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Evaluation of the Pharmacological Effect of Gonadotropin-Based Drugs

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Dedication

To ***my parents,*** the pillars of my life.

To my mother, ***Wassila,*** and my father, ***Ahmed***, who raised me, shaped me, and made me the woman I am today. This work is, above all, the fruit of your upbringing. You have always believed in me and wished for my success.

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This work represents a chapter of my life I will never forget, and after all these years,

I can finally say that I am proud.

List of tables

List of figures

List of Abbreviations

Abbreviation	Signification
BMI	Body Mass Index
CHU	University Hospital Center (Centre Hospitalier Universitaire)
DNA	Deoxyribonucleic Acid
FSH	Follicle-Stimulating Hormone
FSHR	Follicle-Stimulating Hormone Receptor
GnRH	Gonadotropin-Releasing Hormone
hCG	Human Chorionic Gonadotropin
HH	Hypogonadotropic Hypogonadism
HPG axis	Hypothalamic–Pituitary–Gonadal axis
H&E	Hematoxylin and Eosin
ICSI	Intracytoplasmic Sperm Injection
IVF	In Vitro Fertilization
LH	Luteinizing Hormone
LHR	Luteinizing Hormone Receptor
mRNA	Messenger Ribonucleic Acid
PMSG	Pregnant Mare Serum Gonadotropin
r-hCG	Recombinant Human Chorionic Gonadotropin
rFSH	Recombinant Follicle-Stimulating Hormone
rLH	Recombinant Luteinizing Hormone
RNA	Ribonucleic Acid
SD	Standard Deviation
WHO	World Health Organization

Abstract

This experimental study aimed to assess the biological activity of various gonadotropin combinations administered to immature Wistar rats. A total of 75 animals were included, divided into 8 groups for females (n = 40) and 7 groups for males (n = 35), treated with different combinations of FSH, LH, and hCG at three dose levels (low, medium, high).

In females, treatment effects were evaluated through ovarian weight measurement and detailed histological examination of follicles under multiple magnifications. The results showed a significant dose-dependent increase in ovarian weight, along with a clear enhancement of follicular maturation, particularly in the group receiving FSH+LH+hCG.

In males, the impact of the treatments was assessed on seminal vesicles and prostate. Gonadotropin administration induced a dose-dependent hypertrophy of accessory sex organs, accompanied by marked histological changes, including thickening of the secretory epithelium and increased luminal content.

Altogether, these findings demonstrate the biological effectiveness of the gonadotropin treatments and highlight their role in triggering sexual maturation and the development of reproductive structures in the immature rat model.

Keywords : Gonadotropins, hCG, Ovaries, Prostate, Seminal vesicles, Histology, rats

Résumé

Ce travail a porté sur l'évaluation in vivo de l'activité biologique de différentes combinaisons de gonadotrophines administrées à des rats Wistar immatures. L'étude a inclus 75 animaux, répartis en 8 lots pour les femelles (n = 40) et 7 lots pour les mâles (n = 35), traités par des associations variables de FSH, LH et hCG à trois niveaux de doses (faible, moyenne, élevée).

Chez les femelles, l'effet des traitements a été évalué par la mesure du poids ovarien et par une analyse histologique détaillée des follicules à différents grossissements. Les résultats ont mis en évidence une augmentation significative du poids ovarien corrélée à la dose, ainsi qu'une avancée marquée de la maturation folliculaire, en particulier dans les groupes ayant reçu l'association FSH+LH+hCG.

Chez les mâles, les effets ont été étudiés au niveau des vésicules séminales et de la prostate. L'administration des gonadotrophines a induit une hypertrophie dose-dépendante des organes accessoires, ainsi que des remaniements histologiques significatifs, caractérisés notamment par un épaississement de l'épithélium sécrétoire et une augmentation du contenu luminal.

L'ensemble des résultats met en évidence l'efficacité biologique des gonadotrophines utilisées, et souligne leur rôle dans l'induction de la maturation sexuelle et le développement des structures reproductrices chez le rat immature.

Mots-clés : Gonadotrophines, hCG, Ovaires, Prostate, Vésicules séminales, Histologie, rats.

ملخص

يهدف هذا البحث إلى تقييم النشاط البيولوجي لهرمونات الغونادوتروفين عند الجرذان غير الناضجة من نوع ويستار. شملت الدراسة 75 جرذاً، موزعين على 8 مجموعات إناث (40 جرذة) و7 مجموعات ذكور (35 جرذاً). تلقّت جميع الحيوانات تركيبات مختلفة ، وذلك (hCG) ، وهرمون موجهة الغدد التناسلية المشيمائي البشري(LH) ، والهرمون اللوتيني(FSH) من الهرمون المنشط للجريبات .بثلاث جرعات مختلفة: منخفضة، متوسطة وعالية

تم تقييم تأثير هذه العلاجات عند الإناث من خلال قياس وزن المبيض والدراسة المجهرية التفصيلية لمراحل تطور الجريبات تحت تكبيرات مختلفة. وقد أظهرت النتائج زيادة ملحوظة في الوزن مرتبطة بالجرعة، مع تطور واضح في نضج الجريبات، .FSH + LH + hCG خصوصاً في المجموعات التي تلقت مزيج

أما عند الذكور، فقد شمل التحليل الحويصلات المنوية والبروستات. وأسفرت المعالجات عن تضخم واضح في الأعضاء التناسلية .الثانوية بطريقة تعتمد على الجرعة، بالإضافة إلى تغيّرات نسيجية مهمة تمثلت في تضخم الظهارة الإفرازية وزيادة محتوى الغدد

تُبرز هذه النتائج الفعالية البيولوجية للعلاجات الهرمونية المستخدمة، ودورها المحتمل في تحفيز النضج الجنسي وتطوير البنى .التناسلية عند الجرذان غير الناضجة

الكلمات المفتاحية : الغونادوتروفين، هرمون الحمل، المبيض، البروستات، الحويصلات المنوية، الدراسة النسيجية، الجرذان.

__Contents__

Introduction

Introduction

Reproduction is an innate biological drive in animals, including humans, and is essential for the survival of all species. Consequently, for many women, the inability to conceive or to do so within a socially or biologically expected timeframe can be deeply distressing. This condition, referred to as **infertility,** is often perceived as a barrier to the continuation of the family lineage **(Awonuga *et al*., 2025)**.

Infertility is defined as the failure to establish a clinical pregnancy after 12 months of regular and unprotected sexual intercourse. It is estimated to affect between 8 and 12% of reproductive-aged couples worldwide. Males have been found to be responsible for 20-30% of infertility cases, yet they contribute to 50% of cases in general. Secondary infertility, characterized by the failure to conceive after a period of normal fertility, is the most prevalent form of female infertility worldwide, often attributable to reproductive tract infections. The three major factors influencing the spontaneous probability of conception are the time of unwanted non-conception, the age of the female partner, and disease-related infertility. A number of factors have been identified as potential contributors to female infertility, including premature ovarian insufficiency, polycystic ovary syndrome, endometriosis, uterine fibroids, and endometrial polyps. Male infertility may be attributed to testicular and post-testicular deficiencies. Over the years, a decline in semen quality has been observed. This phenomenon may be influenced by various factors, including endocrine-disrupting chemicals and consanguinity (**Vander Borght & Wyns, 2018**).

Medically assisted reproduction (MAR) encompasses a wide range of therapeutic approaches aimed at addressing the diverse etiologies of infertility. These interventions include pharmacological treatments, surgical procedures, and advanced reproductive technologies. Common strategies involve ovulation induction, ovarian stimulation, and ovulation triggering, as well as assisted reproductive technology (ART) procedures such as intracervical and intravaginal insemination using partner or donor semen, uterine transplantation, gamete intrafallopian transfer (GIFT), and zygote intrafallopian transfer (ZIFT). Pharmacological agents used in infertility management primarily function by promoting ovulation. These may be administered orally or via subcutaneous hormone injections. Oral agents such as clomiphene citrate and tamoxifen exert their effects by inhibiting estrogen feedback, thereby enhancing the secretion of gonadotropins. In contrast, injectable agents including gonadotropin-releasing hormone (GnRH) agonists and antagonists act directly on the hypothalamic-pituitary-ovarian axis to stimulate follicular development, prevent premature luteinizing hormone (LH) surges, and improve follicular recruitment **(Fenwick et al., 2023)**.

The primary objective of this study was to assess and compare the biological activity of three gonadotropin-based pharmaceutical formulations used in the treatment of infertility in men and women. These drugs referred to as Drug 1 (follitropin alfa + lutropin alfa), Drug 2 (follitropin alfa), and Drug 3 (menotrophin) using an in vivo model of immature Wistar rats. The evaluation focused on the pharmacodynamic effects of subcutaneously administered gonadotropins, specifically by measuring reproductive organ weights and analyzing their trophic influence on both gonadal and accessory reproductive tissues, in accordance with bioassay protocols established in the British Pharmacopoeia. As a secondary objective, the study aimed to conduct histological examinations of ovarian and testicular tissues to assess morphological changes and cellular differentiation induced by the treatments. Additionally, hormonal profiling was performed to determine circulating levels of key sex steroids, thereby providing a complementary biochemical assessment of the drugs' physiological activity.

. This work is presented in three Chapters:

- Chapter I provides a comprehensive review of the physiological and hormonal regulation of human reproduction, with a particular emphasis on the hypothalamic-pituitary-gonadal axis, the mechanisms of action of gonadotrophins, and the development of gonadotrophin-based medicinal products, including biosimilars.
- Chapter II presents the materials and methods employed in the biological evaluation of the selected gonadotrophin-based drugs. The document meticulously delineates the experimental design, the animal model utilized, the dosing strategy employed, and the array of analyses conducted.
- In Chapter III, the results obtained are discussed, including organ weights, histological findings, and hormonal assays. Furthermore, a comparison is made between the activity of the biosimilar product and that of the reference product. This chapter also includes a critical interpretation of the findings and their implications in the context of biosimilar development.

Chapter I:
Literature review

I.1. The Hypothalamic–Pituitary–Gonadal (HPG) Axis: An Overview

Reproduction is essential for the survival of a species, and its success relies on the coordinated action of various neuropeptides and hormonal systems that regulate both gonadal function and sexual behavior **(Acevedo-Rodriguez *et al*., 2018).**

The hypothalamic–pituitary–gonadal (HPG) axis plays a central role in male reproductive regulation. Pulsatile GnRH secretion from the hypothalamus stimulates the anterior pituitary to release LH and FSH, which act on Leydig and Sertoli cells to regulate testosterone and inhibin B production. These hormones, in turn, exert negative feedback on the axis. Testosterone can also be aromatized to estradiol, further influencing spermatogenesis. Despite its anatomical simplicity, any dysfunction within the HPG axis may lead to significant impairments in male fertility **(Clavijo *et al*., 2018).**

I.1.1. Role of GnRH, FSH, LH in male and female reproductive function

GnRH is the key regulator of the HPG axis, controlling gonadotropin secretion through its pulsatile release. While it is not required for fetal gonadal development, it becomes essential at puberty, initiating spermatogenesis, steroidogenesis, and hormonal feedback mechanisms. Mutations in the GnRHR gene can lead to isolated hypogonadotropic hypogonadism (IHH), which results in impaired reproductive function **(Jin *et al*., 2014).**

LH and FSH are produced by gonadotrope cells in the anterior pituitary and act on the gonads to regulate steroidogenesis and gametogenesis. LH secretion is tightly linked to the pulsatile release of GnRH, while FSH is secreted more continuously. In females, LH and FSH levels fluctuate across the menstrual cycle to support steroid hormone production, follicular development, and ovulation. LH stimulates steroidogenesis in Leydig cells (males) and theca cells (females), with a mid-cycle surge triggering ovulation. FSH supports spermatogenesis in males and promotes follicular development and estrogen production in females through the induction of aromatase **(Coss, 2018).**

I.1.2. Feedback mechanisms in endocrine control

The hypothalamic–pituitary–gonadal (HPG) axis is tightly regulated by sex steroid feedback involving estradiol, progesterone, and testosterone (**Figure1**). Estradiol has a biphasic effect on gonadotropin secretion: it inhibits GnRH and FSH during the early and mid-follicular phases but triggers the LH surge via positive feedback in the late follicular phase. Although

GnRH neurons lack estrogen receptor alpha (ERα), this regulation is mediated by KNDy neurons (co-expressing kisspeptin, neurokinin B, and dynorphin). In the infundibular nucleus, estradiol suppresses kisspeptin and neurokinin B while enhancing dynorphin to mediate negative feedback. Positive feedback, leading to the LH surge, involves kisspeptin neurons in the RP3V region in humans (AVPV in rodents) **(Marques *et al*., 2024)**.

Progesterone, especially during the luteal phase, reduces LH pulse frequency via increased dynorphin expression in KNDy neurons, an effect that depends on estradiol presence. In males, testosterone suppresses LH and FSH mainly through its action on AR-expressing KNDy neurons and its conversion to estradiol, which downregulates Kiss1 and GnRH secretion. Thus, sex steroids regulate GnRH pulsatility and gonadotropin secretion through coordinated hypothalamic and pituitary actions, with KNDy neurons acting as central integrators **(Marques *et al*., 2024)**

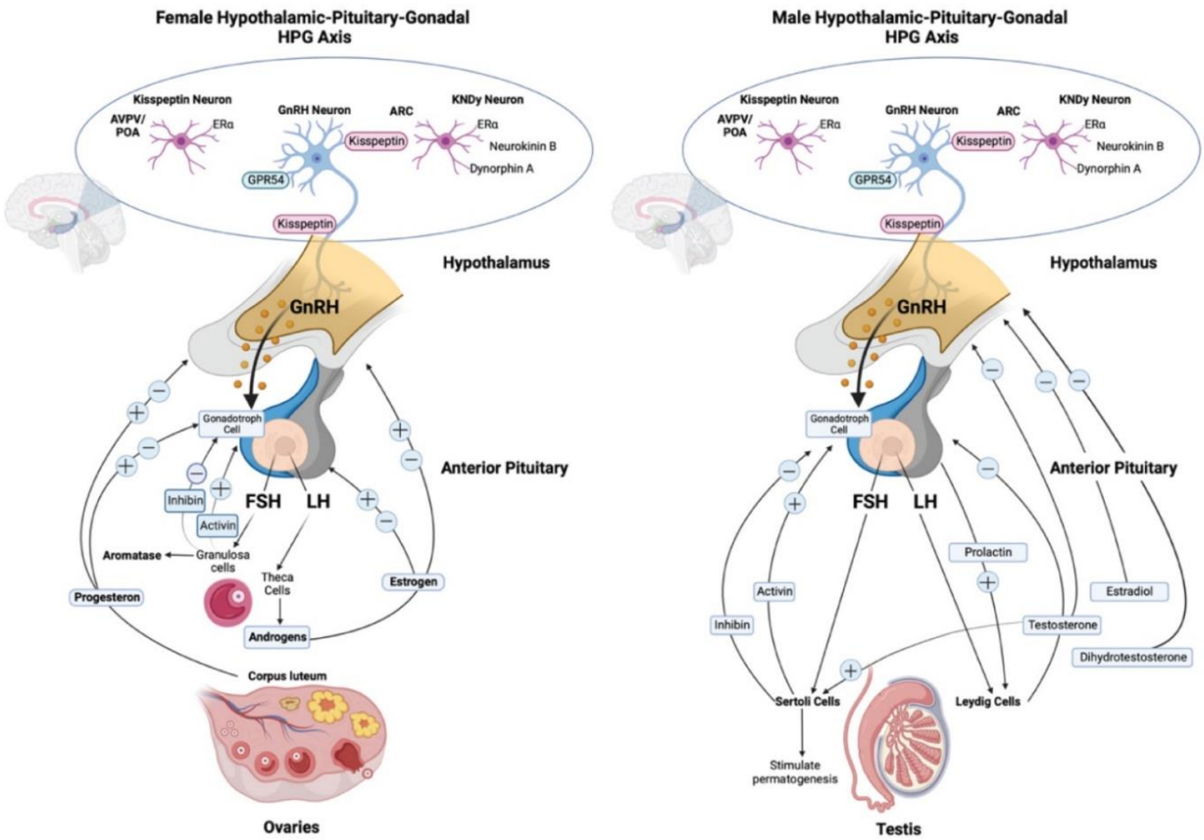

Figure 1: The HPG axis controls reproduction via hormonal signals **(Tammasse & Tamrin, 2023).**

In both sexes, Kiss1 neurons stimulate GnRH release, which regulates pituitary secretion of LH and FSH. In females, LH and FSH act on theca and granulosa cells to produce estradiol and progesterone, with feedback effects on the brain. In males, LH stimulates Leydig cells to produce testosterone, which, along with its metabolites, inhibits GnRH and gonadotropin secretion. FSH promotes spermatogenesis through Sertoli cells. Hormonal feedback differs by sex and cycle phase.

I.2. Physiological Role of Gonadotropins (FSH and LH)

I.2.1. Mechanism of action of FSH and LH

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are glycoprotein gonadotropins secreted by the anterior pituitary, acting via specific G protein-coupled receptors (GPCRs) in the gonads. Their primary roles are to regulate gametogenesis and steroidogenesis via the cAMP-PKA signaling pathway, with important sex-specific functions.

- FSH binds to FSH receptors on granulosa cells in the ovary and Sertoli cells in the testis. In granulosa cells, it stimulates aromatase expression and estradiol production and promotes cell proliferation and the selection of the dominant follicle. In Sertoli cells, FSH induces the production of androgen-binding protein (ABP) and expression of genes essential for germ cell survival, maturation, and spermatogenesis.
- LH acts on theca cells in females to promote androgen synthesis, which granulosa cells then convert into estrogens. A mid-cycle surge in LH triggers ovulation and corpus luteum formation . In males, LH binds to Leydig cells, enhancing testosterone production through cAMP-activated steroidogenic enzymes. Testosterone supports spermatogenesis and provides negative feedback on the hypothalamic-pituitary axis . (**Das & Kumar, 2018).**

I.2.2. Specific roles in spermatogenesis and folliculogenesis

I.2.2.1. Spermatogenesis (Male Reproductive Function)

In males, FSH binds to its receptor on Sertoli cells within the seminiferous tubules, stimulating their proliferation during puberty and maintaining their function in adulthood. FSH promotes the expression of genes necessary for germ cell survival and differentiation and induces the production of androgen-binding protein (ABP), which maintains high intratesticular testosterone levels required for spermatogenesis. FSH also contributes to the regulation of inhibin B, providing negative feedback to the pituitary **(Oduwole *et al*., 2018; McLachlan *et al*., 2002).**

LH targets Leydig cells in the interstitial tissue of the testes, stimulating the production of testosterone via the upregulation of steroidogenic enzymes. This testosterone supports spermatogenesis and is essential for the development of secondary sexual characteristics. LH signaling occurs via the cAMP/PKA pathway, initiating the transcription of key steroidogenic genes **(Ruwanpura *et al*., 2010).**

I.2.2.2. Folliculogenesis (Female Reproductive Function)

In females, FSH acts on granulosa cells within growing ovarian follicles, promoting their proliferation and inducing the expression of aromatase (CYP19A1), which catalyzes the conversion of androgens to estrogens. FSH also stimulates the expression of LH receptors on granulosa cells of dominant follicles, preparing them for the LH surge **(Rosen *et al*., 2021).**

LH binds to its receptor on theca cells, stimulating the production of androgens that serve as precursors for estrogen synthesis in granulosa cells. During the late follicular phase, a surge of LH triggers ovulation, the resumption of oocyte meiosis, and the formation of the corpus luteum, which produces progesterone to support early pregnancy **(Hillier *et al*., 1994; Haisenleder *et al*., 1991).**

I.2.3. Receptors and intracellular signaling pathways

I.2.3.1. FSH Receptor (FSHR) Signaling

FSH binds to its specific receptor (FSHR), a G protein-coupled receptor (GPCR) expressed on Sertoli cells in the testis and granulosa cells in the ovary. The principal signaling pathway activated by FSH is the Gs protein–adenylate cyclase–cAMP–PKA cascade, which leads to the phosphorylation of CREB, and the transcription of genes involved in cell proliferation, steroidogenesis, and germ cell survival. In Sertoli cells, this promotes the expression of androgen-binding protein (ABP) and facilitates germ cell development. In granulosa cells, it induces aromatase (CYP19A1) expression and enhances estradiol production, necessary for follicular maturation **(Das & Kumar, 2018).**

FSHR also activates non-canonical pathways such as PI3K/AKT, MAPK/ERK, and mTOR. These alternative routes are essential for granulosa and Sertoli cell survival, metabolism, and differentiation. For instance, activation of the PI3K pathway in granulosa cells supports protein synthesis and follicular development, while in Sertoli cells it regulates cell junction dynamics and blood-testis barrier function **(Crepieux *et al*., 2001).**

Additionally, FSHR signaling involves β-arrestin–mediated pathways, which contribute to sustained ERK activation independently of G proteins. β-arrestins also mediate receptor internalization and trafficking, which regulate the amplitude and duration of FSH signaling. This mechanism is particularly important in modulating FSH responsiveness during different stages of folliculogenesis **(Sposini *et al*., 2017).**

I.2.3.2. LH Receptor (LHCGR) Signaling

LH binds to its receptor, LHCGR, a GPCR expressed on theca cells in the ovary and Leydig cells in the testis. Like FSHR, LHCGR primarily signals through the Gs–adenylate cyclase–cAMP–PKA axis, which activates transcription of steroidogenic enzymes such as StAR and CYP11A1, essential for the biosynthesis of testosterone in males and androgens in females. These androgens are later converted to estrogens in granulosa cells under FSH influence **(Themmen & Huhtaniemi, 2000).**

LHCGR can also activate MAPK/ERK1/2, p38, and PKC-dependent signaling, particularly in granulosa cells following the LH surge. These pathways contribute to oocyte maturation, ovulation, and luteinization. ERK1/2 signaling, for example, regulates transcription factors required for cumulus expansion and rupture of the follicle wall **(Fan *et al*., 2009).**

Moreover, biased agonism at the LH receptor has been demonstrated: although both LH and hCG bind to LHCGR, they differentially activate downstream pathways. LH preferentially stimulates ERK and AKT for cell proliferation, while hCG elicits a more sustained cAMP/PKA response, favoring progesterone production in luteal cells **(Wehbi *et al*., 2010).**

I.2.4. Natural and exogenous sources of gonadotropins

Endogenous gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are glycoproteins secreted by the anterior pituitary gland in response to pulsatile stimulation by hypothalamic gonadotropin-releasing hormone (GnRH). These hormones consist of a common α-subunit and specific β-subunits that confer biological specificity. The natural proteins are heavily glycosylated, influencing their half-life, receptor affinity, and bioactivity. FSH primarily regulates folliculogenesis in females and spermatogenesis in males, whereas LH stimulates testosterone production in Leydig cells and triggers ovulation and luteinization in females **(Coss *et al*., 2015)**

Exogenous gonadotropin preparations are widely used in assisted reproductive technologies and include two main types: urinary-derived and recombinant. Human menopausal gonadotropin (hMG), obtained from urine of postmenopausal women, contains both FSH and LH activity but has batch variability and potential contaminant proteins. Recombinant gonadotropins (rFSH, rLH, and r-hCG) are produced in mammalian cell lines, offering consistent purity, dosing, and lower immunogenic risk. Clinical studies, including Cochrane reviews, report comparable efficacy between urinary and recombinant products in follicular stimulation; recombinant agents provide logistical advantages and standardized dosing vans **(Wely *et al*., 2011).**

I.3. Pharmacological Use of Gonadotropins and Their Analogues

I.3.1. Clinical and veterinary applications of gonadotropins

Gonadotropins play a pivotal role in both human and veterinary reproductive medicine. Clinically, they are employed to induce ovulation, stimulate follicular growth, and manage hypogonadotropic hypogonadism in both men and women. Recombinant and urinary-derived FSH, LH, and hCG preparations are now integral components of ART protocols and have significantly improved pregnancy rates.

In veterinary medicine, gonadotropins such as equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG), and GnRH analogs are routinely used to induce estrus, control ovulation, and synchronize estrous cycles, particularly in livestock breeding programs. Their application enhances fertility management, facilitates artificial insemination, and supports seasonal breeding regulation across multiple domestic species **(Lunenfeld *et al.*, 2019).**

I.3.2. recombinant or urinary-derived products

Table 1 illustrates the main recombinant and urinary-derived products currently used in clinical practice.

Table 1: Examples of recombinant or urinary-derived products

Type	Product (Trade Name)	Active Substance	Main Clinical Indication(s)	Scientific Reference
Recombinant FSH	Gonal-f®	Follitropin alfa	Controlled ovarian stimulation (COS), ovulation induction	Filicori M. *et al*., 2005.
	Puregon®	Follitropin beta	Same as above	Filicori M. *et al*., 2005.
Recombinant LH	Luveris®	Lutropin alfa	LH and FSH deficiency in women, combined with rFSH	European Medicines Agency.
Recombinant Hcg	Ovitrelle®	Choriogonadotropin alfa	Ovulation triggering in assisted reproduction	Devroey P. *et al*., 2001.
Urinary hMG	Menopur®, Humegon®	Human menopausal gonadotropin (FSH + LH activity)	Ovarian stimulation in ART	Daya S., 2007.
Urinary FSH	Fostimon®, Metrodin®	Purified urinary FSH (uFSH)	Ovulation induction, ovarian stimulation	Broekmans F.J. *et al*., 2009.

Urinary hCG	Pregnyl®, Profasi®	Urinary hCG	Ovulation triggering, luteal phase support	Humaidan P. *et al*., 2005.

I.3.3. Biomedicines vs. traditional hormone therapies

Compared to traditional urinary-derived hormone therapies, recombinant biopharmaceuticals such as gonadotropins offer significant advantages in infertility treatment. These biotechnologically produced hormones have higher specific activity, allowing lower doses via subcutaneous administration and presenting a better safety profile. Although both recombinant and urinary gonadotropins show similar efficacy in achieving pregnancy, recombinant forms improve patient outcomes through enhanced quality indicators such as safety and patient-centeredness. Effectiveness is measured not only by the number of oocytes and embryos produced but also by cumulative pregnancy rates. Current evidence supports the preferential use of biopharmaceutical gonadotropins in assisted reproductive technologies **(Broutin & Watier, 2016; Esteves, 2015).**

I.4. Experimental Models for Studying Gonadotropins

I.4.1. Justification for the use of rats as experimental models

Rats (*Rattus norvegicus*) are widely used in reproductive endocrinology research because their hypothalamic–pituitary–gonadal (HPG) axis exhibits pulsatile GnRH, LH, and FSH secretion patterns and sex-steroid feedback mechanisms similar to those found in humans. Ovariectomized rat models, in particular, replicate many aspects of human reproductive aging and postmenopausal conditions, including altered gonadotropin levels and bone loss dynamics responsive to estrogen therapy. Their short estrous cycle, low maintenance cost, and genetic homogeneity (e.g., Sprague–Dawley, Wistar strains) make them ideal for controlled and reproducible experiments with translational relevance to human reproductive physiology **(Turner *et al*., 2008; Kalu, 1991; Yousefzadeh *et al*., 2020; Sophocleous & Idris, 2014).**

I.4.2. Gender-based differences in response to endocrine modulation

Significant sex-specific differences exist in the response to endocrine modulation in rodents. Classic studies in guinea pigs by **Phoenix *et al*. (1959)** demonstrated that prenatal androgen exposure permanently organized neural circuits, such that female offspring showed male-typical mounting behavior and lacked the estradiol + progesterone induced LH surge highlighting the organizational role of prenatal testosterone via androgen receptors **(Phoenix *et al*., 1959; Blaustein & McCarthy, 2009).** Similarly, intact male rats lack a positive-feedback LH surge in response to estradiol, reflecting the prenatal programming of hypothalamic kisspeptin neurons within regions like AVPV and arcuate nucleus **(Smith & Fink, 1991**). These findings underscore fundamental sex differences in hormonal feedback regulation, determined early in development and sustained into adulthood.

I.4.3. Ethical considerations in hormonal experimentation

Hormonal experiments involving gonadotropins must comply with strict ethical standards based on the 3Rs principle (Replacement, Reduction, Refinement) to ensure both animal welfare and scientific validity. Ethics committees like the IACUC evaluate protocols to verify the necessity of hormonal interventions, appropriate dosing, and humane procedures. Clear guidelines regarding anesthesia, analgesia, and euthanasia are essential to avoid unnecessary suffering or endocrine disruption **(Russell & Burch, 1959; National Research Council, 2011; Festing & Wilkinson, 2007).**

I.4.4. Administration routes and pharmacokinetics in rodents

Substances can be administered to laboratory animals through a wide variety of routes, depending on whether a local or systemic effect is intended. Systemic delivery may follow either the enteral route (via the digestive tract, such as oral administration or gastric gavage) or parenteral routes (bypassing the digestive tract, such as intravenous, subcutaneous, intramuscular, or intraperitoneal injections). Additional administration methods include epicutaneous, transdermal, intradermal, and intraocular routes, as well as delivery into the central nervous system (intracerebral, intrathecal, or epidural). Drugs may also be introduced via intranasal, intratracheal, or inhalation techniques for pulmonary absorption. Other less common routes involve administration via specific body orifices, surgical access points, or species-specific anatomical features (**Figure 2**). The choice of route is primarily guided by the desired pharmacological effect, the properties of the compound, and the experimental design (**Turner et al., 2011).**

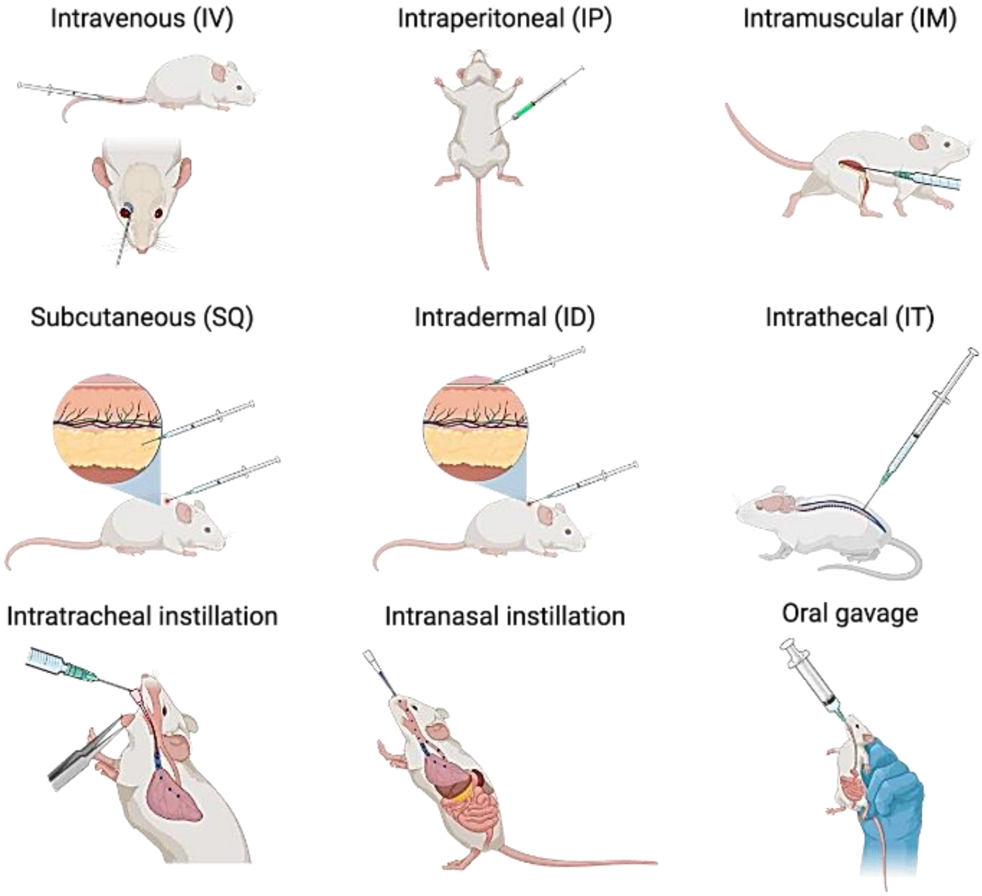

Figure 2: Visual representation of common injection routes in rodents **(Shepard, 2025).**

Chapter II:

Material and methods

The main objective of this study was to evaluate and compare the biological activity of three different gonadotropin-based medicinal products, hereafter referred to as **Drug 1** (follitropin alfa + lutropin alfa), **Drug 2** (follitropin alfa) and **Drug 3** (menotrophin). The assessment was carried out using an in vivo model of immature Wistar rats. This study aimed to investigate the pharmacodynamic response of the reproductive organs to subcutaneously administered gonadotropins, by quantifying organ weights and evaluating the trophic effects on gonadal and accessory reproductive tissues, in accordance with the bioassay protocols described in the British Pharmacopoeia**.**

In addition to gross anatomical measurements, a secondary objective was to perform histological analysis of ovarian and testicular tissues to assess cellular differentiation and structural changes induced by the treatments. Hormonal assays were also included to determine circulating levels of relevant sex steroids, thereby providing a complementary biochemical evaluation of the drugs' biological activity.

II.1. Experimental Setting

This experimental study was carried out over a five-month period, from early February to the end of June 2025. The practical work was conducted across three distinct sites, each corresponding to a specific phase of the experimental process.

The first phase took place at the National Agency for Pharmaceutical Products (ANPP) in Chéraga, Algiers, where the in vivo experimental procedures were performed. This included the preparation of gonadotropin solutions, the subcutaneous administration of the treatments to immature Wistar rats, and all handling related to the practical execution of the protocol.

The second phase was carried out at the Hussein Dey University Hospital Center (CHU Hussein Dey)**,** formerly known as Nafissa Hamoud Hospital (Parnet)**.** This part of the study focused on the histological processing of the collected organs, including tissue fixation, embedding, sectioning, and staining for microscopic analysis.

The final phase involved the hormonal assays, which were conducted in a private medical analysis laboratory equipped for the quantification of circulating sex steroid levels. This allowed for the biochemical evaluation of the pharmacological response to the gonadotropin treatments.

II.1.1. Presentation and organization of national agency for pharmaceutical products (ANPP)

The ANPP is a public institution with legal personality and financial autonomy, operating under the supervision of the Algerian Ministry of Pharmaceutical Industry. Its main responsibilities include (**Figure 3**):

- Evaluation and registration of pharmaceutical products and medical devices.
- Quality control and maintenance of national reference substances.
- Inspections to ensure compliance with Good Manufacturing Practices (GMP).
- Issuance of market authorizations, price certifications, and advertising approvals.
- Scientific assessment of therapeutic value and medico-economic impact.
- Participation in drafting pharmaceutical legislation and maintaining the national formulary.

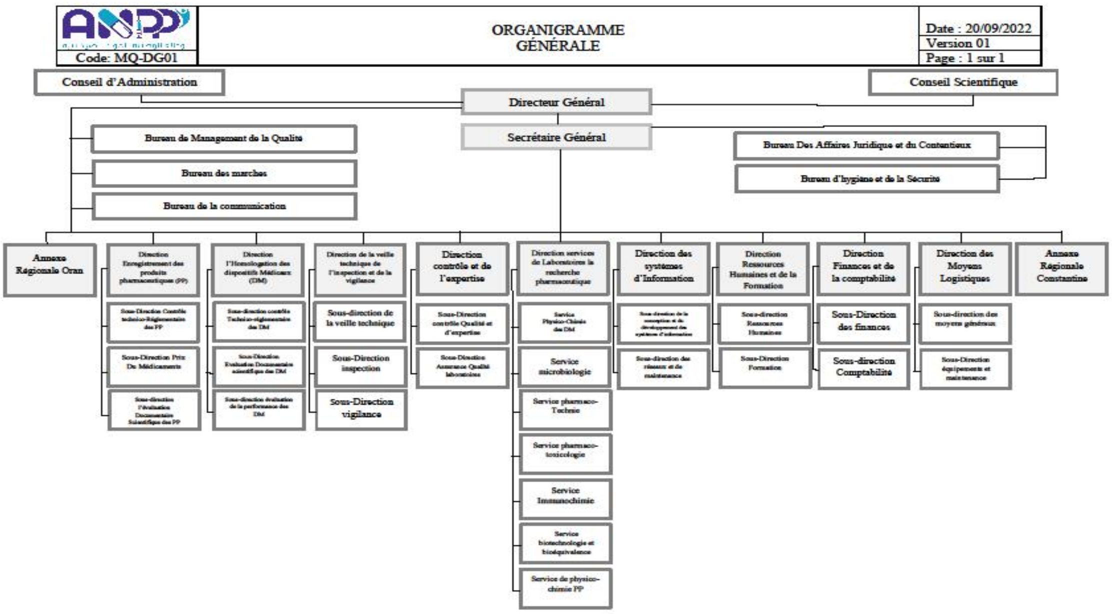

Figure 3: organization of national agency for pharmaceutical products (ANPP).

II.1.2. Nafissa Hamoud University Hospital Center (CHU Parnet)

The CHU Nafissa Hamoud, formerly known as Parnet Hospital, is a major teaching and referral hospital located in Hussein Dey, Algiers. It provides a wide range of medical and surgical services and is affiliated with academic institutions for the training of medical and pharmaceutical students. The hospital is active in clinical research, particularly in endocrinology and pediatrics.

II.2. Material

II.2.1. Biological Material

II.2.1.1. Experimental Animals

The experimental model consisted of **75 immature Wistar rats**, including **35 males and 40 females**, which were used exclusively for the final phase of the study (preliminary trials not included) (**Figure 4**).

Prior to treatment initiation, all rats underwent an acclimatization period under laboratory conditions to minimize stress and ensure stable physiological baselines. Health monitoring was performed daily to assess well-being, behavior, and any signs of distress, in accordance with institutional guidelines for animal welfare and ethical care.

Figure 4: Wistar Rats and Their Experimental Group Assignment.

II.2.2. Non biological material

II.2.2.1. Tested drugs

The medicinal products used in this study were biopharmaceuticals (biological medicinal products) containing gonadotropin hormones of either recombinant or urinary origin. All products were obtained from the ANPP in Chéraga, Algiers, prior to the start of the experimental phase. Four active substances were used, here referred to as Drug 1**,** Drug 2**,** Drug 3, and Drug 4 (hCG)**,** each with a specific formulation and pharmaceutical presentation.

All products were handled under aseptic conditions and prepared in accordance with the manufacturers' recommendations. Detailed information regarding the four drugs is provided in **Appendix 1.**

II.2.2.2. Laboratory equipment and chemical products

All laboratory equipment, reagents, materials, and consumables used during the study are listed in detail in **Appendix 2**.

II.3. Methods

II.3.1. Experimental Design

The experimental design was established to evaluate and compare the biological activity of various gonadotropin-based biopharmaceuticals in both female and male Wistar rats. The study was divided into two independent experimental arms, based on sex, each comprising multiple treatment groups. Animals were randomly assigned to receive one of the tested drug formulations or to a control group. The treatment allocation, dosing protocols, and drug combinations were specifically tailored for each sex to reflect their respective physiological responses to gonadotropins.

II.3.1.1. Female Experimental Groups

The female experimental model included **eight** groups of immature Wistar rats (n = 5 per group). Six groups received one of the two tested gonadotropin-based biopharmaceuticals, **Drug 1** (follitropin alfa + lutropin beta) or **Drug 2** (follitropin alfa) at low, medium, or high doses, all in combination with **Drug 4** (r-hCG)**.** Two control groups were included: one received only the vehicle (buffer), and the other received buffer combined with hCG (**Table 2**).

Each treatment was administered subcutaneously over three consecutive days.

II.3.1.2. Male Experimental Groups

The male experimental model included **seven** groups of Wistar rats (n = 5 per group). Two gonadotropin-based biopharmaceuticals were evaluated: **Drug 1**, a recombinant combination of follitropin alfa and lutropin beta, and **Drug 3**, a highly purified human menotropin of urinary origin. Three different doses were tested for each drug to assess the dose-dependent biological activity.Unlike the female protocol, no chorionic gonadotropin (hCG) was administered in the male groups. A control group receiving only vehicle (buffer) was included (**Table 3**).

All drugs were administered subcutaneously over four consecutive days.

II.3.2. Body Weight Monitoring

Body weight was monitored individually for each rat daily throughout the entire experimental period**.** This monitoring aimed to assess the animals' general health status and to detect any variations potentially associated with the administered treatments.

All measurements were performed under consistent conditions using an electronic analytical balance, with an accuracy of ±0.01 g. Body weight values were recorded in grams (g) and served as an indicator of systemic tolerance and biological response to the tested drugs.

II.3.3. Organ Weight (Ovaries, Seminal Vesicles, Prostate)

At the end of the treatment period (Day 4), all rats were euthanized and dissected under deep anesthesia using a lethal dose of thiopental sodium. The dose was calculated individually based on the animal's body weight to ensure animal euthanasia.

Following euthanasia, the reproductive organs including the ovaries (**Figure 7**) in females and the seminal vesicles and prostate in males were carefully excised. The organs were rinsed in isotonic saline solution (0.9% NaCl) to remove excess blood and tissue debris, then gently blotted dry on sterile gauze.

Each organ was weighed immediately after dissection using a precision analytical balance, and the results were expressed in milligrams (mg). These measurements were used to assess the trophic effects of the administered gonadotropins on the target reproductive tissues.

Figure 7: Rat dissection and ovaries sampling.

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II.3.4. Hormonal Assays (FSH and LH)

Blood samples were collected from each rat at the time of sacrifice. The blood was allowed to clot at room temperature, then centrifuged at 3000 revolutions per minute (rpm) for 10 minutes to separate the serum. The obtained serum samples were carefully aliquoted and stored at appropriate temperatures until analysis.

Hormonal quantification was performed using the Access 2 Immunoassay System (Beckman Coulter®, USA), an automated chemiluminescence immunoassay analyzer. The methodology is based on a two-site immunoenzymatic ("sandwich") assay, where in the LH molecules in the sample bind simultaneously to a solid-phase monoclonal antibody and an acridinium-labeled polyclonal antibody. The resulting chemiluminescent reaction is directly proportional to the LH concentration in the serum.

These hormonal parameters were used to evaluate the endocrine response to the tested gonadotropin treatments.

II.3.5. Histological Examination

Below are the essential steps followed in the preparation of tissues for histological analysis (**Figure 8**):

II.3.5.1. Tissue Collection

The organs were carefully harvested, ensuring no contamination or damage. The organs selected for analysis were the ovaries, prostate, and seminal vesicles.

II.3.5.2. Placement in Cassettes

Each cassette was clearly labeled with the name of the organ and the corresponding animal to prevent any mix-ups during the process.

The information was written using a pencil to ensure it would not be erased or smudged throughout the various steps of the protocol.

II.3.5.3. Fixation of Organs in Formalin

The organs were immediately placed in 10% formalin to fix the tissues, preserving them in a state as close as possible to their living condition. This step is crucial to prevent enzymatic degradation, autolysis, or microbial contamination. Fixation also helps to maintain the tissue's structural integrity and cellular components for further analysis **(Fischer et al., 2008).**

II.3.5.4. Washing and Dehydration

The tissue was first rinsed in clean water, followed by dehydration in an automated tissue processor. The process involved several steps using increasing concentrations of ethanol, and finally, xylene. Dehydration removes water from the tissue, making it suitable for embedding in paraffin wax **(Leica Biosystems, 2023).**

II.3.5.5. Paraffin Embedding

After dehydration, the cassettes containing the tissues were placed in a container holding paraffin wax on a heating plate.

The cassettes were opened, and the tissues were transferred into metal molds for embedding. The upper portion of the cassette was discarded, and only the lower half was used to hold the tissue in place during the embedding process. This ensures uniformity and proper orientation of the tissue for sectioning.

II.3.5.6. Inclusion and Block Formation

The molds were placed onto a cooling plate to allow the paraffin wax to solidify, forming tissue blocks that can be sliced into thin sections for microscopy.

II.3.5.7. Microtomy

Once the paraffin blocks were completely solidified, they were placed into a rotary microtome (Leica RM2235). The microtome was set to produce sections between 5 and 10 microns in thickness. These thin slices of tissue were carefully cut to preserve the cellular structure for subsequent staining.

II.3.5.8. Section Spreading

The tissue sections were floated in a warm water bath (Leica) to spread them evenly and ensure smooth, flat preparation. Once adequately spread, the sections were collected on glass slides, which were etched with the organ and individual labels using a diamond pen.

II.3.5.9. Slide Drying

The slides were then placed in an oven set between 37°C and 60°C to dry them thoroughly before further processing.

II.3.5.10. Deparaffinization and Rehydration

-The sections were deparaffinized by immersing them in xylene for 30 minutes to remove the paraffin wax.

-The slides were then placed in the oven for an additional 5 minutes to ensure complete removal of paraffin.

-Subsequently, the slides were rehydrated by passing them through decreasing concentrations of ethanol: 99%, 85%, and 70%, with 3 minutes in each concentration.

- Finally, the sections were rinsed in clean water for 1 minute.

II.3.5.11. Staining Procedures

After the deparaffinization and rehydration steps, the tissue sections were subjected to a series of stains:

Two different staining techniques were performed in order to evaluate the histological architecture of the collected tissues: **Hematoxylin and Eosin (H&E)** staining and **Masson's Trichrome** staining.

- **Common Initial Step**

After deparaffinization and rehydration, all tissue sections were first stained with Harris's hematoxylin for 45 seconds. This step allowed visualization of cell nuclei. The slides were then rinsed under running tap water for 5 minutes to ensure proper differentiation and bluing of the hematoxylin.

- Following this step, the slides were separated into two groups for the respective staining procedures.

- **Hematoxylin and Eosin (H&E) Staining**

-After hematoxylin staining and the 5-minute rinse, the slides intended for H&E were immediately immersed in eosin for 30 seconds. This step stains the cytoplasm and extracellular matrix in varying shades of pink.

-A quick rinse in clear water was then performed to remove excess eosin without affecting staining intensity.

-After staining, the slides were dehydrated through graded alcohols, cleared in xylene, and mounted with coverslips using a permanent mounting medium.

- **Masson's Trichrome Staining**

-Following the common hematoxylin and rinse steps, the slides designated for Masson's Trichrome were immersed in fuchsin solution for 30 seconds to stain the cytoplasmic components.

-A brief rinse in clear water was carried out.

-Slides were then transferred into phosphomolybdic acid for 5 minutes, which serves to differentiate the tissues and prepare collagen for specific staining.

-Immediately after, the sections were stained with light green for 4 minutes, allowing selective coloring of collagen fibers.

-A quick rinse in water followed.

II.3.5.12. Microscopic Observation and Interpretation of Histological Samples

Tissue sections were observed under a light microscope. Representative images were taken, and a basic interpretation of the morphological features was performed to compare the different treatment groups.

1. Immersion of organs in formalin before cassette

2. Automatic tissue processor for dehydration, clearing, and paraffin infiltration

3. Embedding cassettes immersed in molten

4. Detachment of the upper part of the cassette to access the fixed tissue

5. Orientation of tissue samples during embedding

6. Pouring molten paraffin into the mold

7. Placement of cassettes on the cold plate for paraffin solidification

8. Unmolding of the paraffin block prior to microtomy

9. Cutting thin tissue sections from paraffin blocks using a microtome.

10. Water bath for section flotation after microtomy

11. Stained slides arranged in metal racks before coloration

12. Baths and reagents used in Hematoxylin-Eosin and Masson's Trichrome staining protocols

13. Slides after staining, prepared for microscopic interpretation.

14. Observation of tissue sections under a light microscope

Figure 8: Histological examination and staining.

II.3.6. Statistical Analysis

Statistical analysis was conducted using Microsoft Excel® 2016. All experimental data were expressed as means. Statistical comparisons between groups were performed using the Student’s t-test to assess significant differences. The coefficient of determination (R^2) was calculated to evaluate the correlation between quantitative varibles (organ weights and diffrent drug doses), and the EC_{50} was determined to quantify treatment efficacy. A p-value

Chapter III : Results and Discussion

In this section, the results of the in vivo bioassays are presented and analyzed. The experimental design involved subcutaneous injections of gonadotropin-based formulations in immature Wistar rats, followed by organ collection, histological examination, and comparative assessment of drug effects. The discussion integrates these findings with current knowledge on gonadotropin pharmacology and the expected physiological outcomes in both sexes.

III.1. Results and discussion

III.1.1. Biometrical Results

III.1.1.1. Ovarian Weight in Treated Female Rats

- **Variation of ovaries weight in female treated rats**

Ovarian weight was selected as the principal morphofunctional indicator of the ovarian response to hormonal stimulation in immature female rats.

To evaluate the initial ovarian responsiveness to exogenous gonadotropin stimulation, the mean ovarian weights of the control group (receiving diluent only) and the group treated with the lowest dose of **Drug 2** (2 IU/ml), a reference biotherapy, was compared. The results showed a significant increase ($p < 0.001$) in ovarian weight in the treated group compared to the control group (**Table 4**). These findings confirm the bioactivity of the lowest administered dose of Drug 2 and validate the experimental approach. This supports the use of this group as a reference for subsequent dose–response analyses investigating the pharmacodynamic effects of the tested treatments.

Table 4 : Ovarian Weight: Control vs. Low Dose Follitropin Alfa + hCG.

Parameters/groups	Control	Low dose of drug 1	p value
Ovary weight (g)	45.108 ± 14.46	132.024 ± 19.79	p < 0.001

❖ **Dose–Response Relationship of drug 2 (Follitropin alfa + hCG) on Ovarian Weight**

To evaluate the ovarian response according to the administered dose of **drug 2** (follitropin alfa + hCG), a quantitative dose–response analysis was conducted on three groups of female rats that received increasing concentrations: low (2 IU/mL), medium (4 IU/mL), and high (8 IU/mL). Each group consisted of five animals, and the mean ovarian weight was calculated for each dose **(Table 5).**

A linear regression analysis was performed, plotting the **mean ovarian weight** (y-axis) against the **administered dose** (x-axis). The trendline equation and the **coefficient of determination** (R2) were automatically generated by the software.

The resulting model showed a strong positive linear relationship between the dose and the ovarian response (**Figure 9**), with a high coefficient of determination (R^2=0.9958) indicating an excellent fit of the linear model to the experimental data.

From this regression model, the **half-maximal effective concentration (EC_{50})** corresponding to the dose at which 50% of the maximal biological effect is observed was calculated. The EC_{50} value was estimated at **2.0185 IU/mL** (**Table 6**). These findings highlight a marked dose-dependent ovarian response to drug 2 and confirm its **increasing biological effectiveness** with rising concentrations.

Table 5 : Mean ovarian weights in female rats treated with increasing doses of drug 2 .

Dose UI/ml	average weight
2	132,024
4	186,912
8	272,434

❖ **Dose–Response Relationship of drug 1 (Follitropin alfa + Lutropin alfa + hCG) on Ovarian Weight**

To further characterize the pharmacodynamic activity of **drug 1** (a combination of follitropin alfa, lutropin alfa, and hCG) a dose–response evaluation was performed using a linear regression model. Three escalating doses (2, 4, and 6 IU/mL) were administered to immature female rats, and the resulting mean ovarian weights were measured. These values were plotted against the administered doses (**Table 7**), and a regression analysis was carried out**.**

The resulting curve demonstrated a strong linear relationship between dose and ovarian response (**Figure 10**), with a coefficient of determination (R^2) of 0.9981, indicating excellent goodness of fit. The derived equation allowed estimation of the effective concentration required to reach 50% of the maximal observed effect (EC_{50}), which was calculated to be approximately **1.69 IU/mL** (**Table 8**). These results confirm the high biological potency of the combination therapy, even at relatively low doses, and suggest a synergistic or additive effect among the three gonadotropin components. This justifies its consideration as a clinically relevant formulation for inducing follicular development under controlled experimental conditions.

Table 7 : Mean ovarian weights in female rats treated with increasing doses of drug 1.

Dose UI/ml	average weigh
2	147,608
4	198,876
8	285,5

III.1.1.2.. Seminal Vesicle Weights in Treated Male Rats

❖ **Variation of Seminal Vesicle Weights in Treated Male Rats**

The seminal vesicle weights were selected as reliable indicators of exogenous gonadotropic activity, due to their strong dependence on circulating androgens.

To evaluate the initial seminal vesicle responsiveness to exogenous gonadotropin stimulation, the mean seminal vesicle weights of the control group (receiving diluent only) and the group treated with the lowest dose of **Drug 3** (5 IU/ml), a reference biotherapy, was compared. The results showed no statistically significant difference ($p > 0.05$) in seminal vesicle weight in the treated group compared to the control group (**Table 9**).

The treatment with exogenous gonadotropins in this group did not induce a measurable increase in seminal vesicle weight in immature male rats. This result suggests that, at the tested dose and developmental stage, Drug 3 did not sufficiently stimulate endogenous testosterone secretion, or the vesicles may not yet have reached full androgenic responsiveness.

Table 9 : Seminal vesicle weight: Control vs. Low Dose of drug 3.

Parameters/groups	Control	Low dose of drug 3	p value
Seminal vesicle weight (g)	26.11 ± 6.08	29.95 ± 14.54	$p > 0.05$

- **Dose–Response Relationship of drug 3 (Menotropin) on Seminal Vesicle Weight**

The analysis of the dose–response relationship between drug 3 administration and seminal vesicle weight revealed a progressive increase in organ weight with rising doses of the hormone (5, 10, and 20 IU/mL). The data were plotted on a linear regression curve (**Table 10**), which yielded a coefficient of determination (**R^2 = 0.9515**) (**Figure 11**), indicating a strong and consistent correlation between the administered dose and the observed biological response.

The regression equation obtained from the curve allowed for the estimation of the **half-maximal effective concentration (EC_{50})**, which was calculated to be **4.16 IU/ml** (**Table 11**). This value represents the theoretical dose at which 50% of the maximal stimulatory effect on seminal vesicle weight is achieved.

These results demonstrate that menotropin elicits a clear and dose-dependent effect on the growth of the seminal vesicles, with high linearity and biological responsiveness within the tested dose range.

Table 10 : Administered Doses of Menotropin and Corresponding Mean Seminal Vesicle Weights in Male Rats.

Dose UI/ml	average weight
5	50,95
10	55,716
20	90,19

- **Dose–Response Relationship of drug 1 (follitropin alfa + lutropine alfa) on Seminal Vesicle Weight**

The analysis of the dose–response relationship between combined FSH and LH administration and seminal vesicle weight showed a dose-dependent increase in organ mass across the three tested concentrations (5, 10, and 20 IU/mL). The plotted data were fitted to a linear regression model (**Table 12**), resulting in a high coefficient of determination (**R^2 = 0.9043**) (**Figure 12**), indicating a strong positive correlation between the administered gonadotropin dose and the biological response observed.

The regression equation derived from the model allowed for the calculation of the **half-maximal effective concentration (EC_{50})**, which was estimated at **1.03 IU/ml** (**Table 13**). This value represents the theoretical dose required to induce 50% of the maximal increase in seminal vesicle weight within the experimental conditions.

These findings confirm that the combined administration of FSH and LH induces a marked and dose-proportional trophic effect on seminal vesicle development in immature male rats, as reflected by the high degree of linearity and the low EC_{50} value observed.

Table 12: Administered Doses of FSH + LH and Corresponding Mean Seminal Vesicle Weights in Immature Male Rats.

Dose UI/ml	average weigh
5	72,874
10	73,69
20	110,638

III.1.1.3. Prostate Weights in Treated Male Rats

❖ **Variation of prostate Weights in Treated Male Rats**

To evaluate the potential effect of low-dose of drug 3 (menotropin: 5 IU/mL) on prostate development, a comparaison the mean prostate weight between the control group and the group receiving the low dose of drug 3 was performed. Results revealed no statistically significant difference ($p > 0.05$) in prostate weight between the untreated controls and rats administered the low dose of menotropin (**Table 14**).

This result suggests that, at the tested low dose, menotropin did not exert a measurable or significant stimulatory effect on prostate weight in immature male rats.

Table 14 : Individual Prostate Weights in Control and Low-Dose Menotropin Groups.

Parameters/groups	Control	Low dose of drug 3	p value
Prostate weight (g)	26.76 ± 7.44	21.37 ± 8.50	$p > 0.05$

❖ **Dose–Response Relationship of drug 3 (Menotropin) on Prostate Weight**

The dose–response analysis of prostate weight following menotropin administration revealed a progressive and proportional increase in prostatic mass with escalating doses of the hormone (5, 10, and 20 IU/mL). A linear regression analysis was conducted based on the mean prostate weights corresponding to each administered dose (**Table 15**). The resulting regression model displayed a high degree of linearity, with a **coefficient of determination (R^2) equal to 0.9899**, indicating an almost perfect correlation between the dose and the observed organ response (**Figure 13**).

The fitted regression equation enabled the estimation of the **half-maximal effective concentration (EC_{50})**, calculated to be **3.51 IU/ml** (**Table 16**). This value represents the theoretical dose at which 50% of the maximal prostatic response is achieved under the tested experimental conditions.

The strong linearity of the dose–response relationship and the low EC_{50} value provide robust evidence of the high sensitivity of the immature rat prostate to exogenous gonadotropic stimulation. The effect is consistent with the androgen-dependent nature of the prostate and reflects the stimulatory role of menotropin particularly its LH component, which promotes Leydig cell activation and subsequent testosterone secretion..

Table 16 : Estimated EC_{50} Value for Prostate Weight Following Menotropin Administration.

Dose UI/ml	average weight
5	213,65
10	253,818
20	380,33

- **Dose–Response Relationship of drug 1 (follitropine alfa +lutropine alfa) on Prostate Weight**

The dose–response analysis of prostate weight following administration of combined follitropin alfa (rFSH) and lutropin alfa (rLH) demonstrated a perfectly linear relationship across the tested doses (5, 10, and 20 IU/mL) (**Table 17**), as reflected by a **coefficient of determination (R^2) of 1.000**. This indicates an exact fit of the regression model to the empirical data, with no deviation from linearity.

Despite the strength of the correlation, the **half-maximal effective concentration (EC_{50})** could not be accurately determined from the regression equation. This limitation is likely due to the absence of data points at lower doses, which would be necessary to observe the inflection of the dose–response curve and allow a reliable estimation of the dose producing 50% of the maximal effect. The lack of such sub-threshold values prevents the model from identifying a proper sigmoidal behavior or extrapolating an EC_{50} within the biological range.

These findings suggest that all tested doses of drug 1 fall within the ascending linear portion of the response curve, or possibly even within the maximal efficacy range. Additional experimentation using lower doses would be required to more accurately characterize the full pharmacodynamic profile and determine the threshold of response onset.

Table 17 : Administered Doses of Follitropin Alfa + Lutropin Alfa and Corresponding Mean Prostate Weights in Immature Male Rats.

Dose UI/ml	average weigh
5	258,978
10	291,94
20	356,19

III.1.2. Histological Results

III.1.2.1. Ovarian histology

Histological analyses were performed on ovarian sections stained with hematoxylin-aeosin (H&E), examined under microscope objectifs of ×4 and ×10. This allowed for a detailed assessment of ovarian structural organization and follicular dynamics across the different experimental groups.

- **Ovarian histology in negative Control**

According to **Figure 15** (G × 40)**,** the ovarian histology in negative control (CON-) showed early-stage developing follicles (primary and secondary) are clearly visible, along with a few tertiary follicles. The absence of antral and ovulatory follicles, as well as corpora lutea and cysts, indicates a lack of full follicular maturation and spontaneous ovulation .

In the same manner, the structure of ovaries in rats treated with positive control (T) showed a cortex with preserved tissue architecture. Follicular density is low, with a few structures suggestive of follicles at various developmental stages sparsely distributed throughout the parenchyma. No antral cavities or signs of ovulatory activity are identified.

- **Ovarian histology in female rats treated with drug 2 (Follitropin alpha)**

Depending on the photomicrographs in **Figure 15**, there is a moderate presence of primary, secondary, tertiary, antral, and ovulatory follicles in female group treated with **low dose** of drug 2. Antral (cavitary) follicles are distinctly visible. However, the absence of corpora lutea suggests that ovulation was not fully achieved, despite clear follicular stimulation. When the females are treated with a **medium dose** of drug 2, Primary follicles are absent. Secondary to ovulatory follicles are present, though in limited numbers. Corpora lutea are moderately abundant, and some exhibit features of cystic transformation (thinned wall, dilated lumen). Additional cystic structures are also present, indicating disrupted ovarian activity. Some corpora lutea appear to be undergoing cystic degeneration. There is a complete absence of follicles at any developmental stage in female group treated with a **high dose** of drug 2. Corpora lutea are numerous but do not show signs of cystic transformation. Large, thin-walled cysts occupy most of the cortical area. This pattern is indicative of severe ovarian hyperstimulation leading to suppression of normal follicular growth.

- **Ovarian histology in female rats treated with drug 1 (Follitropin alpha + Lutropin alpha)**

According to the photomicrographs in **Figure 15, n**o primary or secondary follicles are observed in the ovary of rats treated with **low dose** drug 1. The visible follicles are in advanced stages (tertiary and antral). Ovulatory follicles are notably absent. However, corpora lutea are highly abundant, as are cystic structures. This profile suggests partial follicular activation followed by functional disorganization. In feamle rats treated with **medium dose**, there is an abundant presence of primary, secondary, and tertiary follicles. Antral follicles are absent. Ovulatory follicles are moderately represented and accompanied by a large number of corpora lutea and cysts. These findings reflect an effective but imperfectly coordinated stimulation, resulting in partial ovulation. However, the rats ovaries in group treated with **high dose** showed a predominance of ovulatory follicles. All other follicular stages are absent. A few corpora lutea persist, while numerous cystic formations are present. This pattern reflects an extreme ovarian response, suggestive of massive and dysregulated ovulation.

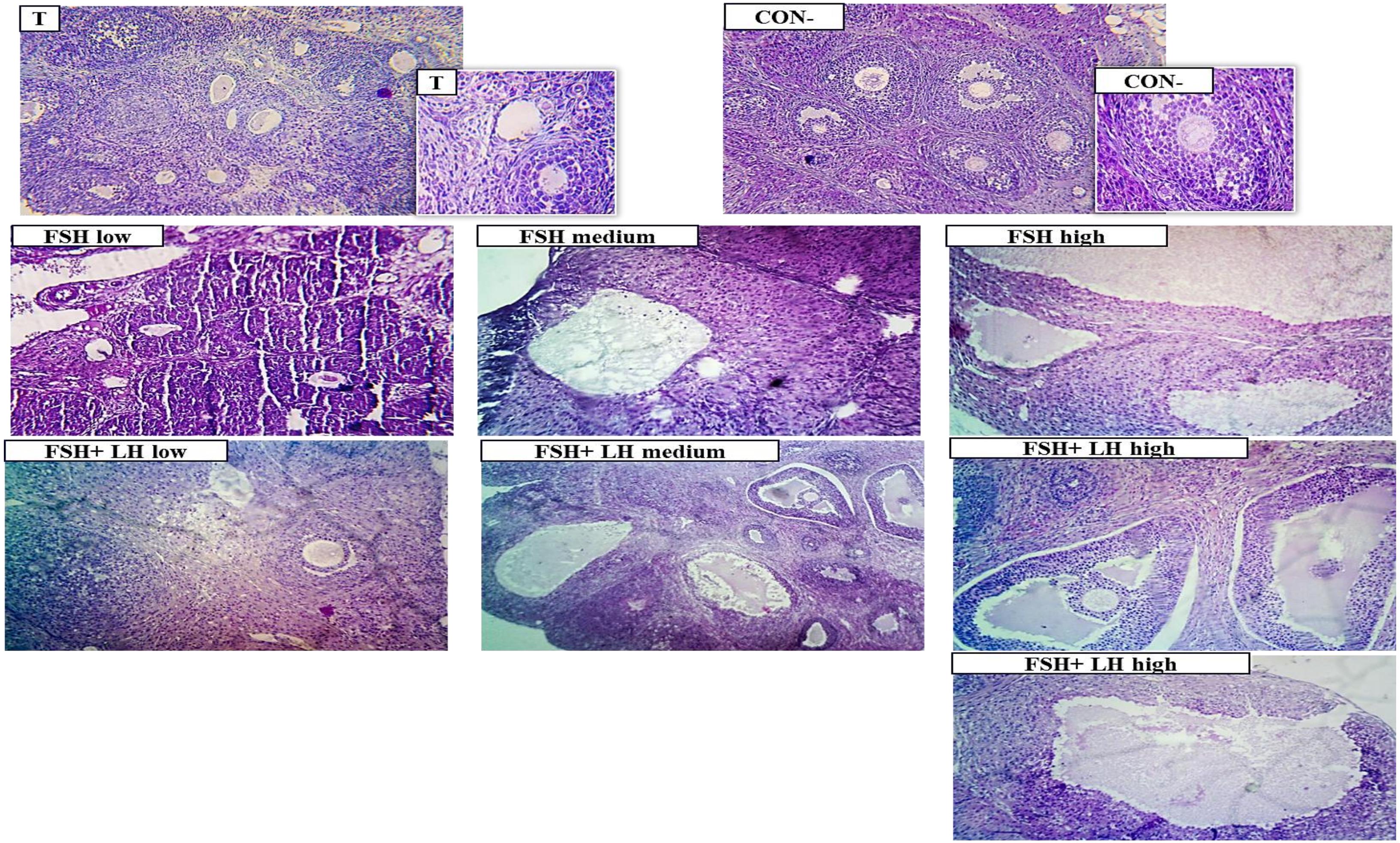

Figure 15 : Photomicrographs of Ovarian sections from all Experimental Groups of female rats. H&E staining (G × 40).

III.1.2.2. Histological observations of prostate in male rats

Histological analysis was conducted on two male accessory reproductive organs: the prostate and the seminal vesicles. As in females, each organ was examined under three magnifications (x4, x10, and x40). The study included a negative control group and two treatment groups: one receiving recombinant gonadotropins (drug 1: FSH + LH), and the other treated with drug 3 (menotropins). Each treatment was administered at three different doses (low, medium, and high), allowing for a detailed comparative assessment of dose-dependent effects on the histoarchitecture of these organs.

❖ **Prostate histology in negative Control**

At low magnification (**Figure 17**), the prostatic tissue displays a well-organized architecture with clearly defined glandular units. The acini are regularly distributed within a loose and uniform stromal matrix. No signs of structural disorganization or inflammation are observed.

❖ **Prosate histology in female rats treated with drug 3 (Menotropin)**

◆ **Low dose**

The prostate parenchyma in low magnification retains a globally organized structure (**Figure 17**). The acinar lumens appear slightly reduced in size compared to the control, while the interstitial space remains open and loose. No marked hypertrophy or hyperplasia is seen at this magnification.

◆ **Medium Dose**

The glandular organization is preserved, but the acinar units appear slightly enlarged. The stroma remains present but begins to show reduced interacinar spacing. No overt disorganization is visible (**Figure 17**).

◆ **High dose**

The glandular tissue appears hyperplastic, with closely packed acini and reduced stromal space. The overall architecture is denser, and the acinar profiles are more irregular compared to lower doses (**Figure 17**).

❖ Prostate histology in female rats treated with drug 1 (Follitropin alpha + Lutropin alpha)

◆ Low dose

The tissue architecture is preserved but with slightly increased acinar size. The stroma is less prominent than in the control, indicating a mild trophic effect (**Figure 17**).

◆ Medium Dose

The prostate exhibits more compact architecture with further reduction in stromal spacing. Acini are more numerous and closely arranged (**Figure 17**).

◆ High Dose

Marked glandular hyperplasia is observed. The acini are enlarged and highly compacted, with a very narrow stromal component. The tissue appears congested with little interstitial separation (**Figure 17**).

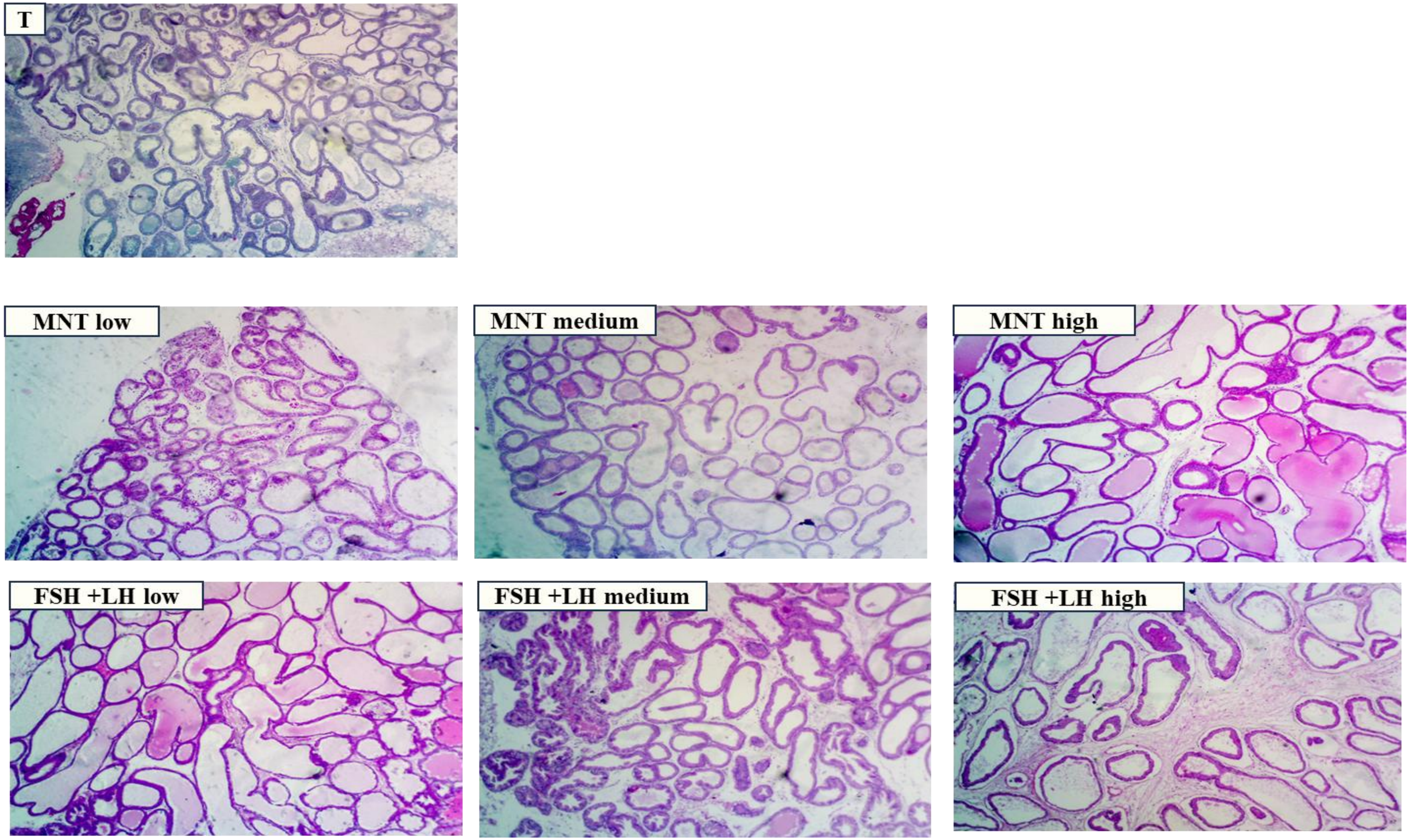

Figure 17 : Photomicrographs of prostate sections from all Experimental Groups of male rats. H&E staining (G × 40).

III.1.2.3. Histological observations of seminal vesicles in male rats

- **Seminal vesicles histology in negative Control**

The seminal vesicles exhibit a simple lobular structure with narrow lumens and limited epithelial complexity. No signs of hypertrophy or glandular stimulation are observed (**Figure 20**).

- **Seminal vesicles histology in female rats treated with drug 3 (Menotropin)**

  - **Low dose**

Slight enlargement of glandular lobules is observed, with moderately dilated lumens and denser tissue organization compared to control (**Figure 20**).

  - **Medium Dose**

Glandular tissue is clearly hypertrophied, with well-developed lobular structures and significantly expanded luminal spaces (**Figure 20**).

  - **High Dose**

Pronounced hypertrophy of the seminal vesicles, with extensively enlarged lobules and lumens filled with dense secretory material (**Figure 20**).

- **Seminal vesicles histology in female rats treated with drug 1 (Follitropin alpha + Lutropin alpha)**

  - **Low dose**

Glandular organization remains similar to control, with minimal increase in lobular size and narrow lumens (**Figure 20**).

  - **Medium Dose**

Moderate glandular development is observed, with more visible lobules and slightly enlarged lumens (**Figure 20**).

◆ **High Dose**

Clear hypertrophy of the seminal vesicles, with enlarged lobular structures and lumens visibly filled with secretory material (**Figure 20**).

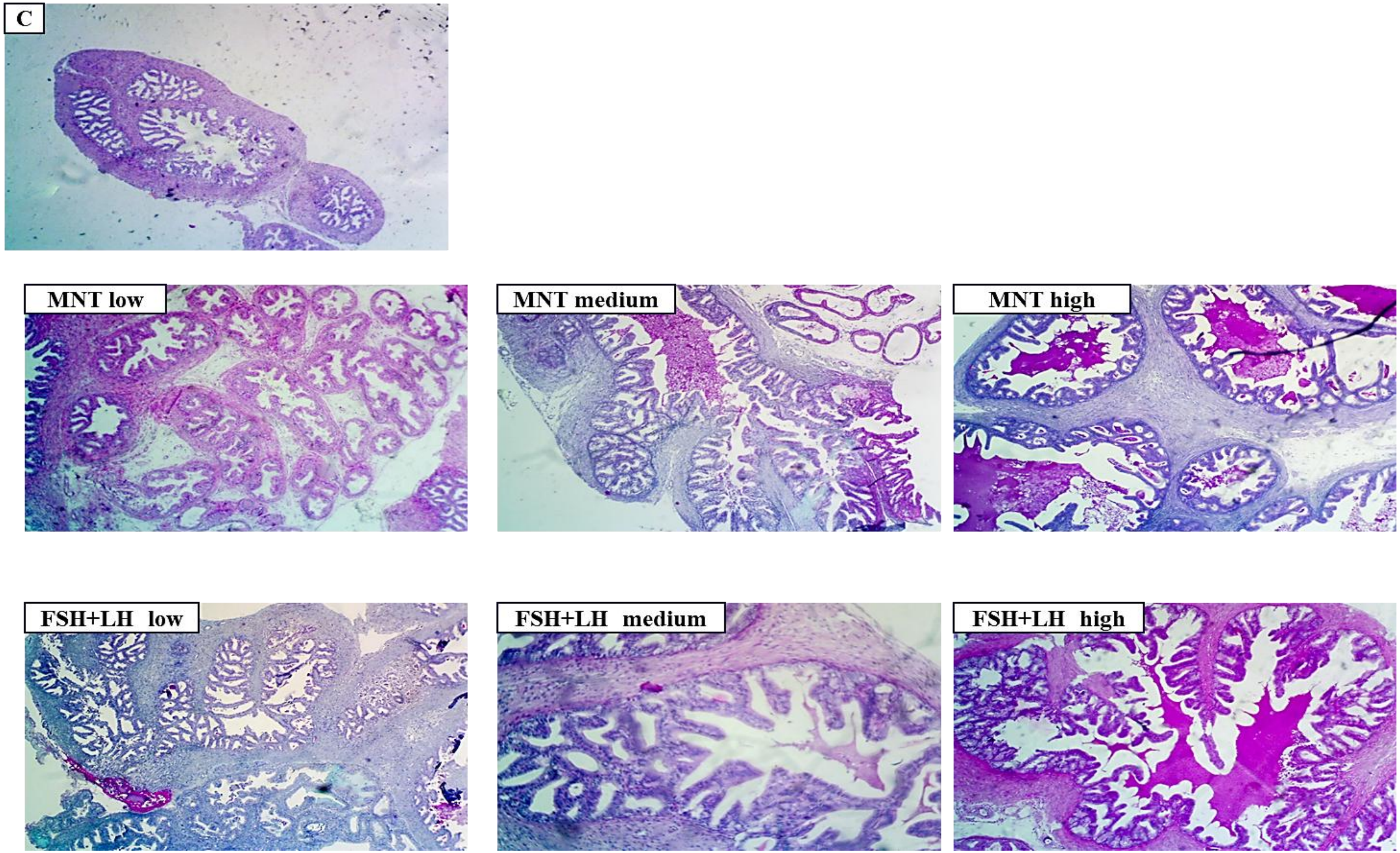

Figure 20: Photomicrographs of seminal vesicles sections from all Experimental Groups of male rats. H&E staining (G × 40).

III.1.3. Hormonal Assay Results

Table 18 presents the effect of different doses of drug 1 (FSH+LH) treatment on LH concentration (IU/L) in male rats. Unfortunatly, the level of LH was analyzed only in three treatment groups of male experimentation. The levels of LH and FSH were not performed in the remaining groups of male experimentation and all groups of female experimentation because the quantity of serum was not sufficient.

According to **Figure 23**, there is a dose-dependent increase in LH concentration with escalating doses of drug 1, suggesting that higher hormonal stimulation leads to greater circulating LH levels.

Table 18 : Serum LH concentrations in male rats after FSH/LH treatment.

Treatment Group	LH Concentration (IU/L)
Drug 1 : Low dose	0.90
Drug 1 : Medium dose	2.28
Drug 1 : High dose	2.95

CONCLUSION

This experimental study provides robust evidence for the biological activity of exogenous gonadotrophins in immature Wistar rats of both sexes. In females, the administration of recombinant FSH, either in combination with LH or hCG, induced marked ovarian maturation, as evidenced by increased ovarian weights, the appearance of advanced follicular stages, and the development of corpora lutea, particularly at higher doses. These findings reflect the synergistic roles of FSH and LH/hCG in stimulating folliculogenesis and ovulation. In males, both menotropins and the combination of FSH with LH significantly stimulated the maturation of accessory sex glands, notably the prostate and seminal vesicles, as demonstrated by histological changes such as epithelial proliferation, glandular expansion, and secretory activation.

Overall, the dose-dependent responses observed in both sexes highlight the pharmacological relevance and tissue-specific actions of gonadotrophins during the prepubertal period. These results validate the use of this animal model for the comparative evaluation of gonadotrophin-based biomedicines and biosimilars. Furthermore, the data underscore the necessity of precisely optimizing dose and hormonal combinations to achieve targeted endocrine outcomes. This work lays the foundation for future investigations into the mechanistic pathways involved and supports the rational design of reproductive therapies.

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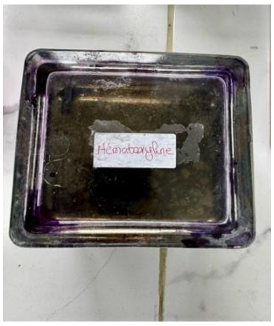
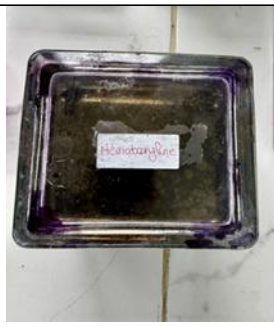
Appendices

Appendix 1

Table 1: Reagents and solutions used

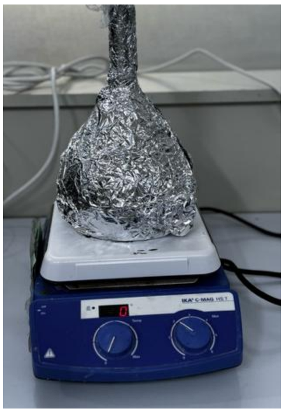
Reagent / solution	Use in the experiment	Figure
Phosphate-albumin buffer	**Prepared to perform accurate dilutions of gonadotropin solutions prior to injection.**	
Gonadotropin solution for female rats		
Gonadotropin solution for male rats	**Use in the experiment**	

Phenol	**Used as a preservative in the phosphate-albumin buffer to prevent microbial contamination.**	
Bovine serum albumine BSA)	**Added to the buffer solution to stabilize proteins and prevent non-specific adsorption during preparation and storage.**	
Formaldehyde	**Used as a fixative to preserve dissected organs (ovaries, prostates, and seminal vesicles) for histological analysis.**	
Hematoxyline	**Used to stain cell nuclei in tissue sections, providing contrast and allowing identification of nuclear morphology.**	

Eosin	**Used as a counterstain with hematoxylin; it stains the cytoplasm, extracellular matrix, and connective tissue in varying shades of pink and red.**	
Light green SF yellowish	**Used in specific staining protocols (e.g., Masson's trichrome) to stain collagen fibers green, aiding in the visualization of connective tissue.**	
Fuchsin	**Used in various histological staining techniques to stain cytoplasmic structures or muscle fibers, often in trichrome stains.**	
Phosphomolybdic acid	**Serves as a mordant or differentiator in complex staining protocols, such as Masson's trichrome, to enhance tissue contrast and color specificity**	
Xylene	**Used as a clearing agent during the histological process, particularly for deparaffinization and preparation of slides for mounting.**	

Paraffin wax	**Used to embed fixed tissue samples, allowing for precise microtome sectioning of the specimens into thin slices for microscopic analysis**	
Eukitt® mounting medium	**Used to mount stained histological tissue sections on microscope slides, ensuring long-term preservation and optimal optical clarity for microscopic examination**	

Table 2: Materials and equipment used during the experiment.

Materials	**Use in the experiment**	**Figure**
Electronic balance	**Used to weigh the rats before administration of the treatment.**	
Analytical balance	**Used to accurately weigh the reagents required for solution preparation, as well as the ovaries, prostates, and seminal vesicles after dissection.**	
PH meter	**Used to adjust the pH of the phosphate-albumin buffer solution during its preparation.**	
Magnetic stirrer	**Used to ensure homogeneous mixing of the prepared solutions.**	

Fume hood	**Used to safely handle formalin and prevent exposure to toxic vapors**	
Water bath	**Used in histological processing to stretch and flatten paraffin tissue sections before placing them on slides.**	
Microtome	**Used to cut thin sections (5 µm) from paraffin-embedded tissue blocks for histological analysis.**	
Laboraty oven	**Used to dry laboratory materials and histological slides before staining.**	

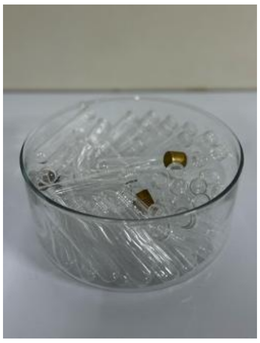
Tissue processor (automated)	**Used for automatic dehydration, clearing, and paraffin embedding of tissue samples during histological processing.**	
Refrigerator	**Used to store prepared solutions at appropriate temperatures to preserve their stability and effectiveness.**	
Light microscope	**Used to observe stained tissue sections mounted on slides for histological evaluation.**	

Table 3: Small Equipment and Laboratory Supplies.

Item	**Use in the experiment**	**Figure**
Micropipettes	**Used during the preparation of solutions requiring precise measurement of very small liquid volumes.**	

Glass pipettes	**Also used for accurately transferring small volumes of liquids, especially when preparing dilutions of reagents.**	
Micropipette tips	**Sterile disposable tips used with micropipettes to ensure accurate volume delivery and to prevent cross-contamination between different solutions.**	
Insulin syringes (1 mL)	**Used for subcutaneous and intraperitoneal injections of gonadotropins and anesthetics in rats, allowing precise administration of small volumes.**	
Syringes (10 ml)	**Used for cardiac puncture during blood collection, and for other procedures requiring the transfer of larger liquid volumes.**	

Microscope slides	**Used to mount histological tissue sections for microscopic examination.**	
Cover slips (microscope coverslips)	**Placed over the mounted tissue sections on slides to protect the sample and allow high-quality microscopic observation.**	
Tubes racks	**Used to support and organize tubes of different volumes during sample preparation, centrifugation, or storage.**	

Microscope slide rack	**Used to hold and organize microscope slides during the staining and drying processes.**	
Petri dishes	**Used to temporarily place and organize dissected organs before weighing or fixation.**	
Dissection tools	**Includes scalpels, scissors, forceps, and probes used for precise dissection and removal of organs.**	
Glass tubes	**Used to store dissected organs in fixatives or preservative solutions for further analysis.**	

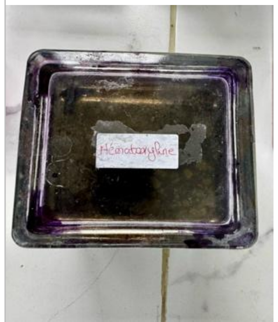
Joseph paper	**Absorbent paper used during dissection to maintain a clean working area and absorb blood or other fluids.**	

Table 4: Chemical products used during the histological study

Product	*Chemical composition*	***Purpose in the protocol***	*Toxicity*	*Precautions*	*Product image*
Phenol	**C6H5OH**	**Employed in tissue processing for its protein-denaturing properties, aiding in the fixation and preservation of .biological specimens**	**Phenol is corrosive and can cause severe burns upon contact . it is toxic if inhaled , ingested, or absorbed through the skin , potentially leading to systemic poisoning**	Utilize in a well-ventilated area or fume hood. Wear appropriate personal protectieve equipement (PPE) , including gloves, lab coat, and eye protection. Avoid inhalation and direct contact with skin and .eyes	

Formaldehyde	**CH_2O**	**Use as a fixative to preserve tissue morphology by cross-linking proteins , essential for subsequent histological . examinations**	**Formaldehyde is a potent irritant and a known human carcinogen. Exposure can cause respiratory distress , skin irritation, and . sensitization**	**Handle within a certified chemical fume hood . Employ PPE such as gloves, safety googgles and lab coat. Ensure proper storage in sealed container to prevent vapor . release**	
Xylene	**C_8H_{10}**	Serves as a clearing agent in histology, facilitating the removal of alcohol and making tissues transparent .before embedding	**Xylene is a flammable and poses health risks upon inhalation, including dizziness , headache , and potential long-term effects on the nervous .system**	**Operate in a fume hood to avoid inhalation. Use flame-resistant lab coats, gloves . and eye protection . Store away from heat sources and .open flames**	

Ethanol	**$C2H5OH$**	Applied in dehydration steps during tissue processing, aiding in the removal of .water before embedding	**Ethanol is flammable and can cause central nervous system depression if ingested in large quantities. Vapors may cause irritation to eyes and respiratory . tract**	**Use in well-ventilated areas, away from ignition sources. Wear appropriate PPE, including gloves and eye protection. Store in tightly closed .containers**	
Eosin y	**$C20H6Br4Na2O5$**	Functions as an acidic dye in histological staining, providing contrast by staining cytoplasmic components and .extracellular matrix	**May cause skin and eye irritation upon contact. Inhalation of dust or fumes can lead to respiratory tract**		

			.irritation		
Hematoxylin	$C_{16}H_{14}O_6$	Utilized as a nuclear stain in histological procedures, particularly in Hematoxylin and Eosin (H&E) staining, .to highlight cell nuclei	May cause eye irritation upon .contact		
Light Green SF Yellowish	$C_{37}H_{34}N_2Na_2O_9S_3$	Serves as a counterstain in histological staining, providing contrast by staining collagen and other .tissue components	May cause skin and eye irritation upon .contact		
Fuchsin	$C_{20}H_{17}N_3Na_2O_9S_3$	Employed in histology for staining cytoplasmic components and connective tissues, aiding in tissue .differentiation	Can cause skin and eye irritation; harmful if swallowed or .inhaled		

EUKITT	Poly(butyl methacrylate-co-methyl methacrylate); contains 45% acrylic resin and 55% xylenes	Used for mounting and preserving stained tissue sections on microscope slides, providing a clear and durable medium.	Flammable; inhalation of vapors may cause dizziness or respiratory irritation.		

PEOPLE'S DEMOCRATIC REPUBLIC OF ALGERIA
MINISTRY OF HIGHER EDUCATION AND SCIENTIFIC RESEARCH
UNIVERSITY OF BLIDA 1

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Department of Biology

Dissertation

Dissertation Submitted to the Department of Biology as a Partial Fulfillment for the Requirement for the Degree of Master in the field of nature and life sciences

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Speciality : **Pharmacotoxicology**

Thème

Evaluation of the Pharmacological Effect of Gonadotropin-Based Drugs

قسم البيولوجيا
المكلف بالدراسات
و المسائل المرتبطة
بالطلبة

Presented by:

Miss Morsi Lydia Fatma Zohra

Defense Date: 08/07/2025

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