin-labeled detection antibody against rat GPX1 is then added, followed by an avidin-HRP conjugate, with incubation steps allowing the binding reactions to occur.

Following incubation, the wells are rinsed to eliminate any unbound substances. A substrate solution is then added to each well, leading to the appearance of a blue color only in wells where the complete immune complex composed of rat GPX1, the biotinylated detection antibody, and avidin-HRP has been formed. The reaction is subsequently terminated by adding the stop solution, which changes the color from blue to yellow. The optical density (OD) is then determined at 450 nm using a spectrophotometer.

3.10.1.2 Assay procedure

1. Separate wells are set for the blank, standards, and diluted samples. 100 μL of the suitable standard solution, blank, or diluted sample is added to it at 37°C for 90 minutes.
2. Once incubation is complete, the liquid is removed from the wells, and the plate is gently blotted dry with clean absorbent paper.
3. Each well is filled with 300 μL of wash buffer and is left to sit for 30 seconds. Then, the liquid is aspirated or poured off, and the plate is patted dry with fresh absorbent paper. This is done three times.
4. One hundred μL of Biotinylated Detection Antibody working solution is added to each well. The plate is covered again with a fresh sealer and incubated at 37°C for 1 hour.
5. The contents of the wells are removed, and the same washing procedure described above is repeated.
5. One hundred μL of HRP Conjugate working solution is dispensed into all wells. The plate is sealed with a new cover and incubated at 37°C for 30 minutes.
6. All wells are emptied, then the wash step outlined in Step 3 is repeated, completing a total of 3 washes.
7. One hundred μL of substrate reagent is added to each well. The plate is covered with a fresh sealer and incubated at 37°C for about 15 minutes in the dark.
8. The reaction is terminated by adding 50 μL of Stop Solution to each well.
10. The optical density (OD) of all wells is read at 450 nm using a microplate reader.

3.10.2 Determination of glutathione reductase (GR) concentration**3.10.2.1 Test principle**

This assay kit uses a sandwich ELISA format. The wells of the supplied micro-ELISA plate are pre-coated with an antibody that specifically binds rat GR. After standards or sample solutions are introduced into the wells, any rat GR present attaches to the immobilized capture antibody. A biotin-labeled detection antibody specific for rat GR is then added, followed by an avidin-HRP conjugate, and the plate is incubated to allow the binding reactions to proceed.

After incubation, the wells are washed to eliminate any unbound materials. A substrate solution is subsequently introduced into each well, resulting in the development of a blue color only in wells where the complete immune complex—comprising rat GR, the biotinylated detection antibody, and avidin-HRP—has been formed. The reaction is then terminated with the stop solution, which causes the color to change from blue to yellow. The optical density (OD) is measured at 450 nm using a spectrophotometer. Because the OD is directly proportional to the concentration of rat GR, the amount present in the samples is determined by comparing the sample absorbance readings with the standard curve.

3.10.2.2 Assay procedure

1. Wells are allocated for samples, blanks, and diluted standards, and 100 μL of each standard, blank solution, and sample dilution is poured into the appropriate wells.
2. The plate is sealed with the cover provided in the kit and incubated at 37°C for 90 minutes. Once incubation is complete, the contents of each well are discarded, and the plate is blotted dry with clean absorbent paper.
3. Three hundred μL of wash buffer is added to each well and allowed to remain for 30 seconds. Then the liquid is aspirated or poured off, and the plate is dried by

gently blotting it with clean absorbent paper. This wash step is repeated three times.

4. One hundred μL of Biotinylated Detection Antibody working solution is dispensed into all wells. The plate is covered with a fresh sealer and incubated at 37°C for 1 hour.

5. The liquid is removed from the wells, and the same washing procedure is repeated.

6. One hundred μL of HRP Conjugate working solution is added to each well. The plate is sealed again with a new cover and incubated at 37°C for 30 minutes.

7. The contents of all wells are discarded, and the previous washing step is carried out again until three complete washes have been performed.

8. One hundred μL of Substrate Reagent is pipetted into each well. The plate is covered with a fresh sealer and incubated at 37°C for approximately 15 minutes, keeping it protected from light.

9. The reaction is stopped by adding 50 μL of Stop Solution to each well.

10. The optical density (OD) of all wells is determined simultaneously at 450 nm using a microplate reader.

3.10.3 Determination of selenoprotein P (SEPP 1) concentration

3.10.3.1 Test principle

This ELISA kit utilizes a sandwich ELISA principle. The supplied micro-ELISA plate is pre-coated with an antibody specific to rat SEPP1. When standards or sample solutions are introduced into the assigned wells, the target antigen binds to the immobilized antibody on the plate.

A biotin-labeled detection antibody specific to rat SEPP1 is added first, followed by an avidin-HRP conjugate, and the plate is then incubated. After

incubation, the wells are washed to remove any unbound substances. A substrate solution is subsequently added to each well.

A blue coloration appears only in wells where the full immune complex consisting of rat SEPP1, the biotinylated detection antibody, and avidin-HRP has been formed. When the stop solution is added, the enzymatic reaction is halted and the color changes from blue to yellow. The optical density (OD) is subsequently measured at 450 nm using a spectrophotometer. Since the OD value is directly proportional to the concentration of rat SEPP1, the concentration in the samples is determined by comparing the sample readings with the standard curve.

3.10.3.2 Assay procedure

1. Separate wells are assigned for the blank, standards, and diluted samples, then 100 μ L of the appropriate standard, blank solution, or sample dilution is dispensed into each designated well.
2. The plate is covered with the sealer provided in the kit and incubated at 37°C for 90 minutes. After incubation, the liquid is discarded from the wells and the plate is gently blotted dry using clean absorbent paper.
3. Three hundred μ L of wash buffer is pipetted into each well, and it is allowed to remain for 30 seconds. After that, the liquid is aspirated or poured, then the plate is patted dry with fresh absorbent paper. This washing step is repeated three times.
4. One hundred μ L of Biotinylated Detection Antibody working solution is added to all wells. The plate is covered with a new sealer and incubated for 1 hour at 37 °C.
5. The wells are emptied, and the same washing procedure is performed once more.
6. One hundred μ L of HRP Conjugate working solution is dispensed into every well. The plate is resealed and incubated at 37°C for 30 minutes.

7. The contents of all wells are discarded, and the washing procedure described in Step 3 is repeated until three complete wash cycles have been performed.
8. One hundred μL of substrate reagent is dispensed into each well, the plate is covered with a new sealer, and incubated at 37°C for about 15 minutes while keeping the plate protected from light throughout the reaction.
9. The reaction is stopped by adding 50 μL of Stop Solution to each well.
10. The optical density (OD) of all wells is read simultaneously at 450 nm using a microplate reader.

3.11 Sperm proteins

3.11.1 Determination of sperm protamine content (PRM) concentration

3.11.1.1 Test principle

This kit measures rat PRM1 through sandwich ELISA. The wells of the microtiter plate are pre-coated with an antibody that specifically targets rat PRM1. Standards or sample solutions are first added to the appropriate wells, followed by biotin-labeled antibody to rat PRM1, and the plate is incubated. Next, avidin-HRP is added to each well and binds to the biotin. TMB substrate solution is then added, so color develops only in wells where the full immune complex with rat PRM1, the biotinylated antibody, and avidin-HRP has formed. The enzymatic reaction is stopped by adding sulfuric acid solution, and the absorbance is read with a spectrophotometer at $450\text{ nm} \pm 10\text{ nm}$. The concentration of PRM1 in the samples is then determined by comparing the sample OD values with the standard curve.

3.11.1.2 Assay procedure

1. Separate wells are assigned for the blank, standards, and samples, including one blank well and seven wells for the standards. 100 μL of Standard Working Solution is added to each standard well, and 100 μL of each sample is added to

the designated sample wells. The plate is covered and incubated at 37°C for 80 minutes. While the solution is dispensed into the bottom of the micro-ELISA wells, touching the inner walls is avoided, and the foam is allowed to form to its maximum during dispensing.

2. The contents of all wells are completely removed. After aspiration, 200 µL of 1× Wash Solution is added to each well, and it is left to stand for 1–2 minutes. The wash solution is discarded, then the plate is inverted and tapped firmly on absorbent paper to remove any remaining liquid. This washing procedure is repeated three times. After the final wash, any residual wash buffer is eliminated by aspiration or decanting, and then the inverted plate is blotted on absorbent paper until dry.

Notes: To avoid contamination, the pipette tip is not left to touch the well wall when the wash solution is dispensed.

The washing solution is added carefully and directly into each well to prevent carryover into neighboring wells.

- A. One hundred µL of Biotinylated Antibody Working Solution is added to each well, the plate is covered and incubated at 37°C for 50 minutes.
 - B. The aspiration and washing procedure described in Step 2 is performed, and it is repeated 3 times.
 - C. One hundred µL of Streptavidin-HRP Working Solution is dispensed into every well, the plate is sealed, and incubated again at 37°C for 50 minutes.
3. The washing step described in Step 2 is performed again, and in this stage, 5 wash cycles are completed in total.
 4. Ninety µL of TMB Substrate Solution is added to each well, and the plate is covered with a new sealer. The plate is incubated at 37°C in the dark for 20 minutes, ensuring that the incubation period of 30 minutes is not exceeded. After the substrate is added, the solution in the wells will gradually turn blue. Before

the OD reading is taken, the microplate reader is allowed to warm up for about 15 minutes.

5. Fifty μL of Stop Reagent is added to each well. Upon addition, the color will change from blue to yellow. The plate is tapped on one side, and the contents are mixed up. If the color is uneven, the plate is hit again until the color is uniform. The Stop Reagent should be applied in the same order as the TMB Substrate Solution.
6. The liquid surface is checked to ensure it is free of air bubbles, and the underside of the plate is wiped to remove any water droplets or fingerprints. The plate is placed in the microplate reader, and the absorbance is immediately recorded at 450 nm.

3.11.2 Determination of acrosin (ACR) concentration

3.11.2.1 Test principle

This kit quantifies rat ACR using a sandwich ELISA technique. The supplied microtiter plate is pre-coated with an antibody that specifically captures rat ACR. After standard or sample solutions are loaded into the wells, a biotin-labeled antibody for rat ACR is added, followed by an avidin-HRP conjugate, which is incubated. Once the TMB substrate has been added, color develops only in wells where the full immune complex forms from rat ACR, the biotinylated antibody, and avidin-HRP. The reaction is then stopped with sulfuric acid, and the absorbance is measured at $450 \text{ nm} \pm 10 \text{ nm}$ with a spectrophotometer. The ACR concentration in the samples is determined by comparing their OD values with the standard curve.

3.11.3.2 Assay procedure

1. The appropriate wells for the blank, standards, and sample solutions are selected (one blank and seven wells for diluted standards). 100 μL of Standard Working Solution is added to each standard well, and 100 μL of each sample

is added to the sample wells. The plate is covered and incubated at 37°C for 80 minutes. When the plate is loaded, the solution is carefully dispensed to the bottom of each micro-ELISA well, and contact of the pipette tip with the inner wall is avoided, and foam is allowed to form if needed.

2. In each well, all the liquid is completely removed. After aspiration, 200 μL of 1 $\times$ Wash Solution is added to each well and incubated for 1–2 minutes. The wash solution is then dumped, and the plate is tapped on absorbent paper to remove any remaining liquid. This wash step is repeated 3 times. After the final wash, any residual buffer is removed by aspiration or decanting, and the inverted plate is blotted on absorbent paper until dry.

Notes:

- To reduce the possibility of contamination, the pipette tip is not allowed to come into contact with the well walls while the wash solution is added.
 - The wash buffer is dispensed very carefully and directly into each well to avoid overflow or carryover into neighboring wells.
3. One hundred μL of Biotinylated Antibody Working Solution is put into each well, the plate is covered, and incubated at 37°C for 50 minutes.
 4. Aspiration and washing are performed again as in Step 2, then three wash cycles are performed.
 5. One hundred μL of Streptavidin-HRP Working Solution is added to every well, the plate is sealed, and incubated again at 37°C for 50 minutes. The washing procedure outlined in Step 2 is performed again for 5 washes.
 6. Ninety μL of TMB Substrate Solution is added to each well, the plate is sealed with a fresh sealer, and the plate is incubated at 37°C in the dark for 20 minutes. It is ensured that the reaction time of 30 minutes is not exceeded. After adding the substrate, the solution turns blue. Before the optical density (OD) is read, the microplate reader should be warmed for approximately 15 minutes.

7. Fifty μL of Stop Reagent is added to every well. Once the solution is added, it will turn yellow. The plate is tapped to ensure the contents are well mixed. If the color is not uniform across the plate, it is tapped once more to ensure everything is mixed. The Stop Reagent should be applied in the same order as the TMB Substrate Solution.
8. It is checked that no air bubbles are present on the liquid surface, and any moisture or fingerprints are removed from the underside of the plate. The plate is then placed into the microplate reader, and the absorbance is recorded at 450 nm.

3.12 Histological exam

3.12.1 Tissue collection and fixation

Tissue samples were collected and immersed in 10% neutral buffered formalin for 48 hours, with the fixative reapplied after the first 24 hours. The specimens were cut into sections approximately 0.5 cm thick and transferred to plastic cassettes for dehydration in an automated tissue processor (Histo-Line ATP700, Italy). The remaining histological procedures were then undertaken using the method of Johnsen (1970), as described below.

3.12.1.1 Dehydration

Preparation: The tissue samples must be properly fixed before the dehydration process is started.

Ethanol Series: The tissue is gradually dehydrated using a series of ethanol solutions with increasing concentrations:

1. Fifty percent ethanol: The tissue is immersed in 50% ethanol for 1 hour.
2. Seventy percent ethanol: The tissue is transferred to 70% ethanol for 1 hour.

3. Ninety-five percent ethanol: The tissue is transferred to 95% ethanol for 1 hour. This step is repeated twice.
4. One hundred percent Ethanol: Finally, the tissue is immersed in 100% ethanol for 1 hour. This step is repeated twice.

3.12.1.2 Clearing

1. Preparation: The tissue must be fully dehydrated before starting the clearing process.
2. Xylene: Xylene is used to clear the tissue:
 - A. First Xylene Bath: The tissue is immersed in xylene for 1 hour.
 - B. Second Xylene Bath: The tissue is transferred to a fresh xylene bath for 1 more hour.

3.12.1.3 Embedding.

1. Preparation: The tissue should be fully cleared before the embedding process is started.
2. Paraffin Infiltration: The tissue is infiltrated with paraffin wax:
 - A. First Paraffin Bath: The tissue is immersed in melted paraffin wax for 1 hour.
 - B. Second Paraffin Bath: The tissue is transferred to a fresh paraffin wax bath for 1 more hour.
3. Embedding: The tissue is carefully positioned inside a metal base mold filled with paraffin wax. The wax is allowed to solidify at room temperature, providing enough rigidity to cut very thin sections.

3.12.2 Sectioning

- 1 .A semiautomatic microtome (Histo-Line MRS3500, Italy) was used to trim and segment the material at a thickness of 4 μm .
2. The sample was put in a water bath (FALC BI, Italy), and a hot plate (K&K HYSH11, Korea) was used to mount it on glass slides.

3.12.3 Hematoxylin & Eosin staining procedure**A. Deparaffinization and Rehydration:**

- 1 .Deparaffinized using two xylene changes, each lasting two minutes.
2. Rehydrated for two minutes at a time using three different ethanol dilutions (100%, 90%, and 70%).

B. Hematoxylin & Eosin(H&E) staining.

- 1 .Stained with Harris's hematoxylin for five to eight minutes after being rinsed with tap water.
- 2 .Spent two minutes washing in tap water.
- 3 .To get rid of too much hematoxylin stain, differentiate in a 1% acid alcohol solution for 20 seconds.
4. Spent a minute washing in tap water.
- 1 .Submerge in a 0.2% ammonia water solution for one minute to blend the hematoxylin stain.
- 2 .Washed for five minutes under running water .
- 3 .Rinsed for 20 seconds with 95% alcohol.
4. Eosin stains were counterstained for five minutes.

3.12.4 Dehydration and Clearing:

1-The tissue sections are dehydrated by immersing them in a series of ethanol solutions with increasing concentrations:

- Seventy percent ethanol: immerse the slides for 2 minutes.
- Ninety percent ethanol: immerse the slides for 2 minutes.
- One hundred percent ethanol: immerse the slides for 2 minutes.

2-The tissue sections are cleared by immersing the slides in two changes of xylene for 2 minutes each.

3.12.5 Mounting:

Using a coverslip and mounting medium, adhere the tissue sections to the coverslip.

1 .Dehydrated for two minutes each using three different ethanol dilutions (70%, 90%, and 100%).

2 .Cleared by two two-minute xylene changes.

3. Examined and observed at 40x, 100x, 200x, 400x, and 1000x magnifications under a light microscope.

3.13 Statistical analysis

One-way ANOVA was used for data analysis. Duncan's Multiple Range Test (DMRT) was used to compare means using statistical software (Cody, 2018; Shoukri,2018).

Chapter four

Results

4.1 Green synthesis and characterization of SeNPs

4.1.1 Visual observation (color changes)

In this study, selenium nanoparticles (SeNps) were synthesized using ascorbic acid as a reducing agent and starch as a stabilizing agent. Color changes were used to determine the properties of the selenium nanoparticles. Over time, the solution changed from colorless to pale yellow, then to dark brown after 4.5 hours on the magnetic stirrer (Figure 4.1).



Figure 4-1: Color change of the solution of SeNPs

4.1.2 Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FTIR) was used to identify different phytochemical constituents and to assess the reduction and stabilization of the nanoparticles during the reaction of sodium selenite at acidic pH (6.8). A spectrum image of FT-IR for SeNPs (Figure 4.2). Fourier transform infrared (FTIR) spectrum of dried powdered SeNPs was obtained over the 400- 4000 cm^{-1} range. The FT-IR spectra showed various peaks and bands at (3434.65, 2924.71, 2854.05, 1732.37, 1631.79, 1109.19, 539.01, and 468.02 cm^{-1}). The FT-IR results provide strong confirmation that the nanoparticles were successfully synthesized. The peak at 3434 cm^{-1} is typical of O–H stretching, indicating that biomolecules are acting as reducing and capping agents. The peak at 1732 cm^{-1} refers to C=O stretching, and the peak at 1631 cm^{-1} refers to C=C. These all point to organic functional groups stabilizing the nanoparticles. However, the real giveaway is the

low-frequency bands at 539 and 468 cm^{-1} , which belong to Se–O or Se–Se vibrations. These strongly indicate the presence of SeNPs in the sample.

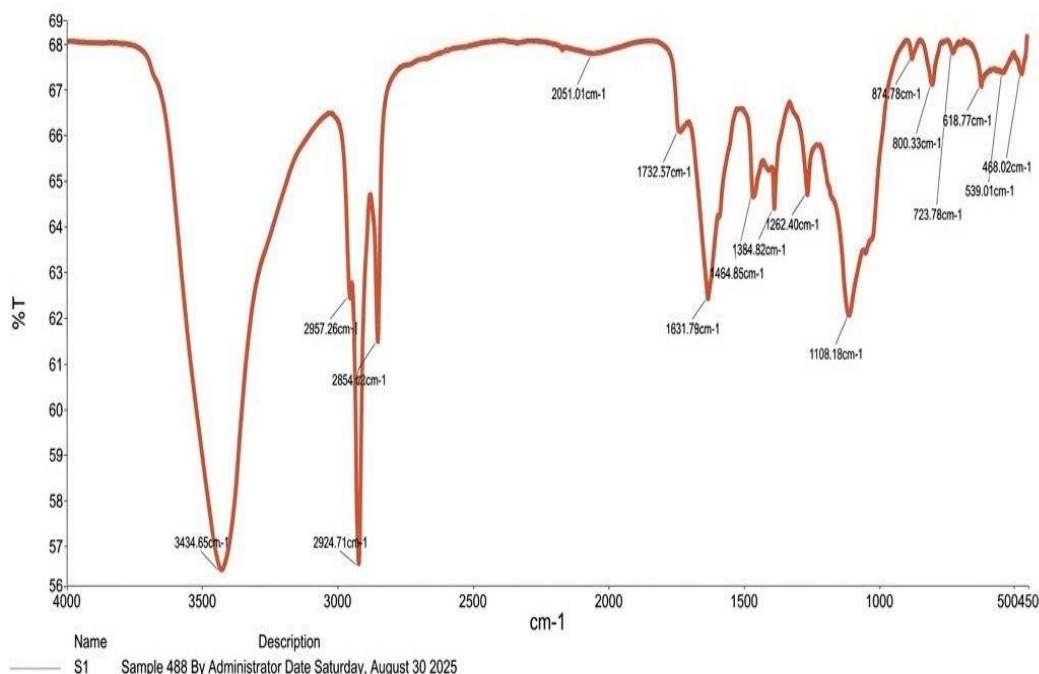


Figure (4-2): FTIR Peaks analysis

4.1.3 Zeta potential

The Zeta potential value refers to the charge that is present on the surface of the particles. When it is higher than ± 30 , this indicates that the synthesized particles will be repellent, highly stable, and will not aggregate in suspension. In the current study, the zeta potential is -58.3 mV (Figure 4-3), indicating that the synthesized SeNPs are highly stable in suspension for a long time.

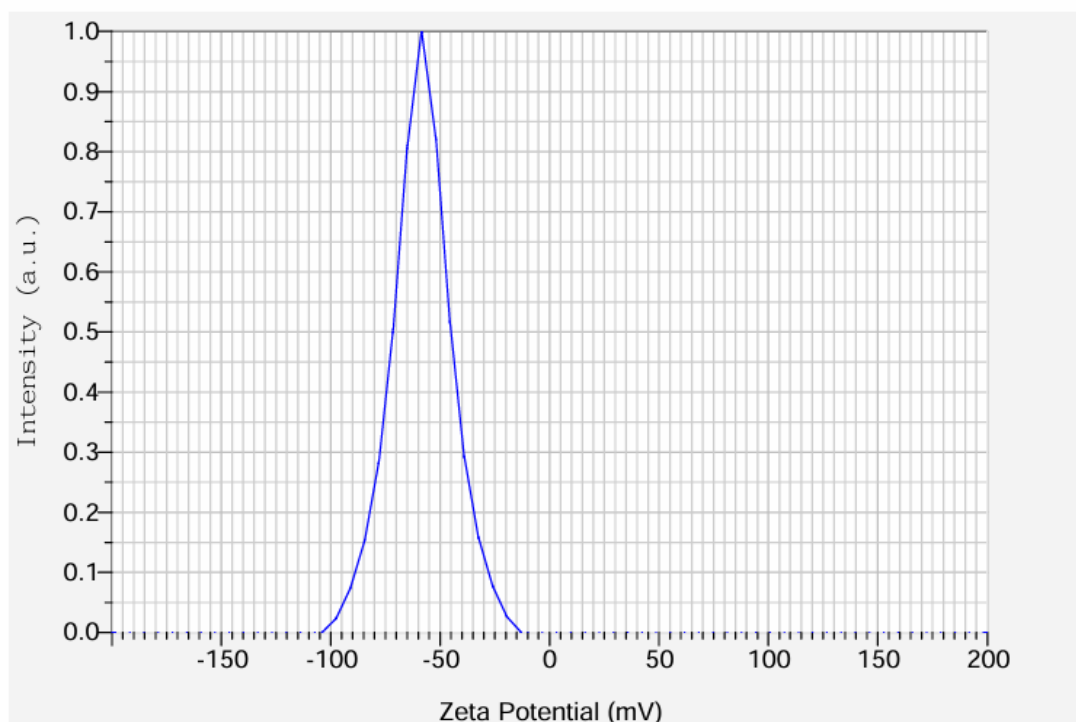


Figure (4-3): Zeta potential (Mv) diagram value

4.1.4 X-RAY diffraction

The effective synthesis of nano-selenium with good crystallinity and phase stability, matching the typical characteristics of elemental selenium, is confirmed by XRD. The crystalline structure and phase purity of the synthesized Selenium Nanoparticles (Se-NPs) were characterized using X-ray diffraction (XRD). The sample's diffraction pattern is shown in Figure 4-4. The XRD pattern exhibits sharp, well-defined peaks, confirming the crystalline nature of the synthesized nanoselenium.

The most intense diffraction peak is observed at $2\theta = 29.4^\circ$, corresponding to the (101) reflection plane. This is the characteristic mark of the Trigonal Selenium phase (t-Se). The absence of any other peaks related to selenium oxides and other impurities indicates that the prepared selenium nanoparticles have high chemical purity. The widening of the diffraction peaks clearly shows the particles' nanoscale size.

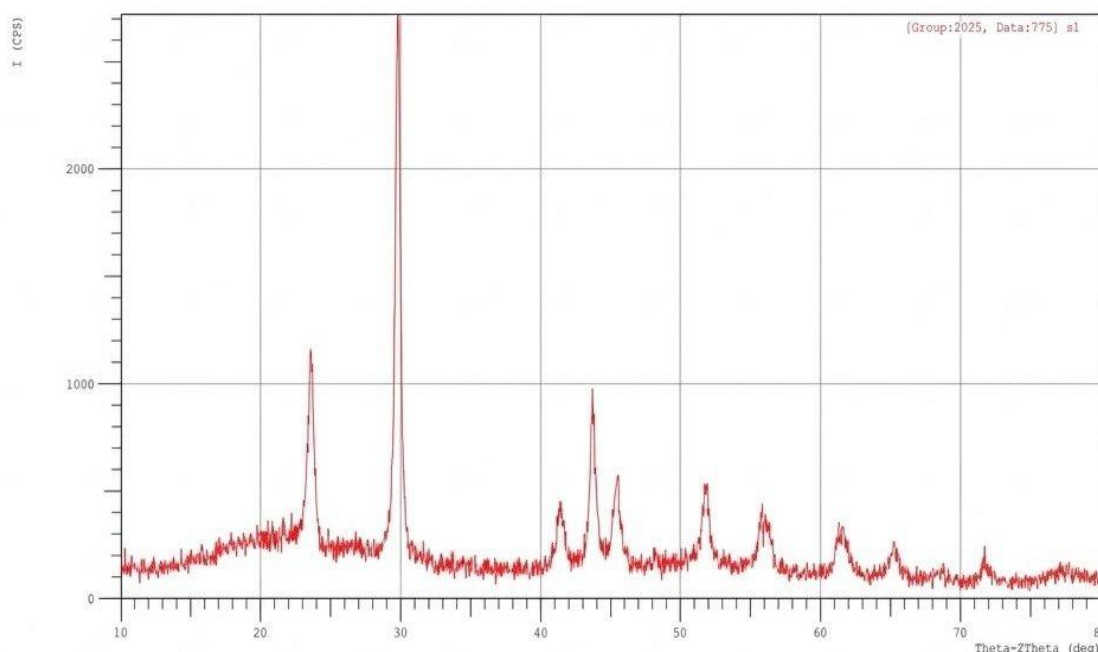


Figure (4-4): XRD analysis peaks

4.1.5 Field Emission Scanning Electron Microscopy (FESEM)

The Scanning electron microscopy is used to determine the shape and size of green-synthesized selenium nanoparticles. The images in this study showed that the particles formed an oval or semicircular shape and had diameters of 27-40 nanometers (27.69, 30.72, 31.66, 31.94, 38.85, 39.23), as shown in Figures 4-5, indicating successful nanoparticle formation (Srivastava, 2015). The FESEM results in the current study confirm the successful green synthesis of SeNPs using starch as a stabilizing agent and ascorbic acid as a reducing agent. The morphology and stability of the manufactured particles agree with those reported by Kazemi (2021).

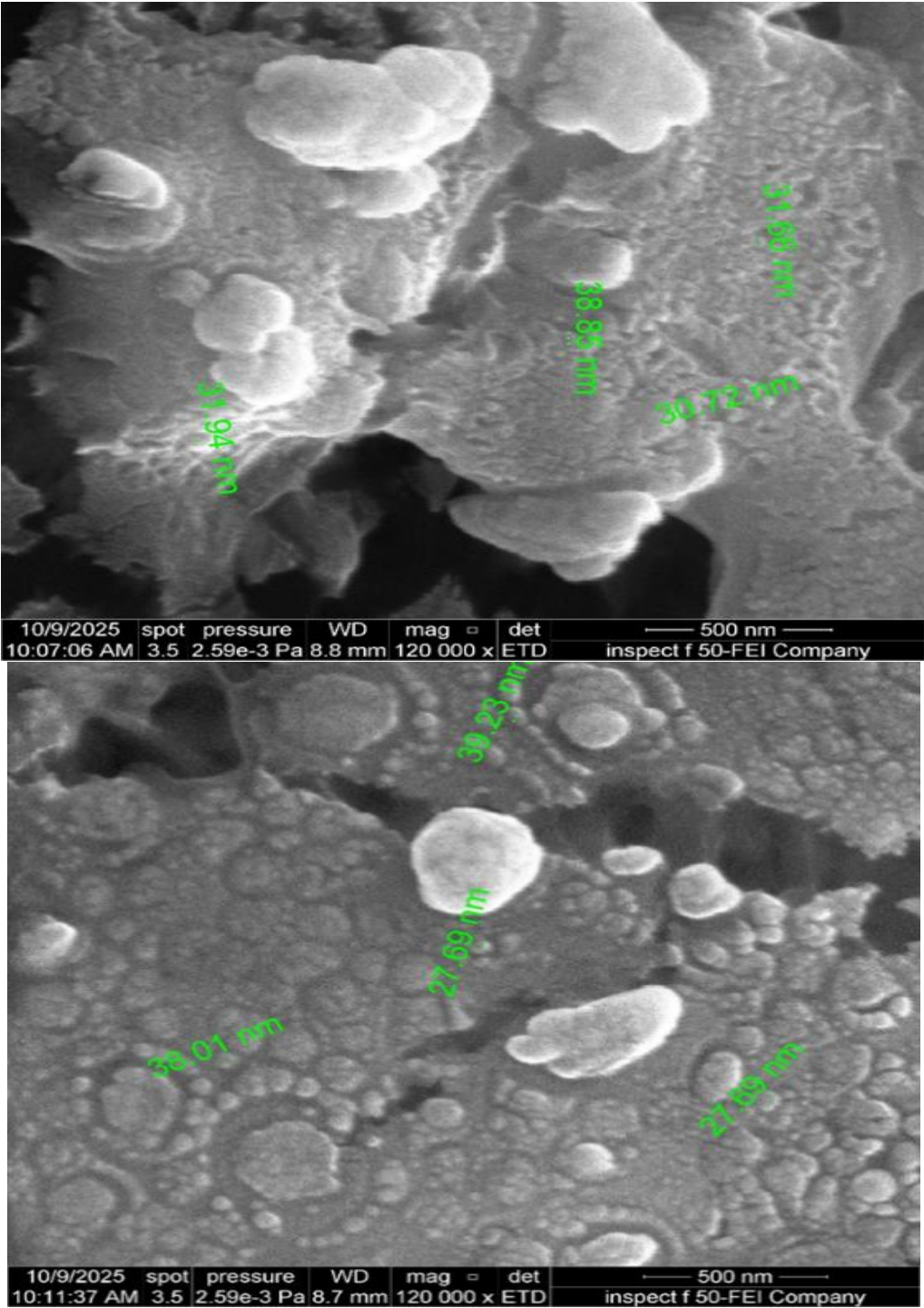


Figure (4-5): FESEM of synthesized SeNPs size

4.2 Hormonal assays

Table 4-1 Effect of SeNPs administration on hormone levels in experimental rats.

Groups	Testosterone (ng/ml) N=8 (Mean±SE)	LH (mIU/ml) N=8 (Mean±SE)	FSH (mIU/ml) N=8 (Mean±SE)
G1	14.059 ± 1.164 A	2.361 ± 0.392 DC	13.791 ± 1.022 C
G2	9.489 ± 0.224 C	6.055 ± 0.848 A	23.909 ± 1.232 A
G3	10.886 ± 0.224 BC	4.680 ± 0.542 AB	18.363 ± 1.297 B
G4	12.540 ± 0.531 AB	3.666 ± 0.423 BC	13.305 ± 0.733 C
G5	14.638 ± 1.011 A	2.010 ± 0.229 D	11.563 ± 1.161 C
Sign. Level	**($P \leq 0.01$)	**($P \leq 0.01$)	**($P \leq 0.01$)

In the same column, Means with different superscript letters differ significantly ($P \leq 0.01$) **

Table 4-1 presents the concentrations of the studied hormones across all groups, revealing a significant ($P \leq 0.01$) decrease in Testosterone concentration in group G2 compared with the G1, G4, and G5 groups. At the same time, the G3 group did not show any significant ($P > 0.01$) changes compared to the G2 and G4 groups. Also, the G4 group showed non-significant ($P > 0.01$) differences with the control, G3, and G5 groups.

Luteinizing hormone concentration increased significantly ($P \leq 0.01$) in groups G2 and G3 compared with the control and G5 groups; in the G4 group, LH concentration showed a non-significant ($P > 0.01$) difference compared with the control and G3 groups. The G5 group showed a non-significant change compared with the control group ($P > 0.01$) and a significant decrease compared with the G2, G3, and G4 groups ($P < 0.01$).

The FSH level in the current study showed a significant ($P \leq 0.01$) increase in the G2 and G3 groups compared to the G1, G4, and G5 groups. At the same time, the level of FSH in the G2 group was significantly higher ($P \leq 0.01$) than in

the G3 group. The concentration of the studied hormone showed no significant ($P>0.01$) differences among the G1, G4, and G5 groups, as shown in Table 1.

4.3 Antioxidant assays

Table 4- 2 Effect of administered SeNPs on antioxidant levels in experimental rats.

Groups	Glutathione reductase (pg/ml) N=5 (Mean±SE)	Glutathione peroxidase(pg/ml) N=5 (Mean±SE)	Selenoprotein P (ng/ml) N=5 (Mean±SE)
G1	346.440 ± 11.351 A	1670.00 ± 86.023 A	1.600 ± 0.109 BC
G2	268.460 ± 33.471 B	1650.00 ± 128.452 AB	1.120 ± 0.245 C
G3	184.480 ± 8.057 C	1444.00 ± 83.88 AB	2.440 ± 0.519 AB
G4	205.500 ± 26.034 BC	1490.00 ± 85.732 AB	0.940 ± 0.188 C
G5	228.660 ± 19.027 BC	1340.00 ± 107.703 B	2.920 ± 0.389 A
Sign. Level	**($P\leq 0.01$)	*($P\leq 0.05$)	**($P\leq 0.01$)

In the same column, Means with different superscript letters differ significantly ($P\leq 0.01$) **, ($P\leq 0.05$) *

Table 4-2 shows the effects of SeNPs on the antioxidant enzymes. At a significant level ($P\leq 0.01$), the concentration of Glutathione reductase decreased significantly ($P\leq 0.01$) in all groups in comparison with the G1 group. The G3 group showed a significant ($P\leq 0.01$) decrease compared with the G2 group. Also, the G4 and G5 groups did not show significant differences ($P>0.01$) compared with the G2 and G3 groups. The Glutathione peroxidase level study revealed a significant ($P\leq 0.05$) decrease in the G5 group when compared with the G1 group. However, the G2, G3, and G4 groups showed no significant differences ($P>0.05$) compared with the G1 and G5 groups. In addition to the above parameters, the

concentration of the selenoprotein studied showed a significant ($P \leq 0.01$) increase in the G5 group compared with other groups, except the G3 group, which showed non-significant ($P > 0.01$) changes compared with the G1 and G5 groups. The G2 and G4 groups decreased significantly ($P \leq 0.01$) compared with the G3 and G5 groups, but not significantly ($P > 0.01$) compared with the G1 group.

4.4 Sperm protein assays

Table 4-3 Effect of administered SeNPs on sperm protein levels in experimental rats.

Groups	Acrosin (ng/ml) N=5 (M±SE)	Protamine (ng/ml) N=5 (M±SE)
G1	0.780 ± 0.058 C	6.920 ± 1.084 A
G2	1.360 ± 0.172 C	4.280 ± 0.265 B
G3	0.680 ± 0.115 C	3.160 ± 0.163 BC
G4	4.460 ± 0.911 B	1.660 ± 0.215 C
G5	6.940 ± 0.855 A	1.760 ± 0.307 C
Sign. Level	**($P \leq 0.01$)	**($P \leq 0.01$)

In the same column, means with different superscript letters differ significantly ($P \leq 0.01$) **

Table 4-3 illustrates the effects of selenium nanoparticle doses on serum acrosin and protamine levels in the different groups compared with the G1 group. It shows a significant increase ($P \leq 0.01$) in acrosin levels in the G4 and G5 groups compared to the G1, G2, and G3 groups. No significant difference ($P > 0.01$) was observed among the G1, G2, and G5 groups. Finally, G4 showed a significant ($P > 0.01$) increase compared with G4. As for protamine, it showed a significant decrease ($P \leq 0.01$) in the G2, G3, G4, and G5 groups compared to the G1 group,

as well as a significant decrease ($P \leq 0.01$) in the G4 and G5 microgram groups compared to the G2 group. The G3 group showed non-significant changes compared with the G2, G4, and G4 groups ($P < 0.01$).

4.5 Male Reproductive Parameters Assays:

Table 4-4 Effect of administered SeNPs on Male reproductive traits in experimental rats.

Groups	Sperm count $\times 10^6$ N=8 Mean $\pm$ SE	Live/dead ratio (%) N=8 Mean $\pm$ SE	Testicle weight/(g) N=8 Mean $\pm$ SE
G1	1.83 $\pm$ 0.217 C	92.750 $\pm$ 1.359 B	1.316 $\pm$ 0.021 C
G2	2.72 $\pm$ 0.086 C	97.750 $\pm$ 1.221 A	1.673 $\pm$ 0.048 B
G3	3.62 $\pm$ 0.151 C	98.750 $\pm$ 0.839 A	1.833 $\pm$ 0.035 A
G4	6.19 $\pm$ 0.933 B	97.500 $\pm$ 1.636 A	1.812 $\pm$ 0.037 A
G5	13.38 $\pm$ 1.665 A	99.750 $\pm$ 0.250 A	1.627 $\pm$ 0.042 B
Sign. Level	**($P \leq 0.01$)	**($P \leq 0.01$)	**($P \leq 0.01$)

In the same column, Means with different superscript letters differ significantly ($P \leq 0.01$) **

Table 4-4 shows a clear and significant ($P \leq 0.01$) increase in the G4 and G5 groups as compared to the other groups with respect to sperm count in the semen, and no significant ($P > 0.01$) difference between the G2 and G3 groups as compared to the G1 group. There is a significant ($P \leq 0.01$) increase in the live/dead ratio in all treated groups as compared with the G1 group. There was a significant ($P \leq 0.01$) increase in testicle weight in all treated groups as compared with the G1 group, and there was a significant ($P \leq 0.01$) increase in G3 and G4 groups compared with G2 and G5 groups. No significant ($P > 0.01$) difference

between the G2 and G5 groups, and also no significant difference ($P > 0.01$) between the G3 and G4 groups.

4.6 Histological study of the testis

The G1 group showed a normal histological architecture of the testicular tissue. About 50% of the examined tissue showed immature seminiferous tubules, characterized by an incomplete spermatogenic layer with a narrow lumen. In contrast, the mature seminiferous tubules exhibit an organized germinal layer, with stages of spermatogenesis; however, the tubular lumen contains a low concentration of spermatozoa. Leydig cells are evident in the interstitial tissue between seminiferous tubules, with eosinophilic cytoplasm and round, centrally located nuclei.

The G2 group showed a normal histological architecture of the testicular tissue. However, approximately 50% of the examined tissue area shows immature seminiferous tubules, characterized by incomplete spermatogenic stratification and relatively narrow lumina. In contrast, the mature seminiferous tubules exhibit an organized germinal epithelium with identifiable stages of spermatogenesis; however, the tubular lumen contains a low density of spermatozoa.

The G3 group showed normal histological architecture of testicular tissue. The majority of seminiferous tubules appear mature, with an organized germinal layer and clearly identifiable stages of spermatogenesis, including spermatogonia along the basement membrane, primary spermatocytes, and round-to-elongated spermatids progressing to the tubular lumen. Despite complete spermatogenic layering, a relatively low sperm density is observed within the lumina. The thickness of the interstitial tissue is increased compared with the G1 and G2 groups, suggesting interstitial expansion consistent with an increased number of Leydig cells.

The G4 group showed normal histological architecture of the testicular tissue. All seminiferous tubules were mature, exhibiting a well-organized germinal layer with clearly identifiable stages of spermatogenesis, including spermatogonia along the basement membrane, primary spermatocytes, and round-to-elongated spermatids progressing toward the tubular lumen. The concentration of spermatozoa in the lumen is higher than in the G1, G2, and G3 groups. Additionally, the thickness of the interstitial tissue is greater than that of the G1 and G2 groups, indicating interstitial expansion consistent with an increased number of Leydig cells.

The G5 group showed that normal testicular histological tissue architecture is demonstrated. All seminiferous tubules are mature and active, showing a well-organized germinal layer with different stages of spermatogenesis, including spermatogonia on the basement membrane, primary spermatocytes, and round-to-elongated spermatids toward the tubule lumen. The lumina contain a higher density of spermatozoa than the G1, G2, and G3 groups. Interstitial tissue is also thicker than in the G1 and G2 groups, reflecting expansion associated with an increased number of Leydig cells.

4.6.1 Histological examination of the G1 group

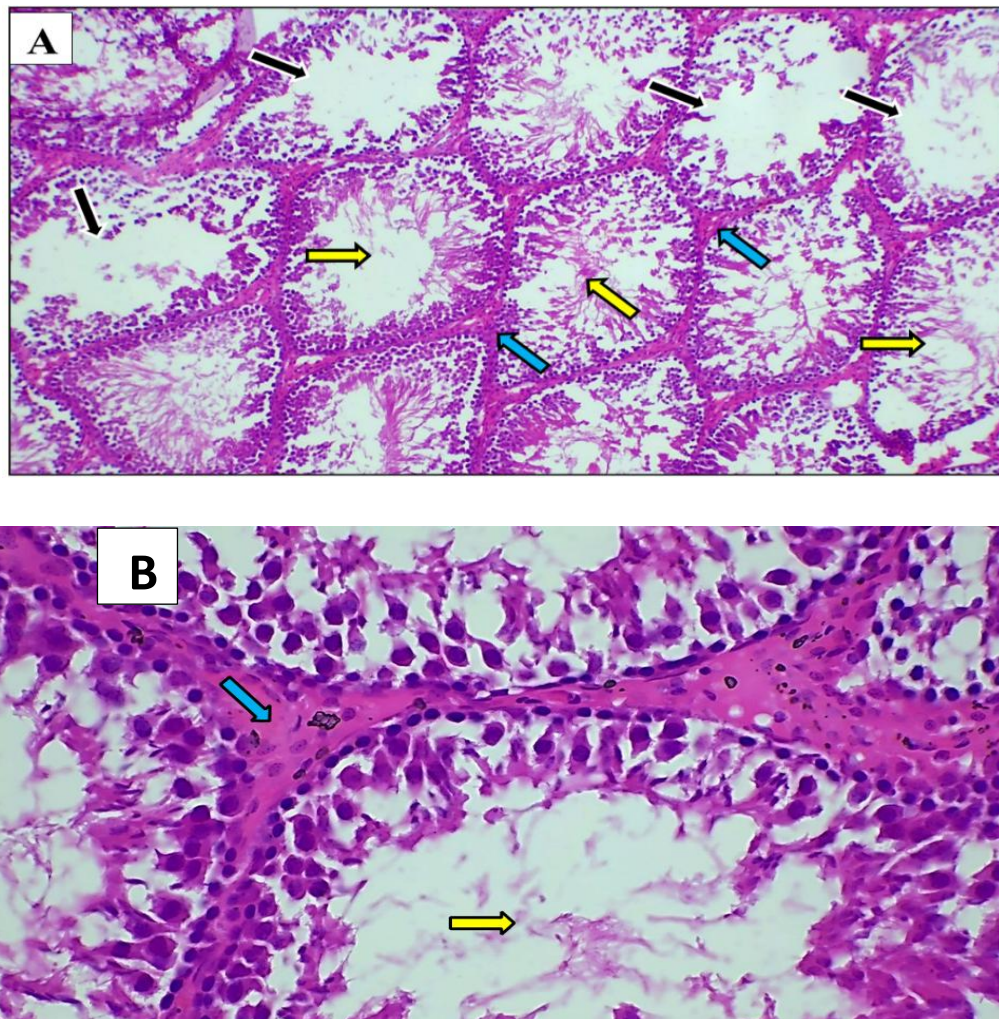


Figure 4-6: Photomicrograph of the testis of a G1 group rat. Received 0.2 mL of D.W. for 30 days of treatment. Seminiferous tubules (black arrows), spermatozoa (yellow arrows). Leydig cells (blue arrows). H&E. A: 10x and B: 40x.

The G1 group showed normal architecture, with about 50% mature seminiferous tubules and a low concentration of spermatozoa in the lumen.

4.6.2. Histological examination of G2 group

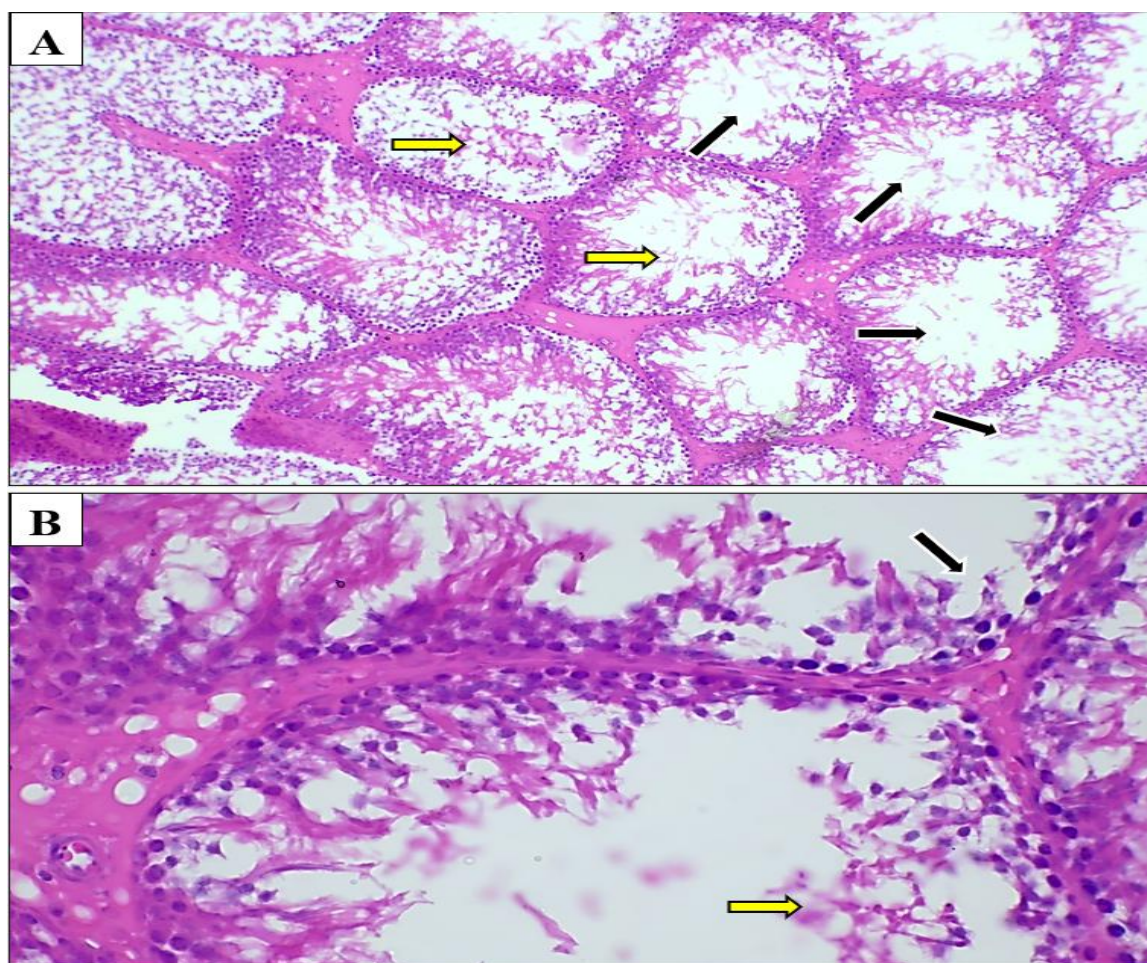


Figure 4-7: Photomicrograph of the testis of the G2 group that received orally 50 $\mu\text{g/kg}$ B.W of SeNPs for 30 days of treatment. Seminiferous tubules (black arrows), spermatozoa (yellow arrows). H&E. A: 10x and B: 40x.

The G2 group showed normal histological architecture, with approximately 50% of the examined tissue being mature seminiferous tubules, and there was a low concentration of spermatozoa.

4.6.3. Histological examination of the G3 group

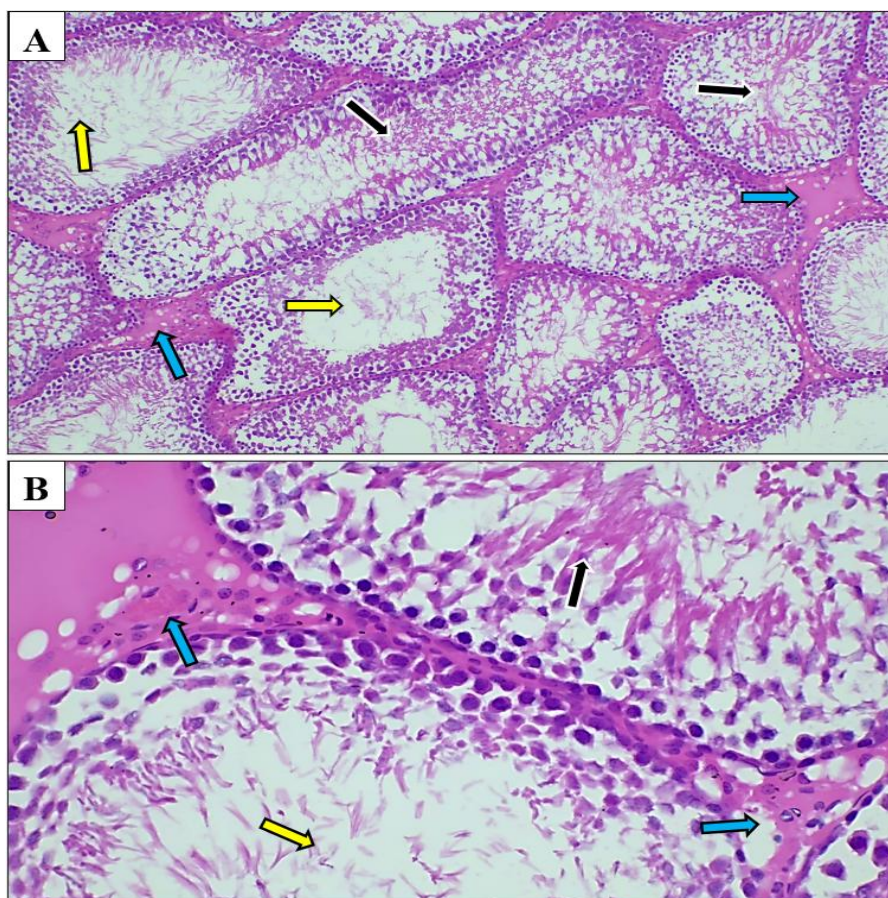


Figure 4-8 Photomicrograph of the testis of a G3 received orally 100 $\mu\text{g/kg}$ B.W of SeNPs for 30 days of treatment. Seminiferous tubules (black arrows), spermatozoa (yellow arrows), interstitial tissue (blue arrows).H&E. A: 10x and B: 40x.

The G3 group showed normal histological architecture of testicular tissue with a high percentage of mature seminiferous tubules and relatively low sperm density within the lumen.

4.6.4. Histological examination of G4 group

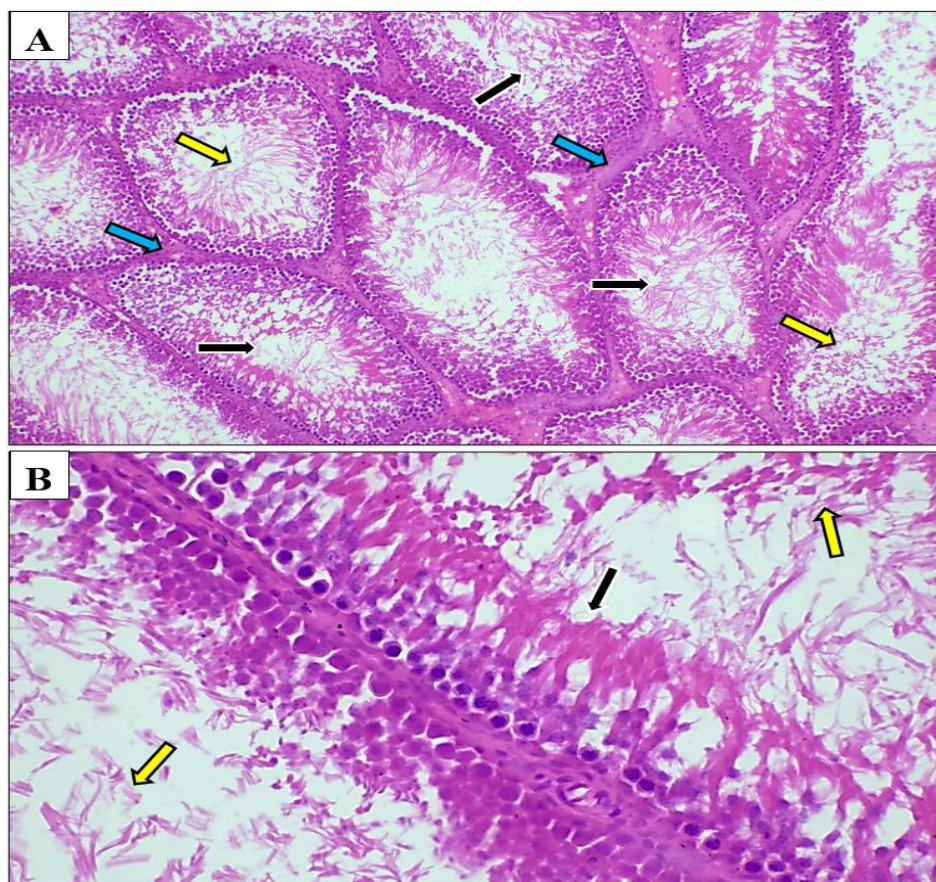


Figure 4-9 Photomicrograph of the testis of a G4 received orally 200 $\mu\text{g/kg}$ B.W of SeNPs for 30 days of treatment. Seminiferous tubules (black arrows), spermatozoa (yellow arrows), and interstitial tissue (blue arrows). H&E. A: 10x and B: 40x.

The G4 group showed normal histological architecture of testicular tissue, and all seminiferous tubules were mature; the concentration of spermatozoa was higher than in the G1, G2, and G3 groups.

4.6.5 Histological examination of G5 group

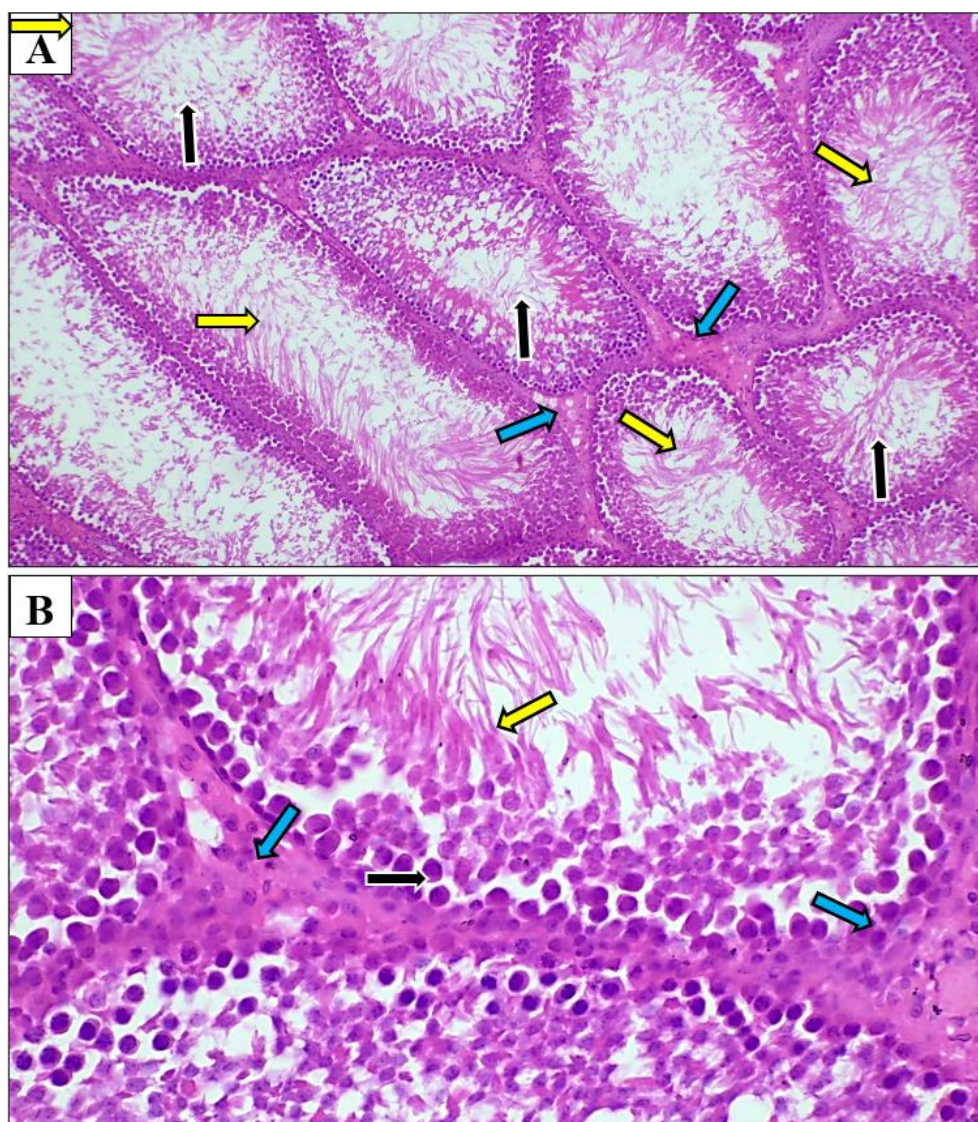


Figure 4-10 Photomicrograph of the testis of a G5 received orally 400 $\mu\text{g/kg}$ B.W of SeNPs orally for 30 days of treatment. Seminiferous tubules (black arrows), spermatozoa (yellow arrows), and interstitial tissue (blue arrows). H&E. A: 10x and B: 40x.

The G5 group showed normal architecture, and all seminiferous tubules were mature and active, with a high density of spermatozoa in the tubule lumen

The effect of SeNPs treatment on the maturation of seminiferous tubules

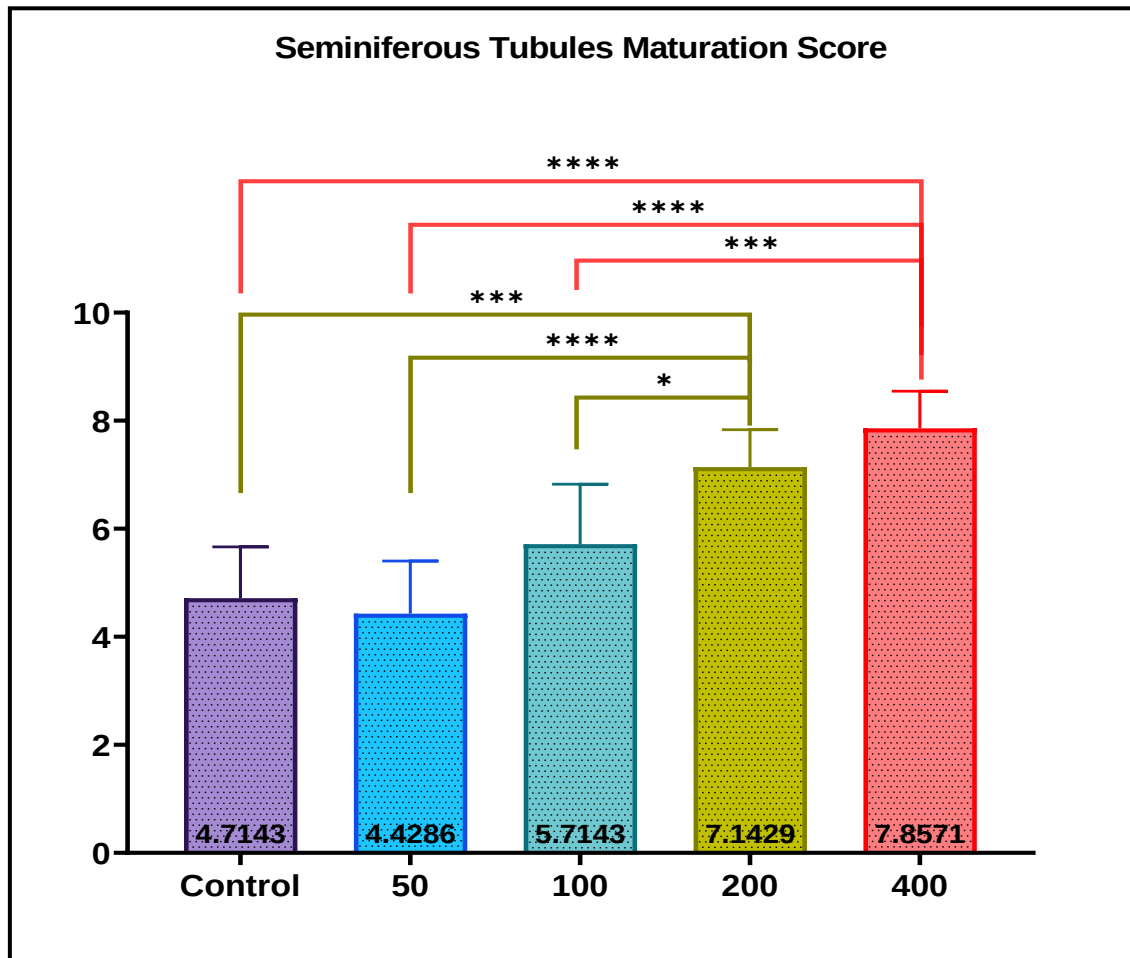


Figure 4-11: The effect of SeNPs treatment on the maturation of seminiferous tubules

Figure 4-11 shows a significant increase ($p < 0.0001$) in the rate of seminiferous tubule maturation in G5 when compared to the G1 and G2 groups. And a significant increase ($p < 0.001$) in G5 in comparison with the G3 group. And no significant difference between G5 and G4. Also, there is a significant increase ($p < 0.0001$) in G4 compared with the G2 group. Moreover, a significant increase ($p < 0.0001$) in G4 compared with the G1 group, and a significant increase ($p < 0.05$) in the G4 group compared with the G3 group.

The effect of SeNPs treatment on seminiferous tubules diameter (mm)

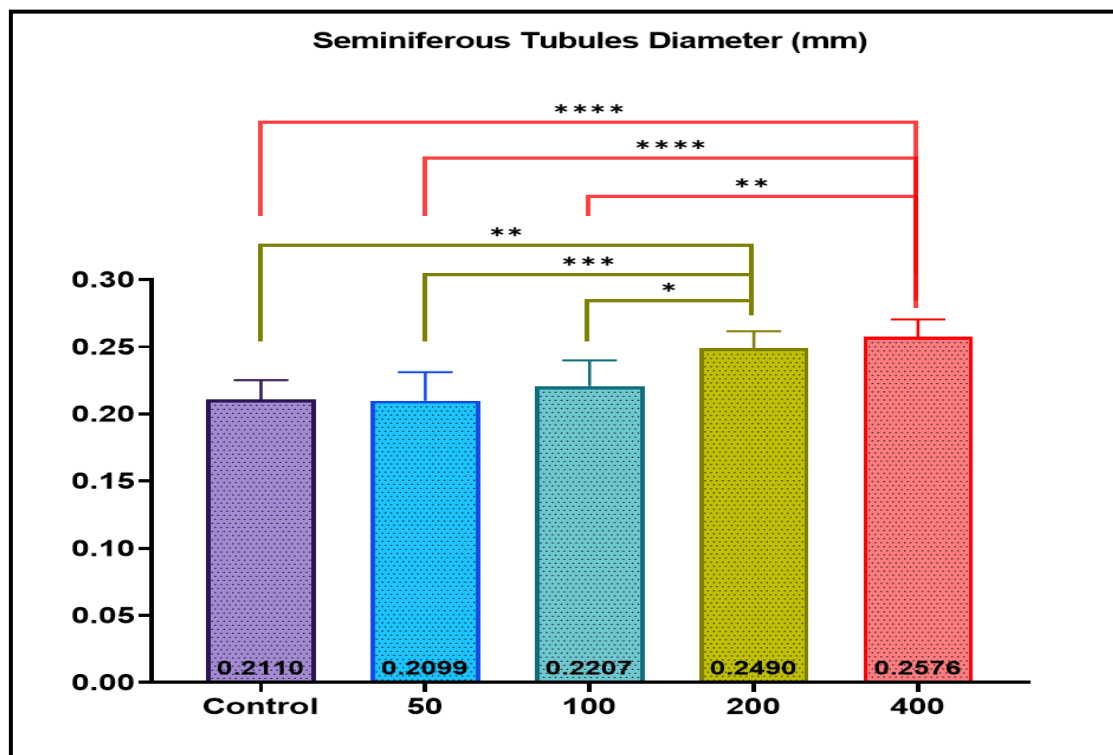


Figure 4-12: The effect of SeNPs treatment on seminiferous tubule diameter (mm)

Figure 4-12 shows a significant increase in seminiferous tubule diameter with increasing dose across the different groups compared to the G1 group. There is a significant increase ($p < 0.0001$) in G5 compared with the G1 and G2 groups. and a significant increase ($p < 0.01$) in G5 compared with G3 group. There is no significant difference between the G5 and G4 groups. Also, there is a significant increase ($p < 0.001$) in G4 compared with the G2 group. Moreover, a significant increase ($p < 0.01$) in G4 compared with the G1 group. and a significant increase ($p < 0.05$) in G4 compared with G3 group.

Effect of SeNPs treatment on germinal layer thickness

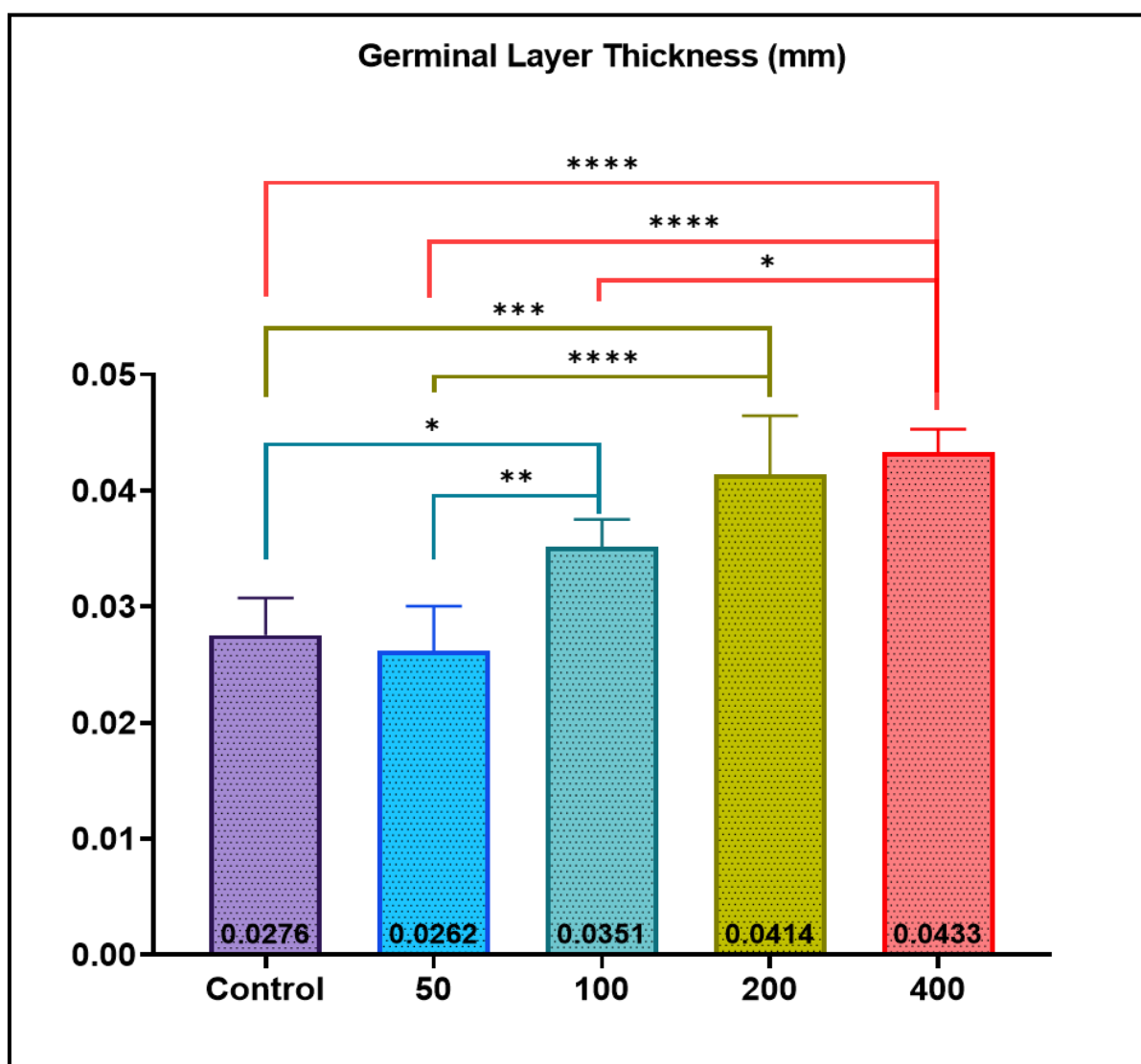


Figure 4-13 Effect of SeNPs treatment on germinal layer thickness(mm)

Figure 4-13 shows that germinal layer thickness is significantly increased ($p < 0.0001$) in G5 compared with the G1 and G2 groups, and significantly increased ($p < 0.05$) in G5 compared with the G3 group. No significant difference between the G5 and G4 groups. Also, there is a significant increase ($p < 0.0001$) in G4 compared with the G2 group, and a significant increase ($p < 0.001$) in G4 compared with the G1 group. In addition, there is a significant increase ($p < 0.01$) in G3 compared with the G2 group, and a significant increase ($p < 0.05$) in G3 compared with the G1 group.

Chapter Five

Discussion

5. Discussion

5.1 Biological importance and toxicity of SeNPs

Selenium is an essential element for good health, required for many important functions in the body, and considered part of the body's powerful antioxidant defense system. Its deficiency can lead to decreased function in some body tissues, especially in the male reproductive system, due to its important role in the production of sex hormones and in sperm health, production, motility, and vitality (Zambonino *et al.*, 2023).

The study indicated that the key factor in choosing the nanoform of selenium over its traditional forms is that selenium compounds have a narrow therapeutic or nutritional window, with low absorption and a narrow safety margin. In contrast, selenium nanoparticles exhibit low toxicity and the ability to enhance biological properties. This was further confirmed by Khurana *et al.* (2019), who demonstrated that nano-selenium could mitigate toxicity and improve biological activity.

Some studies indicate that toxicity may vary depending on the dose, preparation method, and type of animal used in the experiment. A well-established principle in toxicology confirms that toxicity is not solely dependent on the dose used, but is closely linked to the properties of the particles and the preparation method. For example, biosynthetic particles are coated with active compounds, which increases their stability and reduces their toxicity (Ryabova *et al.*, 2024).

The selenium nanoparticles also demonstrated greater bioavailability than other conventional forms. This is due to their small particle size, which enables better absorption across cell membranes and tissues. Furthermore, nano selenium exhibits greater biological activity, as demonstrated by Shi *et al.* (2010), who showed improved semen quality, antioxidant enzyme activity, and enhanced testicular structure in male goats. Despite the many advantages of SeNPs, important considerations must be taken into account when discussing their potential field use in animal nutrition supplements, the most important being the

narrow therapeutic window, as noted by Khurana *et al.* (2019) and Zambonino *et al.* (2023).

Shi *et al.* (2010) also emphasized the crucial role of selenium in fertility, maintaining oxidative balance, and thyroid function through selenoproteins. They also cautioned against its narrow therapeutic window, noting that excessive doses could be harmful even in animals with sufficient or high selenium levels. This explains observations in other studies, such as those by Ryabova *et al.* (2024), who showed toxicity at some doses but not others, depending on the dose, preparation method, and animal species used. In the current study, we used specific doses of SeNPs that showed good efficacy without signs of toxicity. This is consistent with the study by Eleouny *et al.* (2025), who used a dose of 200 microgram/kg B.W. and reported good results in antioxidant activity and hormone balance. This is also consistent with the study by Rashad *et al.* (2018), who used two doses (200, 500 microgram/kg B.W.) and showed improvement in various parameters. That means the use of an appropriate dose gives good results by a good balance between oxidative stress and the body's antioxidant defense. The difference in study results does not necessarily indicate a contradiction, but may be due to differences in experimental conditions and the physical and chemical properties of the manufactured material. Results cannot be generalized because a range of factors influence the outcome, such as animal species, age, general health, preparation method, particle size, dosage administration method, and experiment duration. These factors combined can affect the study's result.

5.2 Selenium nanoparticles (SeNPs) biosynthesis characterization

5.2.1. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum of the SeNPs was recorded over 400-4000 cm^{-1} . This process allows the recognition of structural changes in the molecular bond between the stabilizer and the Selenium atoms.

The detected bands at 3440 cm^{-1} , 2920 cm^{-1} , and 1630 cm^{-1} are associated with O-H stretching, C-H stretching, and C-O bending vibrations of the O-H

group, respectively. These peaks suggest the presence of a biopolymer associated with the SeNPs. The starch structure, indicating the presence of a stabilizer on the surface of the SeNPs, is evidenced by an extra band at 1109cm^{-1} . All these results are consistent with those of other researchers (Kazemi *et al.*, 2021), who synthesized the colloid selenium NPs (Se-NPs) through the utilization of Na_2SeO_3 , in which starch has the role of stabilizer and capping agent, as the functionality of green reducing mediums is taken by glucose and ascorbic acid. (Vasanthakumar *et al.*, 2024) who determined the functional units at bands 3275cm^{-1} , 2922cm^{-1} and 1631cm^{-1} peaks. confirming the successful biosynthesis of SeNPs and demonstrating that starch effectively acts as a coating agent for the synthesized SeNPs. and ascorbic acid as effective and successful reducing agents in the biosynthesis of SeNPs.

5.2.2. Zeta potential exam:

This is the process of measuring the electrostatic potential at the electrical double layer surrounding a nanoparticle in a colloidal solution. This potential is called the zeta potential (Honary and Zahir, 2013). Nanoparticles with a zeta potential of $+10$ or -10 mV are considered nearly neutral, while those with a zeta potential of $+30$ or less than -30 mV are considered strongly cationic or anionic. The greater the negative charge, the stronger the repulsion between nanoparticles, preventing them from clumping over time and resulting in greater stability. (Zheng *et al.*, 2011). The results of the zeta potential test of the selenium nanoparticles prepared in the current experiment showed that the charge was -58.3 mV ; this high charge means there is a strong electrical repulsive force between the particles, preventing them from clumping over time. This ensures they remain in their effective nanoscale form for a long period, allowing for efficient storage and use without losing their vital properties. This indicates the high stability of the nanomaterial and the stability that allows it to be used for a long period without the occurrence of aggregation of the particles. These results are similar to a previous study of Sarkar *et al.* (2011).

5.2.3. X-ray diffraction (XRD)

The X-Ray Diffraction analysis technique determines the structural and crystalline nature of SeNPs (phase and degree of crystallinity) and can provide an approximate crystallite size. The green SeNPs may show crystalline selenium reflections in some systems, while other preparations appear weakly crystalline or predominantly amorphous; thus, XRD supports phase/structure identification, not morphology. (Mekky *et al.*, 2025). It works by directing X-rays at a sample and analyzing how they scatter when they interact with the crystal lattice. In the current manufacturing process, X-ray diffraction results indicate the formation of nanocrystalline selenium particles. The X-ray diffraction pattern revealed sharp, clear peaks at 23°, 30°, 41°, 44°, and 45°, which are almost identical to those reported by Kazemi *et al.* (2021). The main peak at 30 indicates high crystallinity, and the absence of any unusual peaks indicates the sample is pure, free of impurities from the synthesis process. This was consistent with the study by Saravanan *et al.* (2025), who reported that X-ray diffraction analysis of SeNPs revealed a crystalline structure with a prominent peak at $2\theta = 33.63^\circ$. This peak confirms the crystalline nature of the SeNPs and indicates a well-defined atomic arrangement within the particles (Saravanan *et al.*, 2025). The XRD pattern shows good agreement with the results of the field-emission scanning electron microscopy (FESEM) images. Comparing the results of this study with previous studies reinforces the reliability of this method for preparing SeNPs and the success of the biosynthesis process.

5.2.4. Field Emission Scanning Electron Microscope (FESEM)

Scanning electron microscopy is used to determine the shape and size of particles resulting from the biosynthesis of selenium nanoparticles via green synthesis using ascorbic acid as a reducing agent and starch as a stabilizing agent (Al-Ghareebawi *et al.*, 2021). The images in this study showed that the particles took an oval or semicircular shape and had diameters of 30-40 nanometers (30.72, 31.66, 31.94, 38.85), indicating successful nanoparticle formation (Srivastava and Mukhopadhyay, 2015).

5.3 Effects of SeNPs on male reproductive hormones

The current study showed a significant decrease in testosterone concentration in the G2 and G3 groups, which received 50 and 100 µg/kg B.W of SeNPs, whereas there were no significant differences among the groups that received 200 and 400 microgram/kg B.W doses of SeNPs during the period of study, as compared with the G1 group. This is not consistent with (Abdallah *et al.*, 2023) and agrees with (Hamza and Diab, 2020), who reported a decrease in testosterone levels in rats receiving nano selenium doses. resulted from the effect of Monosodium glutamate (LD and HD), which induced a decrease in testosterone hormone levels and markedly elevated oxidative stress markers. The drop in testosterone alongside improved sperm quality, this can be explained by how testosterone works in the body. Spermatogenesis relies mainly on testosterone levels inside the testes rather than in the blood. So, even if blood testosterone drops—possibly because the body is using it up quickly or due to the treatment's effect on Leydig cells—the local testosterone inside the testes was still enough to support healthy sperm production (Talebi *et al.* 2021).

Regarding luteinizing hormone (LH), the current study showed a significant increase in LH in the groups that received 50 and 100 µg/kg B.W. This result is consistent with (Yuan *et al.*, 2024). As for Follicle-Stimulating Hormone (FSH), the study showed a significant increase in the G2and G3 groups, which

received 50 and 100 µg/kg B.W of SeNPs during the study period, compared to the G1, G4, and G5 groups. The G2 group also showed a significant increase compared to the G3 group. This is consistent with the findings of Dkhil *et al.* (2016). These results suggest that selenium does not directly increase testosterone, LH, and FSH. But it increases production by improving the health of testicular cells. That is in agreement with Zečević *et al.* (2025), who say that the common assertion that selenium "directly stimulates testosterone" should be qualified: selenium appears to influence testosterone biosynthesis and spermatogenesis by decreasing oxidative stress and improving testicular cell function, but direct human evidence remains limited.

5.4 Effects of SeNPs on antioxidant enzymes

The current study shows a significant decrease in Glutathione reductase activity in all treated groups compared with the G1 group. These results agree with what Talebi *et al.* (2021) observed. As sperm cells mature and go through the final stages of development, they lose much of their cytoplasm—and with it, their natural antioxidant defenses. This means antioxidants are heavily used up during the critical process of DNA packing. Also, there was no significant difference in Glutathione peroxidase between the G2, G3, and G4 groups, whereas the G5 group showed a significant decrease. As for selenoprotein, the study showed a significant increase in its concentration in the G5 group. These results are consistent with those of Nasirpour *et al.* (2017), who attribute this to antioxidant consumption counteracting oxidative stress in rats, which is the opposite of the results reported by Shi *et al.* (2010) and Zhou *et al.* (2009). Those who found that there was an increase in these enzymes when nano selenium was taken. This difference in the results of the current study and another may be attributed to the fact that the biological effects of SeNPs depend on dose, route of administration, and environmental factors (Li *et al.*, 2024). Antioxidants such as vitamin E may influence selenium status and its retention and distribution in the body (Fujihara and Orden, 2014).

5.5 Effects of SeNPs on sperm proteins (Acrosin, Protamine)

The current study shows a significant increase in Acrosin levels in the groups that received 200 and 400 µg/kg B.W., which enhances sperm fertilization and sperm penetration of the ovum membrane. This agrees with Horky *et al.* (2023) and Talebi *et al.* (2021), who showed that giving SeNPs improves male fertility. The current study also showed a significant decrease in protamine levels across all groups, likely due to the high doses used, which affect protamine levels during sperm formation. This means that selenium acts on the external functional aspects of sperm and its ability to fertilize, while at the same time requiring careful dose adjustment to maintain the integrity of the genetic material. This was also evident in the study of (Khalil *et al.* 2025; Piri *et al.* 2024).

5.6 Effects of SeNPs on male reproductive traits

Testicular weight and size can affect hormones and sperm production due to the increase in Sertoli cells and Leydig cells, depending on maturation of the reproductive system. Degeneration of testicular tissue and seminiferous tubules may also occur in cases of low testicular weight and Se deficiency. Consistent with the increase in testicular weight in animals treated with SeNPs, an increase in sperm count and live/dead ratio was also observed. The current study showed a significant increase in sperm count, especially in the G4 and G5 groups, compared to the other groups. This is consistent with the findings of Talebi *et al.* (2021). The study also showed a significant increase in the percentage of live sperm in all groups compared to the G1 group. This is consistent with Zhang *et al.* (2019), who also showed a significant increase in testicular weight across all groups compared to the G1 group. This is consistent with El-Kazaz *et al.* (2020). They confirmed that adding nano selenium to feed increases the weight of reproductive organs, stimulates sexual behavior, protects testicular tissue, and improves overall reproductive function. Selenium supplementation at 200 microgram/day for six months was associated with significant improvements in

sperm concentration, motility, and seminal antioxidant capacity, as well as reduced sperm DNA fragmentation (Alahmar, 2023).

In another study involving men with chronic autoimmune thyroiditis and abnormal semen parameters, 83 µg/day for six months was associated with improvements in sperm concentration, progressive motility, and morphology, alongside lower DNA fragmentation. However, this was a small, selected cohort, and findings should not be generalized to all infertile men (Asghari-Moghadam and Mehri, 2025). However, current evidence supports a non-linear (bell-shaped or U-shaped) relationship, indicating that both selenium deficiency and excess may adversely affect sperm quality and fertility outcomes. This has important clinical implications, as indiscriminate supplementation may not benefit men with adequate selenium status (Zečević *et al.*, 2025).

5.7 The histological examination

This study showed that 50-60% of seminiferous tubules were immature, with low sperm count and relatively narrow seminiferous ducts in the G1 and G2 groups. In the group that received 100 µg/kg B.W (G3), the majority of seminiferous tubules were mature, with a very regular germ layer; however, sperm count was low, and interstitial tissue thickness was increased compared to the G1 and G2 group, which received 50 µg/kg B.W of SeNPs. In the group that received 200 µg/kg B.W, all seminiferous tubules were mature, with a very regular germ layer, clear stages of spermatogenesis, increased sperm count, and increased interstitial tissue volume. The G5 group of the current study showed a significant increase in the maturity and activity of all seminiferous tubules, with a well-organized germinal layer displaying distinct stages of spermatogenesis, including spermatogonia along the basement membrane, primary spermatocytes, and round-to-elongated spermatids toward the tubular lumen. Furthermore, the lumina contain a higher density of spermatozoa compared to the G1 group. Interstitial tissue is also thicker than in the G1 group, which agrees with Abd-Allah and Hashem (2015), Gan *et al.* (2019), and Dkhil *et al.* (2016).

The results of this study also showed a clear improvement in testicular structure in the nano-treated groups compared to the G1 group. This is consistent with the study by Vatanpour *et al.* (2024) and their colleagues, which showed a significant improvement in histological parameters in a model of infertile mice. It is also consistent with Samy *et al.* (2026) and their colleagues, who confirmed that nano-treatment preserved the histological structure of the reproductive glands.

In general, the results of this study are partially consistent with other studies showing that nano-selenium can improve reproductive function when used in appropriate doses that do not cause adverse effects or toxicity in testicular cells. These studies also indicated that it enhances testosterone production and increases the production of antioxidants and other biomarkers. This may be attributed to the animal's age, the dose used, and the nature of the continuous exposure over a period of thirty days, as shown in studies of Khalaf *et al.* (2019) and Zecevic *et al.* (2026). Recent studies also confirm that selenium is a substance with a bidirectional effect and not a linear effect, as a deficiency leads to a disturbance in fertility, while an excess or increase may lead to tissue or hormonal toxicity, as shown in the study by Liu L *et al.* (2017). In this study, it was clearly shown that continuous exposure to nanoselenium particles in the pre-puberty stage may lead to a decrease in testosterone and antioxidant levels, despite the presence of indications of accelerated maturation and improved sperm parameters, sperm count, and testicular tissue maturation, which confirms the need for studies to determine safe doses and monitor hormonal and antioxidant indicators.

Conclusions and Recommendations

6. Conclusions and recommendations

6.1 Conclusions

1. The study showed a significant increase in sperm count, live/ dead ratio, and testicular weight.
2. This demonstrates the ability of nano selenium to stimulate reproductive development in the pre-puberty stage. Thus, SeNPs can be considered an effective dietary supplement for improving male fertility in early developmental stages.
3. The study demonstrated improved levels of testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) at specific doses, resulting in significantly enhanced male reproductive function in immature rats.
4. The study demonstrated a significant dose–dependent modulation of the hypothalamic-pituitary-gonadal axis, characterized by improved levels of LH and FSH, accompanied by a significant reduction in testosterone concentration, particularly at higher dosages.
5. The study also showed elevated levels of Selenoprotein P, an antioxidant that protects testicular tissue and supports the growth and development of the male reproductive system. Furthermore, acrosin levels gradually increased with increasing doses administered to each group, significantly improving sperm penetration during fertilization.
6. The study demonstrated an improvement in the percentage of mature seminiferous tubules, reaching approximately 100% in the G5 group compared to 50% in the G1 group, as well as an increase in interstitial tissue thickness, basement membrane thickness, primary spermatocyte density, and greater spermatogenesis. Indicating an improvement in testicular tissue as a result of using SeNPs.
7. Selenium nanoparticles significantly decreased protamine levels in the treated groups, while acrosin levels increased at higher doses, particularly in G3 and G4 groups.

6.2 Recommendations

1. Conduct long-term studies to evaluate reproductive performance, safety, and potential genetic effects of SeNPs.
2. Expand the studies to include female animals and other animal models, such as sheep, goats, and cattle, because they may exhibit different physiological responses.
3. A comparison study between biologically and chemically synthesized SeNPs at equivalent doses.
4. Assess safety and efficacy over the long term before starting field application.
5. Study the economic possibility of adding nano-selenium to animal feed for practical use.

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الخلاصة :

يُعد تسريع نضج الجهاز التناسلي الذكري من النقاط المهمة في تربية الحيوانات. هدفت هذه الدراسة الى تقييم مرحلة نضج الجهاز التناسلي الذكري للجرذان غير الناضجة باستخدام جسيمات السلينيوم النانوية المصنعة حيويًا باستخدام النشا.

فُسِّمَت التجربة إلى جزأين: تناول الجزء الأول تصنيع جسيمات السلينيوم النانوية باستخدام النشا كعامل مُثَبِّت وحمض الأسكوربيك كعامل مُخْتَزَل. بعد التصنيع، تم توصيف المادة النانوية المُنتجة باستخدام مطيافية الاشعة تحت الحمراء بتحويل فورييه وحيود الاشعة السينية وقياس جهد زيتا والتصوير بواسطة المجهر الضوئي الماسح. ويتضمن الجزء الثاني من التجربة استخدام 40 جرذاً غير ناضج مقسمة الى خمسة مجموعات تلقت المجموعة الأولى (المجموعة الضابطة) الماء المقطر فقط والمجموعة الثانية 50 مايكروغرام/ كغم من وزن الجسم من جسيمات النانو سلينيوم المصنعة حيويًا عن طريق الفم وتلقت المجموعة الثالثة 100 مايكروغرام/كغم من وزن الجسم، وتلقت المجموعة الرابعة 200 مايكروغرام لكل كيلو غرام من وزن الجسم والمجموعة الخامسة تلقت 400 مايكرو غرام لكل كيلو غرام من وزن الجسم. استمر الجزء الثاني من التجربة لمدة 30 يوماً. بعد ذلك تم تخدير حيوانات التجربة بالاستنشاق لأخذ عينات الدم لتحليل تركيز الهرمونات (التستوستيرون و الهرمون المحفز للجريب والهرمون اللوتيني) وتركيز بروتينات الحيوانات المنوية (الاكروسين والبروتامين)، وتركيز مضادات الاكسدة (كلوتاثيون بيروكسيداز وكلوتاثيون ريدكتاز والسلينيوبروتين) كما تم فحص خصيتي ذكور جرذان التجربة لدراسة وزن الخصية وعدد الحيوانات المنوية ونسبة الحيوانات المنوية الحية الى الميتة. والتغيرات النسيجية بما في ذلك درجة نضج الانابيب المنوية وقطر الانابيب المنوية وسماكة الطبقة الجرثومية.

أظهرت نتائج التجربة الحالية زيادة معنوية ($p \leq 0.05$) و ($p \leq 0.01$) في مستويات السلينيوبروتين والاكروسين وعدد الحيوانات المنوية ونسبة الحيوانات المنوية الحية الى الميتة ووزن الخصية ونسبة نضج الانابيب المنوية وقطرها وسماكة الطبقة الجرثومية وبالإضافة الى ذلك ظهر نقص معنوي في تركيز التستوستيرون و مضادات الاكسدة والبروتامين في مجموعات مختلفة من التجربة .

اما التغيرات النسيجية فقد أظهرت زيادة في مستوى نضوج الانابيب المنوية.

وخلصت التجربة إلى أن استخدام جزيئات السلينيوم النانوية المصنعة بيولوجيًا يساعد في تسريع نضج الخصيتين لدى ذكور الجرذان، مع بعض الحذر في بعض الجرعات التي تمت دراستها.



جمهورية العراق

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كلية الطب البيطري

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المعاملة بجسيمات السلينيوم النانوية المحضرة حيويًا باستخدام النشا

رسالة مقدمة الى مجلس كلية الطب البيطري – جامعة الشطرة وهي جزء من متطلبات نيل

درجة ماجستير في علوم الطب البيطري (الفلسفة)

من قبل

عبد الستار محمد عفراوي

بكلوريوس طب وجراحة بيطرية

1990

اشراف

أ.م.د. عامر مكطوف عبد الغريباي

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