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ETHINYL ESTRADIOL–LEVONORGESTREL CONTRACEPTIVE BAIT IMPAIRS TESTICULAR FUNCTION AND INDUCES GERM-CELL APOPTOSIS IN MALE RATS

By

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A thesis submitted in fulfilment of the requirements of the degree of Doctor of Veterinary Medicine

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ETHINYL ESTRADIOL–LEVONORGESTREL CONTRACEPTIVE BAIT IMPAIRS TESTICULAR FUNCTION AND INDUCES GERM-CELL APOPTOSIS IN MALE RATS

ABSTRACT

Hormonal baiting is being studied as an innovative fertility control method in rodents, with most studies focusing on quinestrol + levonorgestrel (EP1). However, no study has investigated ethinylestradiol-levonorgestrel (EE-LNG) bait formulation for male rats, and no studies have reported investigating multi-organ tissue integrity using AO/PI, DAPI, and H&E staining for histopathological analysis. The objective of the present study is to investigate the toxicological and antifertility effects of an EE-LNG bait in male rats. Eighteen adult male rats were divided into three groups, namely control, low dose, and high dose. They were fed the EE-LNG bait for 14 days. Serum testosterone levels were quantified using ELISA. Furthermore, histopathology and the DNA and nuclear integrity of the testis, liver, and kidney were carried out using H&E, DAPI, and AO/PI staining methods. The results revealed that both EE-LNG doses result in the reduced weight of the testes and epididymis relative to the control and significantly suppressed serum testosterone levels in a dose-dependent manner ($P<0.00000001$). Testicular, hepatic, and renal tissues showed degenerative histopathology, with fragmented DNA and nuclear condensation confirmed by the H&E, AO/PI, and DAPI staining. This study represents the first research on an EE-LNG contraceptive bait in male rats, revealing that even 14 days of bait feeding is sufficient to suppress testosterone levels and impair multi-organ integrity. The innovative application of AO/PI and DAPI provided evidence of tissue-level toxicity, supporting the potential of EE-LNG bait as a candidate for fertility control.

Keywords: Ethinyl Estradiol- Levonorgestrel (EE-LNG), Endocrine disruption, Germ-cell apoptosis, Spermatogenesis, Testicular toxicity

KESAN UMPAN ETHINYL ESTRADIOL–LEVONORGESTREL TERHADAP FUNGSI TESTIKULAR DAN APOPTOSIS SEL GERMINAL TERHADAP TIKUS JANTAN

ABSTRAK

Penggunaan umpan hormon sedang dikaji sebagai kaedah inovatif bagi mengawal populasi tikus dan kebanyakan kajian memberi tumpuan kepada gabungan hormon quinestrol dan levonorgestrel (EP-1). Namun begitu, sehingga kini, tiada kajian yang meneliti formulasi umpan hormon ethinylestradiol dan levonorgestrel (EE-LNG) terhadap tikus jantan dan tiada kajian dilaporkan dalam menilai integriti tisu organ dengan menggunakan pewarnaan AO/PI, DAPI, dan H&E bagi analisa. Objektif kajian ini adalah untuk menyiasat kesan toksikologi dan antifertiliti umpan hormon EE-LNG dalam tikus jantan. Lapan belas ekor tikus jantan dewasa dibahagikan kepada tiga kumpulan iaitu kumpulan kawalan, kumpulan dos rendah, dan kumpulan dos tinggi. Mereka diberikan umpan EE-LNG selama 14 hari. Paras testosteron serum dinilai menggunakan ELISA dan histopatologi serta integriti DNA dan nuklear bagi tisu organ dinilai menggunakan pewarnaan AO/PI, DAPI, dan H&E. Hasil kajian menunjukkan bahawa umpan hormon EE-LNG menyebabkan pengurangan berat testis dan epididimis ($p=0.086$) berbanding kumpulan kawalan dan paras testosteron serum juga berkurang ($p<0.00000001$). Tisu organ juga menunjukkan perubahan degeneratif, frakmentasi DNA, dan kondensasi nuklear. Kajian ini merupakan penyelidikan pertama mengenai umpan hormon EE-LNG pada tikus jantan, menunjukkan bahawa tempoh 14 hari pemberian umpan hormon sudah mencukupi untuk menekan paras testosteron serum dan menjejaskan integriti tisu organ.

Kata kunci: Ethinyl Estradiol dan Levonorgestrel (EE-LNG), Ketoksikan testikular, Spermatogenesis, Gangguan Endokrin, Apoptosis sel germinal

CERTIFICATION

This is to certify that we have read this research paper entitled **"Ethinyl Estradiol–Levonorgestrel Contraceptive Bait Impairs Testicular Function And Induces Germ-Cell Apoptosis In Male Rats"** by **Norlaila binti Najib Paarthiban,** and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirements for the course DVT 55204 – Research Project.

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DEDICATIONS

In the name of Allah, the Most Gracious and Most Merciful. All praise and gratitude belong to Allah for giving me the strength and guidance to complete this thesis entitled, "Ethinyl Estradiol–Levonorgestrel Contraceptive Bait Impairs Testicular Function and Induces Germ-Cell Apoptosis in Male Rats."

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LISTS OF ABBREVIATIONS

ABP	-	Androgen-binding protein
AOPI	-	Acridine Orange/Propidium Iodide
DAPI	-	4′,6-diamidino-2-phenylindole
DF	-	Degrees of Freedom
EE	-	Ethinyl Estradiol
ELISA	-	Enzyme-Linked Immunosorbent Assay
EP-1	-	Combination of Quinestrol and Levonorgestrel
ER	-	Estrogen receptor
F (DFn, DFd)	-	F-statistics with numerator and denominator DF
FSH	-	Follicle-stimulating hormone
GnRH	-	Gonadotropin-releasing hormone
HD	-	High-Dose
HIT	-	Hyalinized Interstitial Tissues
HPG	-	Hypothalamic-pituitary-gonadal
LC	-	Leydig Cell

LD	-	Low-Dose
LH	-	Luteinizing hormone
LNG	-	Levonorgestrel
MS	-	Mean Square (SS–DF)
P-value	-	Probability value
PS	-	Primary Spermatocytes
QE	-	Quinestrol
R^2	-	Coefficient of determination
SC	-	Sertoli Cell
SD	-	Standard Deviation
SE	-	Standard Error
SEM	-	Structural Equation Modelling
SG	-	Spermatogonia
SS	-	Sum of Squares
ST	-	Seminiferous Tubules

CHAPTER 1

INTRODUCTION

Rodent infestations in urban and agricultural settings present a serious threat to infrastructure, food security, and public health. Traditional methods of pest control rely primarily on deadly poisons, particularly second-generation anticoagulant rodenticides (SGARs), which have significant environmental and welfare consequences despite their effectiveness. These persistent poisons bioaccumulate in the environment and frequently cause secondary poisoning in non-target wildlife that consumes poisoned rodents (Stuart *et al*., 2024). Modern pest management ethics, meanwhile, call for more humane solutions as they push for control measures that reduce animal suffering, as rodents are increasingly considered sentient creatures (De Ruyver *et al*., 2023). Given this, the contraception program has become a viable non-lethal approach, which may lower rodent populations while reducing animal suffering and without endangering other species or causing death.

Male contraceptive studies are gaining more attention in human and veterinary medicine as an innovative method for controlling animals recognised as pests, such as rodents (Selemani *et al*., 2022). Traditional population control methods are reported to have ecological and ethical implications and hormonal contraceptive bait is advocated as an alternative population control (Mikail *et al*., 2024). Previous studies have reported the suppression of male reproductive functions, such as reduced sperm count, motility, and organ weight, following treatment with combined hormonal contraceptives (EP-1) (Massawe *et al*., 2017). These findings suggest that hormonal contraceptive bait may impair fertility in rodents through endocrine-mediated mechanisms (Selemani *et al*., 2022). However, the cellular effects of such treatment are not well

understood. Hence, it is important to evaluate the impact on male reproductive tissues for safety, efficacy, and welfare compliance.

Testosterone is a secreted androgen hormone responsible for the development and maintenance of the male reproductive system and normal spermatogenesis (Das *et al.*, 2023; Weinbauer & Nieschlag, 1998). Changes in testosterone levels, testicular morphology, and germ-cell viability are key indicators of reproductive infertility. While extensive studies have been conducted on female hormonal contraception (Krapf & Goldstein, 2024), the effect of Ethinyl Estradiol and Levonorgestrel (EE-LNG) formulations on male reproductive organs has not been studied. Although this hormonal agent is reported to induce apoptotic responses in various tissues in female animals (Mikail *et al*., 2024), there is a lack of evidence to suggest such an effect in males exposed to EE-LNG bait. These gaps in knowledge limit our understanding of the potential reproductive risks associated with hormonal contraceptive baits in male animals.

1.1 Research Problem Statement

Traditional rodent control methods, such as poisons and traps, have negative environmental consequences and are becoming increasingly ineffective. Fertility control using oral contraceptives offers a promising alternative, but most research has focused on female fertility (Selemani *et al*., 2021). Relatively few studies have investigated how combined hormonal baits affect male rodents at the endocrine and tissue levels. In particular, the impact of feeding male rats with a combination of hormones of estrogen and progesterone bait on their hormone levels, reproductive organ histology, and sperm parameters is not well characterised. To advance ecological fertility control, it is necessary to test whether a combined hormonal bait can

effectively suppress male reproduction and further understand its physiological effects. This gap in knowledge constitutes the main problem addressed by this study.

1.2 Research Questions

1. Does combined hormonal bait (EE-LNG) cause significant changes to the hormonal profile of male rats after consumption?
2. Does combined hormonal bait (EE-LNG) cause histopathological changes in the reproductive tissues of male rats after consumption?
3. Does combined hormonal bait (EE-LNG) cause germ-cell apoptosis in the reproductive tissues of male rats after consumption?

1.3 Research Hypothesis

1. **Null Hypothesis (H_0):** Combined hormonal bait (EE-LNG) does not affect the hormonal profile or reproductive tissues of male rats.
2. **Alternative Hypothesis (H_1):** Combined hormonal bait (EE-LNG) significantly induces degenerative changes in male reproductive tissues and suppresses spermatogenesis.

1.4 Research Objectives

1. To determine the hormonal profile of male rats after consumption of combined hormonal bait (EE-LNG).
2. To evaluate tissue alterations in male reproductive tissues, including histopathological changes and germ-cell apoptosis, after combined hormonal bait (EE-LNG) treatment.

CHAPTER 2

LITERATURE REVIEWS

2.1 Normal Hypothalamic Regulation of Gonadotropins and Testicular Function

The regulation of male reproductive function mainly relies on the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus releases gonadotropin-releasing hormone (GnRH) in pulses. This pulsatile release is crucial for the pituitary to produce and release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). If GnRH stimulation is continuous or disrupted, it can lead to reduced LH production and changes in FSH levels (Acevedo‐Rodriguez *et al.*, 2018).

FSH targets Sertoli cells, boosting spermatogenesis by increasing cyclic adenosine monophosphate's (cAMP) levels and downstream signalling pathways. It also encourages the production of androgen-binding protein (ABP) and aromatase for making estrogen in the testicles. LH affects Leydig cells, activating enzymes that turn cholesterol into testosterone. This process generates high levels of testosterone in the testicles, which are necessary for normal sperm production. Testosterone provides negative feedback to both the hypothalamus and pituitary, reducing GnRH and LH secretion. FSH levels are significantly influenced by inhibin B, produced by Sertoli cells. Inhibin B lowers FSH release and adjusts how activin stimulates FSH. Together, these mechanisms ensure proper testosterone levels, Sertoli cell function, and spermatogenesis (Corradi *et al.*, 2016).

2.2 Mechanisms of hormonal impact on male fertility

Male hormonal contraception works mainly by suppressing the HPG axis. Exogenous steroids such as androgens, estrogens or progestins give feedback on the hypothalamus and pituitary to

lower GnRH, LH, and FSH secretion. This leads to a profound drop in intratesticular testosterone, which is essential for spermatogenesis. In practice, combining androgens with progestins or estrogens achieves more rapid and complete suppression than androgens alone, according to the study. These endocrine mechanisms impair sperm production, which, for instance, chronic estradiol administration in male rodents disrupts Sertoli-cell function and germ-cell development via estrogen receptor (ER) pathways, causing germ‑cell depletion. Consequently, male fertility is inhibited by central feedback via gonadotropin suppression and the direct impact of hormones on Leydig and Sertoli cells (Bania *et al.*, 2025).

2.3 Combined estrogen-progesterone contraceptives in rodents

A study demonstrated that levonorgestrel (LNG), quinestrol (QE), and their combination (EP-1) significantly impacted reproductive function in black rats following seven days of bait consumption. The female reproductive organs displayed noticeable swelling due to oedema, with an increase in uterus and ovary weights. In males, the mass of reproductive organs decreased, along with a significant drop in sperm production. Sperm counts decreased from 119.8 million/mL in the control group to 11.1 million/mL with QE at 50 ppm and 27.9 million/mL with EP-1 at 50 ppm. Sperm motility also fell, from 77.5% to 17.3% and 34%, respectively. Fertility outcomes mirrored these bodily changes. Untreated pairings produced a 70% pregnancy rate and eight pups per litter. In contrast, pairings with untreated females and treated males had only a 30% pregnancy rate and four pups per litter. Importantly, females that took QE at 50 ppm or EP-1 at 50 ppm had zero pregnancies, regardless of the male's treatment, showing complete short-term infertility caused by the combined estrogen and progestin contraceptive approach (Selemani *et al.*, 2021).

Another study was also conducted to look at the effects of levonorgestrel (LNG), quinestrol (QE), and their combination (EP-1) on male Sprague-Dawley rats. It found that a seven-day oral regimen caused quick and reversible subfertility. This phenomenon was marked by large drops in caudal sperm counts and mating success at Day 21. Recovery occurred by Day 42. The treatment led to shrinkage of seminiferous tubules, reduced androgen receptors, fewer Sertoli cells, and a significant increase in germ-cell apoptosis. These changes suggest impaired support from Sertoli cells and disrupted sperm production. Meanwhile, testosterone levels stayed mostly the same, which suggests the estrogen component acted locally in the testicles. These findings indicate that combined hormonal baits can disrupt testicular function and trigger germ-cell apoptosis in males. This result supports their potential use as reversible contraceptives for controlling rodent populations (Liu, Wan, *et al*., 2011).

Another study had shown that short-term exposure to quinestrol (QE), levonorgestrel (LNG), and their combination (QE+LNG) in bait at concentrations of 10, 50, and 100 ppm significantly harmed reproductive function in *Mastomys natalensis*. Bait intake dropped significantly at higher doses, with reductions of about 45% at 50–100 ppm. Treatment led to dose-dependent decreases in body mass, with nearly 20% loss in groups receiving QE and QE+LNG. In male rats, QE and QE+LNG caused serious suppression of the reproductive system. Testicular, epididymal, and seminal vesicle weights fell by around 60-80%. Sperm parameters were greatly harmed, with over 95% reductions in sperm concentration and motility, along with a notable increase in morphological abnormalities. In females, treated groups showed significant uterine oedema, and reproductive trials indicated that fertility was greatly reduced. Pregnancy did not occur in any pairing with males treated with QE or QE+LNG. Litter size also significantly decreased in surviving pregnancies. Overall, the findings suggest that QE and the

QE+LNG combination are very effective at suppressing reproductive capacity by disrupting both male and female reproductive physiology. The evidence supports their potential use as hormonal fertility-control agents for managing rodent populations (Massawe *et al.*, 2017).

2.4 Implications for humane rodent population control

Controlling pest rodents through fertility suppression is an emerging strategy as an alternative to lethal poisons. Fertility control is often hailed as a humane, ecologically safe pest management tool. A study noted that reducing rodent reproduction can lower populations while avoiding the environmental hazards of rodenticides to non-target wildlife, children, or the environment (Massei *et al*., 2023). Recent field and lab trials suggest that if broadly adopted and integrated into pest management strategies, hormonal contraception could effectively suppress rodent populations while preserving biodiversity and ecosystem services (Nakayama *et.al,* 2019). Thus, fertility control emerges as a viable, ethical, and ecologically sound tool for long-term rodent population management. Combined hormonal bait (EE-LNG) could be formulated similarly to a rodent contraceptive bait, designed to be orally palatable and to safely reach urban rat or mouse populations. If effective, such a method would reduce the production rate of the rats and mice, which would slow population growth without animal killing or toxic residues.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Ethical Considerations

This research study adhered to the ethical guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan (UMK), under approval No. **UMK/FPV/IACUC/AUP-R001/2025 (As shown in Appendix 1).**

3.2 Experimental design and treatment

The study was conducted using adult male Sprague-Dawley rats (n=18; 195 to 220 g, 8 weeks old), divided into three groups (n=6 per group), which are the control (no hormone in feed), low-dose (LD), and high-dose (HD) groups, respectively. These rats were housed individually in standard rat laboratory cages at the Animal Research Facility of the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, with controlled temperature (23–24°C), with a 12-hour light-dark cycle. All experimental rats were acclimatised for 14 days and provided with food and water *ad libitum*. The treatment period was conducted for 14 days in total, where the rats were fed with two different types of baits, each for seven days, with daily consumption measured in grams per kilogram of body weight per day (g/kg/day). However, the control group was fed with bait without hormones, and the low-dose treatment group was fed with bait containing 0.32 mg of combined hormones (0.28 mg LNG + 0.04 mg EE). The high-dose treatment group was fed with bait containing 1.08 mg of combined hormones (0.9 mg LNG + 0.18 mg EE). We did not provide other food types or sources during the treatment period, but we provided all groups with water *ad libitum.*

3.3 Bait Formulation

We formulated two different baits to represent sweet and savoury types of food-bait. For bait A (the savoury), we produced 21 units each weighing 50 g using the following ingredients: 200 mL of water, 25 mL of cooking oil, 250 g of anchovies, and 250 g of maize (corn) starch powder. We prepared the bait by adding 200 ml of water and 25 ml of oil to a cooker, then gently boiled it to 100 °C. After reaching the desired temperature, we added the 250 g of anchovies and simmered the mixture for five minutes. Next, we reduced the heat and mixed 100 g of maize starch in a separate cup with a small quantity of water, gradually adding it into the cooker while stirring to avoid lumps. This mixture was cooked for 15 minutes to form a cohesive paste. Once the paste was formed, we added another 150 g of maize starch powder, mixing continuously until we obtained a smooth consistency. We then allowed the paste to cool to a comfortable hand-warm temperature, and incorporated the dissolved hormone powder, mixing vigorously for one to three minutes and scraping the sides of the bowl to ensure uniformity. Finally, we portioned the paste into 50 g peanut moulds, gently demolded them, and arranged them on a tray to set at room temperature as shown in Figure 1. We then incubated the baits to let them dry. We repeated the same procedure to produce the required quantity of the bait, along with the control for the experiments.

Figure 1: Showing Bait A made up of anchovies, starch and ethinyl estradiol-levonorgestrel contraceptives

For bait B (the sweet food), we used sliced bread (Gardenia®, Malaysia) and Coconut Jam spread (Kaya) (Gardenia®, Malaysia). Each slice of bread weighed 15 g, and we formulated the bait by forming a jam-hormone mixture. Using a mortar, we combined the calculated volume of hormones and mixed them until fully dissolved. We then spread the jam-hormone mixture evenly over the surface of each 15g bread slice. After allowing two to five minutes for surface tack to diminish, we then used the 17.5g bait immediately, as shown in Figure 2. We prepared an identical bread unit containing jam without hormones for the control group.

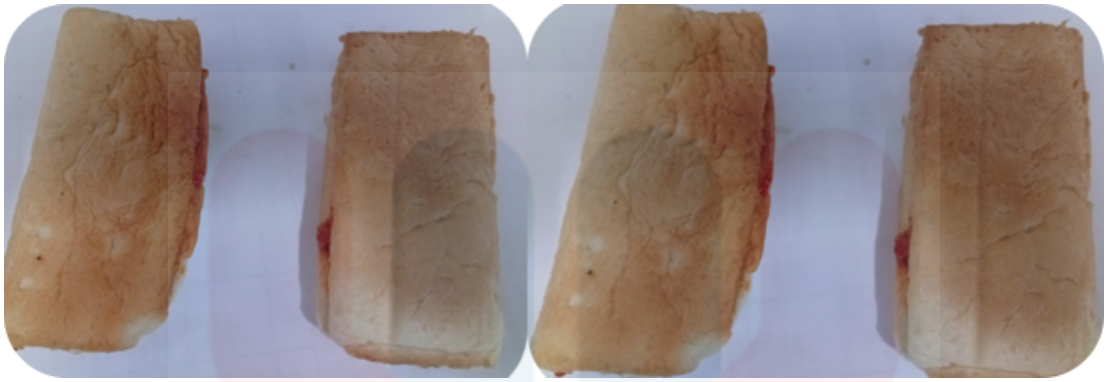

Figure 2: Showing Bait B made up of Gardenia® Bread, Kaya Spread, and ethinyl estradiol-levonorgestrel (EE-LNG) contraceptives

3.4. Blood and Tissue Sampling

On day 14, the rats were fasted overnight in preparation for blood sampling on day 15. Blood samples were collected through rapid decapitation as described by Farhan *et al*., (2021). Trunk and cardiac blood were collected immediately into a plain tube and EDTA tube for serum biochemistry, serum testosterone analysis, and complete blood count. We collected approximately 4 to 6 mL of blood per rat. Blood samples in both type of tubes were then taken to the clinical pathology laboratory at the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in an ice-packed flask. Serum was extracted through centrifugation at 4000g for 15 min and stored at -20 °C for hormonal analysis. Testes, liver, and kidney tissues were collected for histopathological and morphometric measurements (Su *et al*., 2019; Opuwari & Monsees, 2020).

We chose decapitation followed by trunk and cardiac blood collection to avoid anaesthetic-related endocrine artefacts and minimise pre-sampling delays for hormone endpoints. Fasting status (12 hr), housing, weight, and age were standardised across the group.

3.6 Testosterone analysis

Serum was extracted by centrifuging the blood at 4000 g for 15 min using an ELISA kit (Elabscience Biotechnology, Inc., USA) according to the manufacturer's instructions. Before initiating the assay, all reagents were equilibrated at room temperature and mixed gently by vortexing. The reagents were mixed and labelled accordingly, and the samples were labelled based on the animal ID. For the testosterone assay, a standard was prepared with concentrations of 100, 50, 25, 6.25, 3.13, 1.57, and 0 ng/ml. The testosterone assay kit demonstrated high specificity, with a sensitivity of 0.64 ng/ml and a detection range of 1.56-100 ng/mL.

3.7. Histopathological analysis

Following euthanasia, the collected tissues were analysed for histopathologic changes using H&E staining. The collected tissues (testes, liver, and kidney) were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. Sections measuring 5 μm were prepared using the automatic microtome and stained with Haematoxylin & Eosin. The stained slides were then analysed using the digital slide scanner and slide viewer software (Case Viewer, 3DHISTEC).

3.8 Apoptotic analysis

The paraffin-embedded tissues were deparaffinized by washing them in xylene, followed by dehydration through different concentrations of ethanol (100%, 95% and 90%) for 10 minutes each. The slides were then rinsed in phosphate-buffered saline (PBS). The tissue slides were stained with acridine orange and propidium iodide (AO/PI) to evaluate cell viability and apoptosis. The sections were incubated with a 10 μg/mL solution of AO (Elab Science) in PBS at room temperature. AO is a permeable dye that stains viable cells with green fluorescence, while

apoptotic cells show orange-red fluorescence. After AO incubation, we washed the tissue sections with PBS and then incubated with 5 µg/mL of PI (Elab Science) for 5 minutes. After staining, the slides were mounted with a coverslip and examined under a fluorescence microscope (Mikail *et al*., 2024).

3.9. Statistical analysis

The statistical analysis was performed using GraphPad Prism. We analysed the weight of the testes and epididymis using the Kruskal-Wallis H test, which is a non-parametric test for comparing the median of three independent groups. For pairwise comparison, we used the Dunn post hoc test. For the analysis of the serum testosterone concentration, we analysed the data using one-way analysis of variance (ANOVA) to compare testosterone concentrations across the treatment groups: Control, Low dose, and High dose. To determine pairwise differences, we used Tukey's post hoc test.

CHAPTER 4

RESULTS

4.1 Testis and Epididymis

The weights of the testes and epididymis were analysed using the Kruskal–Wallis H test due to the violation of normality in the high-dose group. The overall Kruskal–Wallis test was not statistically significant ($H = 8.747$, $p = 0.086$), indicating that the differences among groups did not reach significance. Despite this, Dunn's post hoc test was performed to explore pairwise differences. The post hoc analysis showed a significant difference between the control and high-dose groups ($p = 0.0094$), while no significant differences were observed between control vs. low-dose or low-dose vs. high-dose groups, as summarised in the Table 1 and Table 2.

Table 1: Showing the Mean±SD of the testes and epididymis weight (g) of the male rats for the control, low-dose and high-dose treatment groups (n=18)

Group (n=18)	Weight of Testes and Epididymis (Mean±SD)
Control (n=6)	3.50±1.05
Low-Dose (n=6)	2.33±1.03
High-Dose (n=6)	1.50±0.55

Table 2: Showing Dunn's multiple comparison test of the weight of the testes and epididymis across the group.

Dunn's multiple comparison test	Mean Diff.	Z	Summary	Adjusted P-Value
Control vs LD group	4.750	1.604	ns	0.3264
Control vs HD group	8.750	2.954	***	0.0094
LD group vs HD group	4.000	1.350	ns	0.5307

Note: * indicates statistical significance.**

4.2 Serum Testosterone Concentration

The mean testosterone levels decrease across the treatment from the control group (6.89 ng/mL) to the low dose (4.24 ng/mL), with the lowest level in the high-dose group (1.17 ng/mL), as shown in Table 3 and Figure 4.

Table 3: Showing the mean testosterone across the groups. Data are presented as mean, SD, and SEM.

Group	n	Mean (ng/mL)	SD	SEM
Control	6	6.89	0.66	0.27
Low Dose	6	4.24	0.78	0.32
High Dose	6	1.17	0.53	0.22

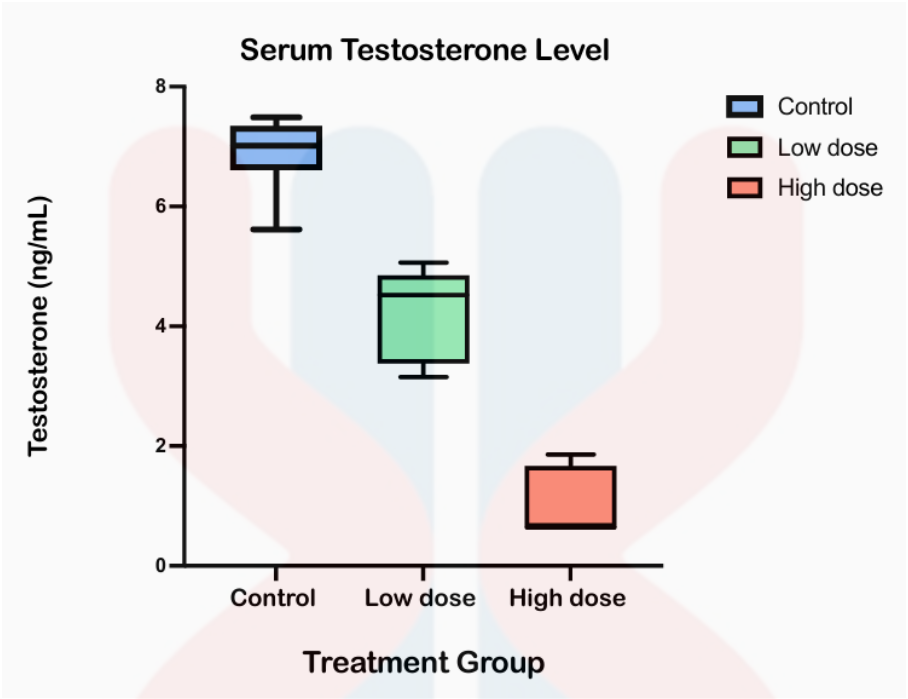

Figure 3. Mean ± SD serum testosterone levels (ng/mL) in control, low-dose, and high-dose groups (n = 6 per group). A significant dose-dependent reduction was observed (one-way ANOVA, $p < 0.001$; Tukey's post hoc, $p < 0.001$).

The one-way ANOVA revealed that EE-LNG treatment had a statistically significant effect on testosterone levels, $F(2, 15) = 114.9$, $p < 0.00000001$ as summarised in Table 4. Tukey's post hoc test revealed significant differences among all pairwise group comparisons ($p < 0.001$), indicating that each treatment dose produced a distinct reduction in testosterone. Effect size analysis showed that the treatment accounted for 93.9% of the variance in testosterone levels ($R^2 = 0.9387$), demonstrating a strong dose-related effect of EE-LNG bait.

Table 4: Summary of the one-way ANOVA that compares the mean of the testosterone (ng/mL) between the parameters.

Source	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	104.4	2	52.21	F (2, 15) = 114.9	P < 0.0001
Residual (within column)	6.817	15	0.4544		
Total	111.2	17			

Abbreviations: SS = Sum of Squares; DF = Degrees of Freedom; MS = Mean Square; F = F-statistic; P = Probability value; ng/mL = nanograms per millilitre.

Table 5: Showing Tukey multiple comparison tests of the serum testosterone across the group.

Tukey's comparison test	Multiple means differ.	SE of Diff.	Below Threshold	Summary	Adjusted P-Value
Control vs LD	2.648	0.3892	YES	***	P < 0.0001
Control vs HD	5.890	0.3892	YES	***	P < 0.0001
LD vs HD	3.242	0.3892	YES	***	P < 0.0001

Note: * indicates statistical significance**

4.3 Histopathological analysis: H&E staining

The histopathological analysis of the testes and epididymis showed significant changes across the groups of treatment groups. For the control group, overall testes showed a normal architecture of the testis with regular seminiferous tubules (ST) and showed normal interstitial tissue structures where Leydig cells can be observed. The tubules also showed narrow lumens filled with mature spermatozoa. ST was lined with germinal epithelium that contains spermatogenic cells at different stages of sperm development. Sertoli cells were observed to be adhered to a continuous basement membrane with spermatogonia (SG) along with primary spermatocytes (PS).

In the low-dose treatment group, the testes showed irregular seminiferous tubules (ST) separated by interstitial oedema (IE). Some degree of congested blood vessels can also be observed in the interstitial region of the testes. While the same findings can be observed in the high-dose treatment group, we can also observe the sloughing of the basal membrane into the ST lumen, where germ cells can be appreciated in the lumen.

For the kidneys, both dose groups showed moderate glomerular atrophy and interstitial congestion. Inflammatory cells infiltrate around the kidney tubules and show tubular degeneration.

In the liver, the control group showed normal liver architecture with hepatocytes radiating from the central vein; sinusoidal spaces and the portal triad were intact. While in the low- and high-dose treatment groups, there is mild cellular infiltration, with vacuolations suggestive of hydropic degeneration.

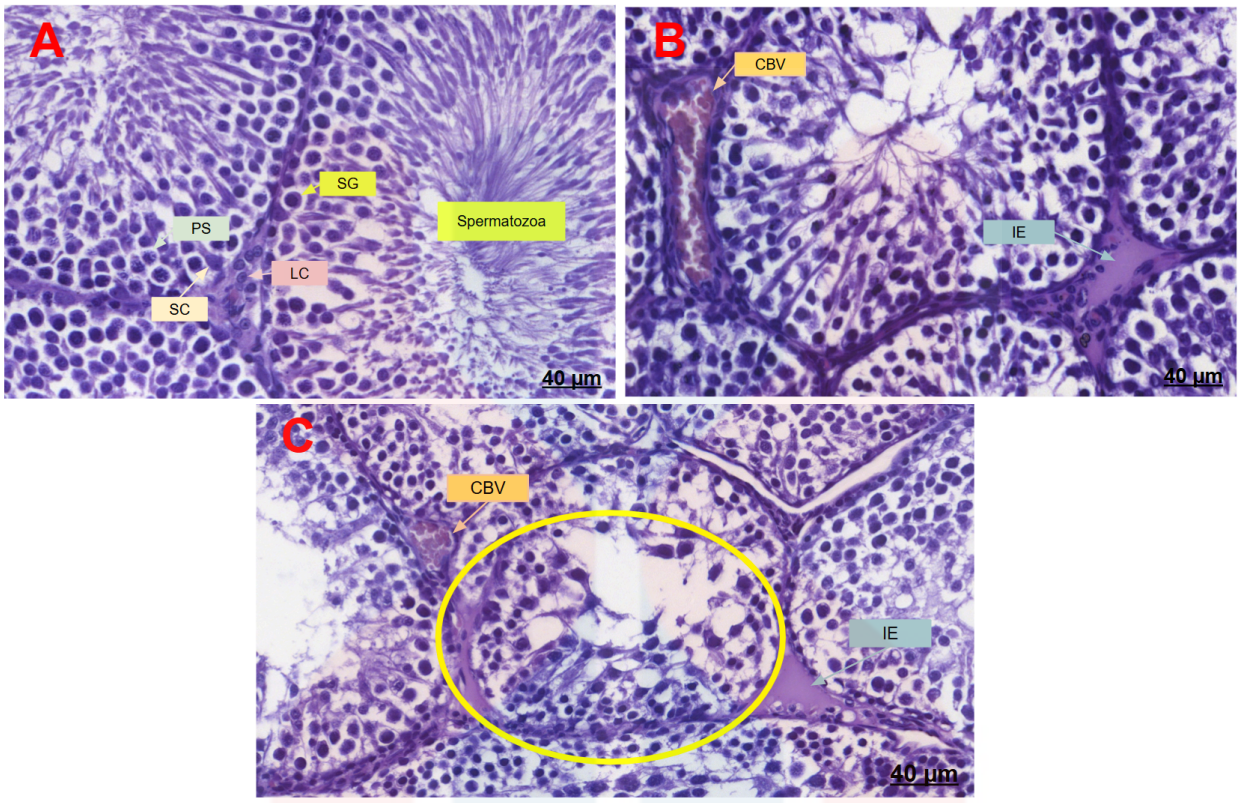

Figure 4. Representative photomicrographs of testicular sections stained with Hematoxylin and Eosin (H&E) from the control (A), low-dose (B), and high-dose (C) groups. The control group (A) shows normal testicular architecture with well-organised seminiferous tubules and complete spermatogenic cell layers. The low-dose group (B) demonstrates interstitial oedema and vascular congestion within the interstitial spaces. The high-dose group (C) exhibits more pronounced histopathological alterations, including disruption of seminiferous tubule architecture and sloughing of germinal epithelium into the lumen. Scale bar = 40 µm.

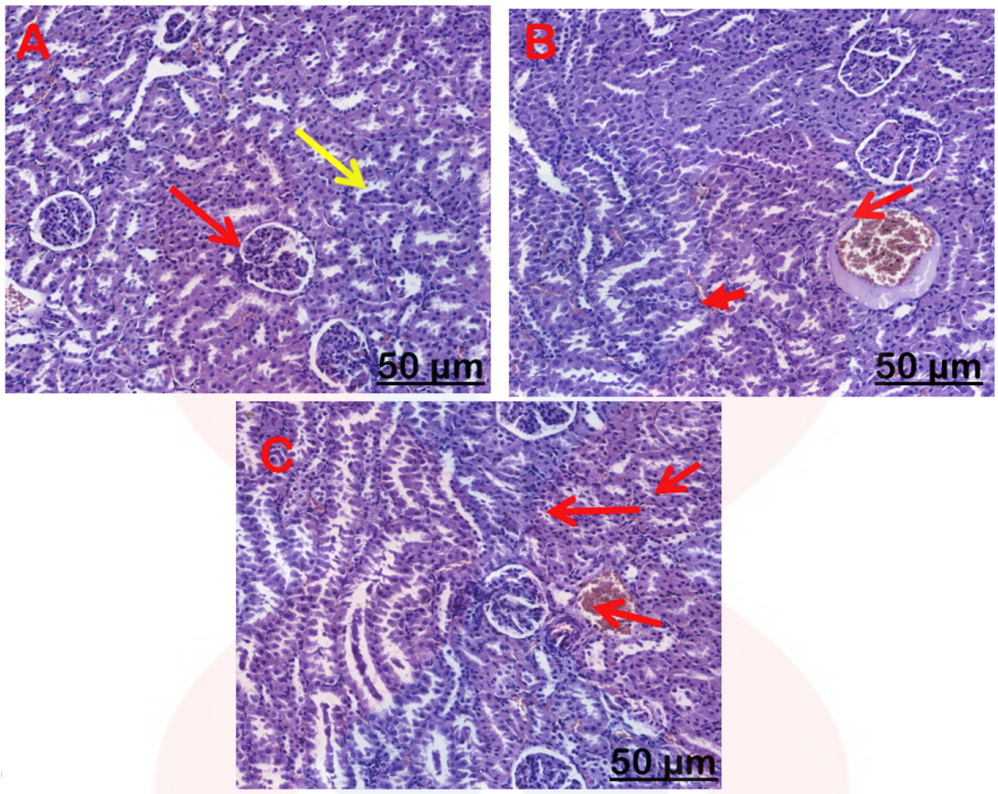

Figure 5: Showing representative histopathological analysis of kidney sections stained with Hematoxylin & Eosin (H & E), the control, low-dose and high-dose groups

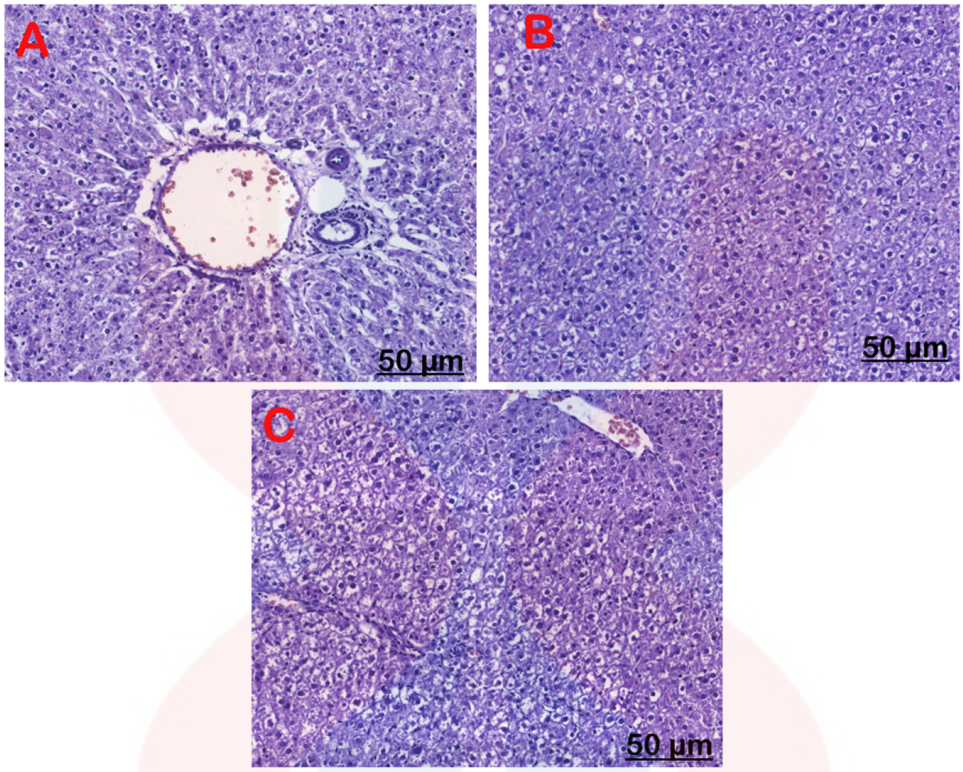

Figure 6: Showing representative histopathological analysis of liver sections stained with Hematoxylin & Eosin (H & E) in the control, low-dose and high-dose groups.

4.4 Nuclear Morphological Analysis (DAPI staining)

DAPI staining was used to evaluate the nuclear integrity across the treatment groups. In the testes of the control group, the nuclei of spermatogonia, spermatocytes, spermatids and Sertoli cells were evident with organised layers from the basal to the luminal areas, which indicates active and healthy spermatogenesis. In the low-dose group, there was a slight reduction in nuclear density in the luminal region, indicating early germ-cell depletion with no evident chromatin condensation, but the nuclear dispersion suggests mild nuclear stress. In contrast, testes from the high-dose group showed prominently irregular and fragmented nuclei, which are

suggestive of apoptosis. These results showed a dose-dependent fragmentation of germ-cell nuclei, suggesting the cytotoxic effects of the EE + LNG bait on the testicular nuclei.

In the control group liver, the nuclei were round, suggestive of preserved hepatocellular structure and healthy nuclear morphology. In the low-dose liver group, there was a mild nuclear dispersion suggestive of nuclear stress. In the high-dose liver group, there was a fragmented nucleus, which is suggestive of nuclear damage. The changes in nuclear patterns suggest a dose-dependent hepatotoxic effect of the treatment.

For the kidney tissue, the control group showed uniformly round DAPI-positive nuclei with regular morphology. In the low-dose group, the nuclei showed signs of condensation, suggestive of mild nuclear stress. However, the high-dose group showed an increased presence of condensed and fragmented nuclei. These results suggest that nuclear damage is probably a result of dose-dependent toxicity.

Table 6: Summary of the nuclei morphology of the testes, kidney, and liver tissues

Group	Nuclear Morphology	DAPI Intensity	Chromatin Condensation
Control	Regular, Round Nuclei	Strong & Uniform	Absent
Low-Dose	Slight Condensation	Slightly Reduced	Mild
High-Dose	Fragmented	Reduced	Present

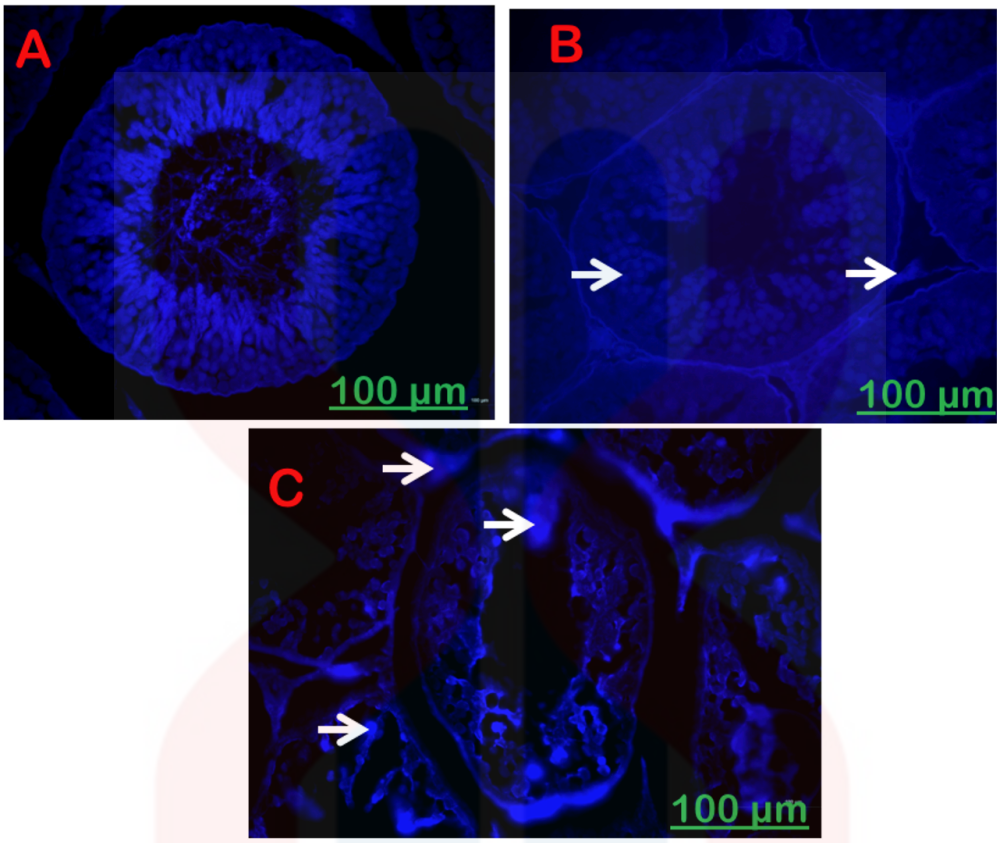

Figure 7. Representative fluorescence photomicrographs of testicular sections stained with DAPI in the control (A), low-dose (B), and high-dose (C) groups. The control group (A) shows well-organised layers of spermatogenic cells extending from the basal membrane toward the lumen, indicating normal spermatogenesis. The low-dose group (B) demonstrates a mild reduction in nuclear density, particularly within the luminal region. The high-dose group (C) exhibits irregular seminiferous tubule architecture with fragmented and condensed nuclei suggestive of apoptotic changes. Scale bar = 100 µm.

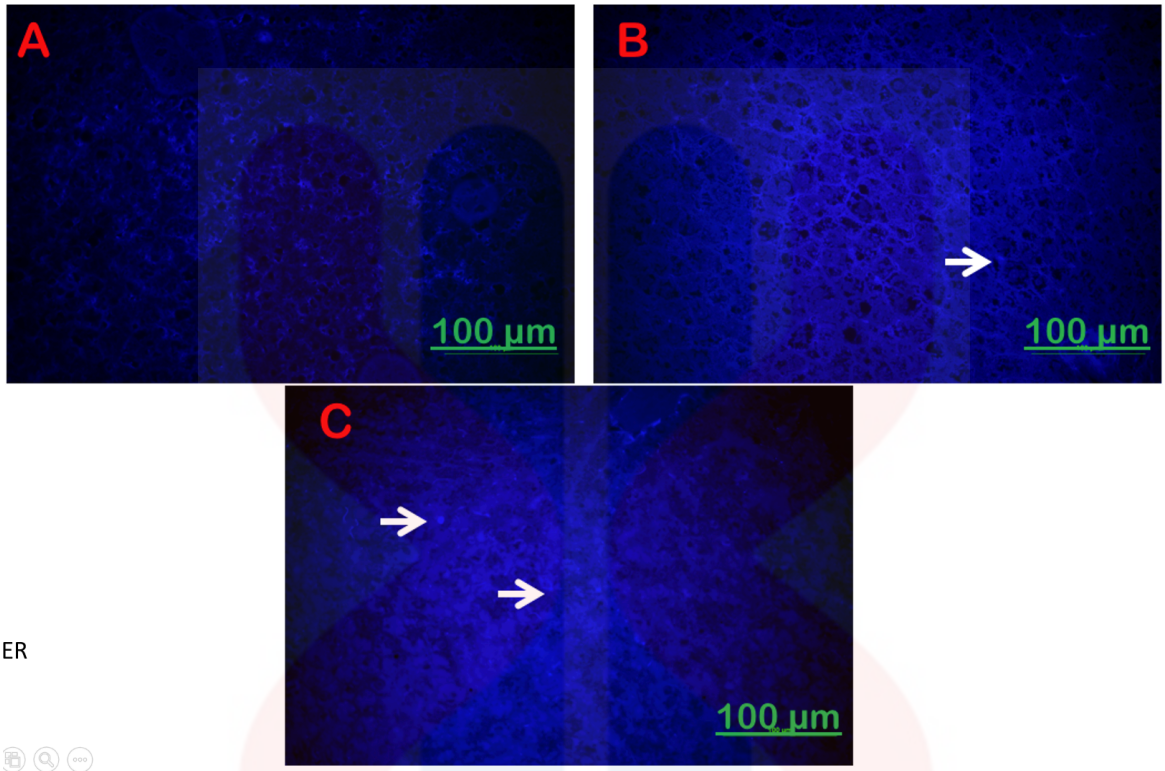

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Figure 8. Representative fluorescence photomicrographs of liver sections stained with DAPI in the control (A), low-dose (B), and high-dose (C) groups. The control group (A) shows preserved hepatocellular architecture with normal, uniformly stained nuclei indicating healthy nuclear morphology. The low-dose group (B) demonstrates mild nuclear dispersion suggestive of early nuclear stress. The high-dose group (C) exhibits marked nuclear fragmentation and irregular nuclear morphology consistent with nuclear damage. Scale bar = 100 µm.

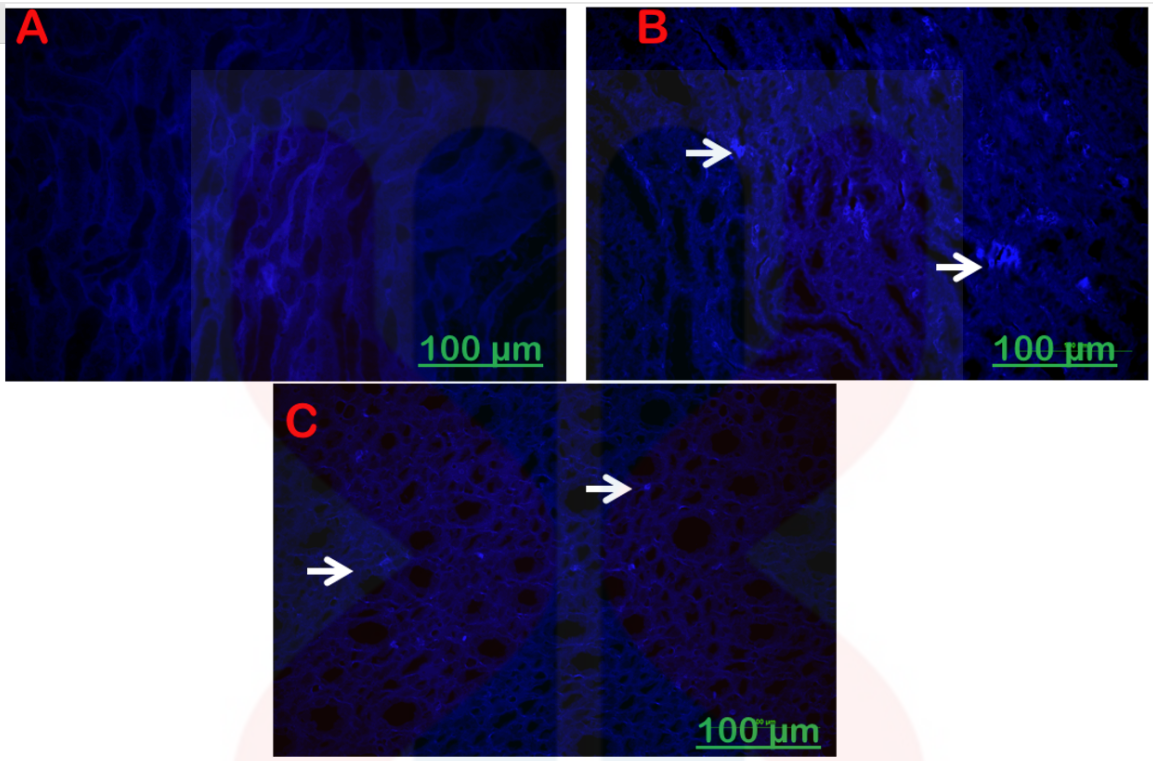

Figure 9. Representative fluorescence photomicrographs of kidney sections stained with DAPI in the control (A), low-dose (B), and high-dose (C) groups. The control group (A) shows uniformly round, DAPI-positive nuclei with regular morphology, indicating normal renal cellular architecture. The low-dose group (B) demonstrates nuclear condensation suggestive of mild nuclear stress. The high-dose group (C) exhibits an increased presence of condensed and fragmented nuclei, indicative of nuclear damage. Scale bar = 100 µm.

4.5 Cell Viability Analysis (Acridine orange (AO) and propidium iodide (PI) staining)

The AO/PI staining of testis sections showed a dose-dependent testicular cytotoxicity. The control group revealed abundant green fluorescence suggestive of viability and normal spermatogenesis. The low-dose group revealed the presence of apoptotic cells with intact tissue architecture. while the high-dose group showed extensive apoptosis and necrosis within the

seminiferous tubules. The observed germ-cell loss suggests impaired testicular function in the high-dose treatment group.

For the liver tissue, the control group showed uniform green fluorescence with minimal apoptosis. In the low-dose group, there is a moderate increase in apoptotic hepatocytes, while the high-dose group showed extensive hepatocyte apoptosis and necrosis. The loss of viable cells suggests liver injury at high doses.

The control kidney sections exhibited predominantly viable cells without indications of necrosis; conversely, the low-dose group displayed both viable cells and a moderate increase in apoptotic cells. The low-dose group displayed yellow/orange fluorescence, indicating acute or sub-lethal toxicity. In contrast, the high dose showed extensive apoptosis and necrosis (red fluorescence) with loss of viable cells, suggestive of apoptosis due to high-dose exposure.

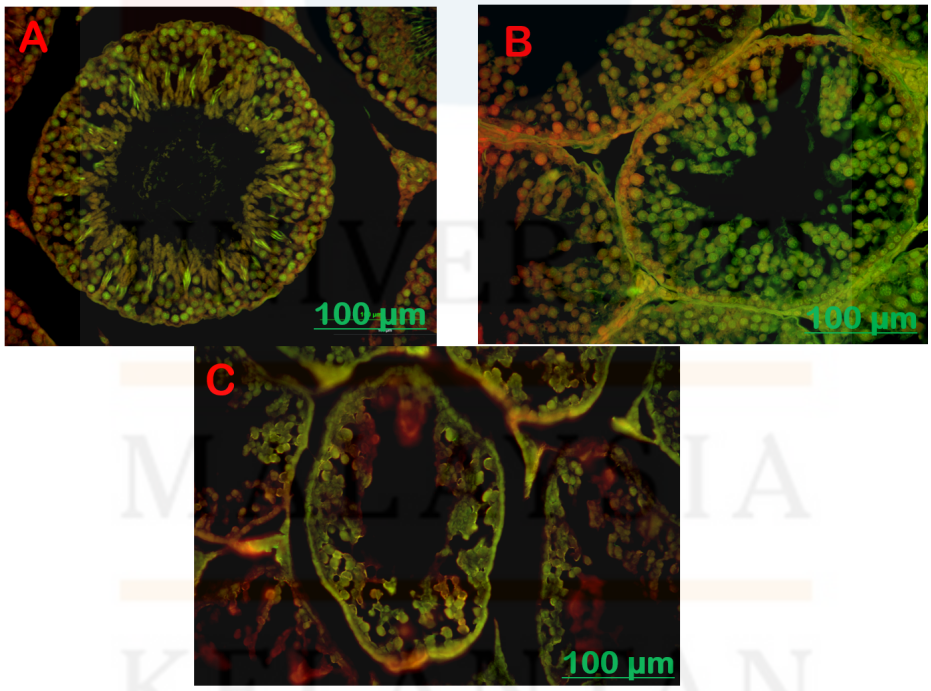

Figure 10. Representative fluorescence photomicrographs of testicular sections stained with Acridine Orange/Propidium Iodide (AO/PI) in the control (A), low-dose (B), and high-dose

(C) groups. The control group (A) demonstrates high cellular viability with well-defined seminiferous tubules and predominantly green fluorescence, with only a few yellow/orange apoptotic cells, consistent with normal spermatogenesis. The low-dose group (B) shows mild apoptotic activity indicated by an increased presence of yellow/orange fluorescent cells. The high-dose group (C) exhibits extensive cell death with pronounced germ-cell apoptosis and increased red/orange fluorescence, indicating severe testicular toxicity. Scale bar = 100 µm.

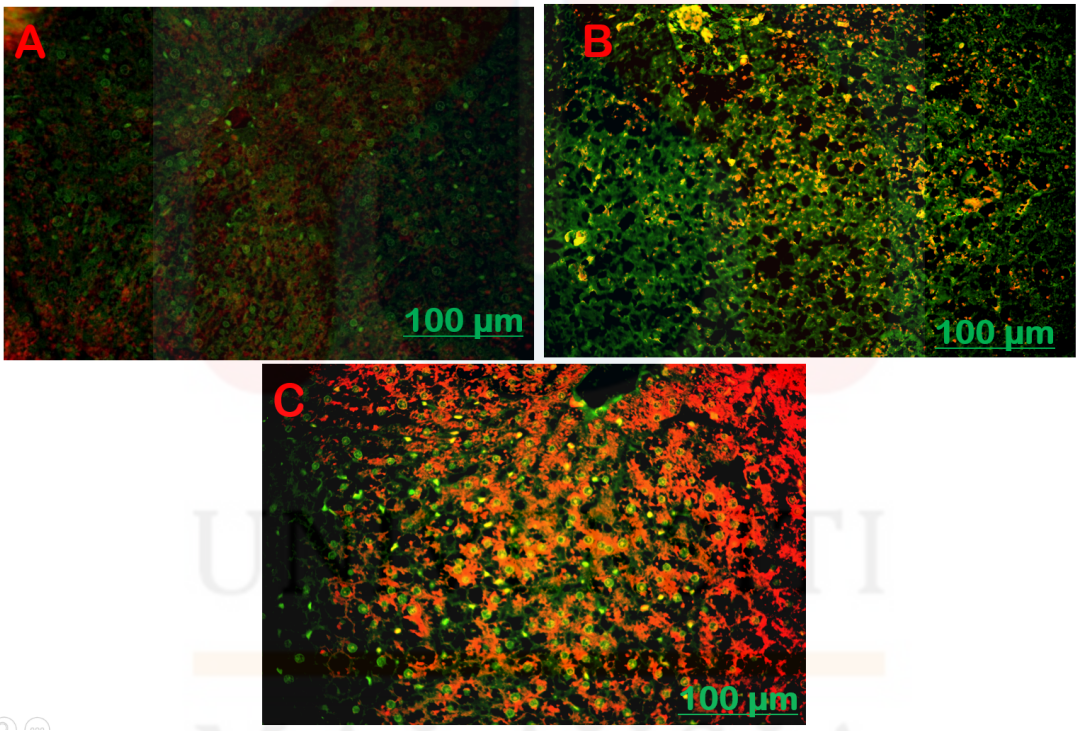

Figure 11. Representative fluorescence photomicrographs of liver sections stained with Acridine Orange/Propidium Iodide (AO/PI) in the control (A), low-dose (B), and high-dose (C) groups. The control group (A) shows predominantly viable hepatocytes with strong green fluorescence and minimal red/yellow fluorescent cells, indicating negligible apoptosis. The low-dose group (B) demonstrates focal areas of orange/yellow fluorescence suggestive

of early apoptotic changes. The high-dose group (C) exhibits a marked reduction in viable cells with increased apoptotic and necrotic cells, characterised by dominant orange and bright red fluorescence, consistent with severe hepatocellular toxicity. Scale bar = 100 µm.

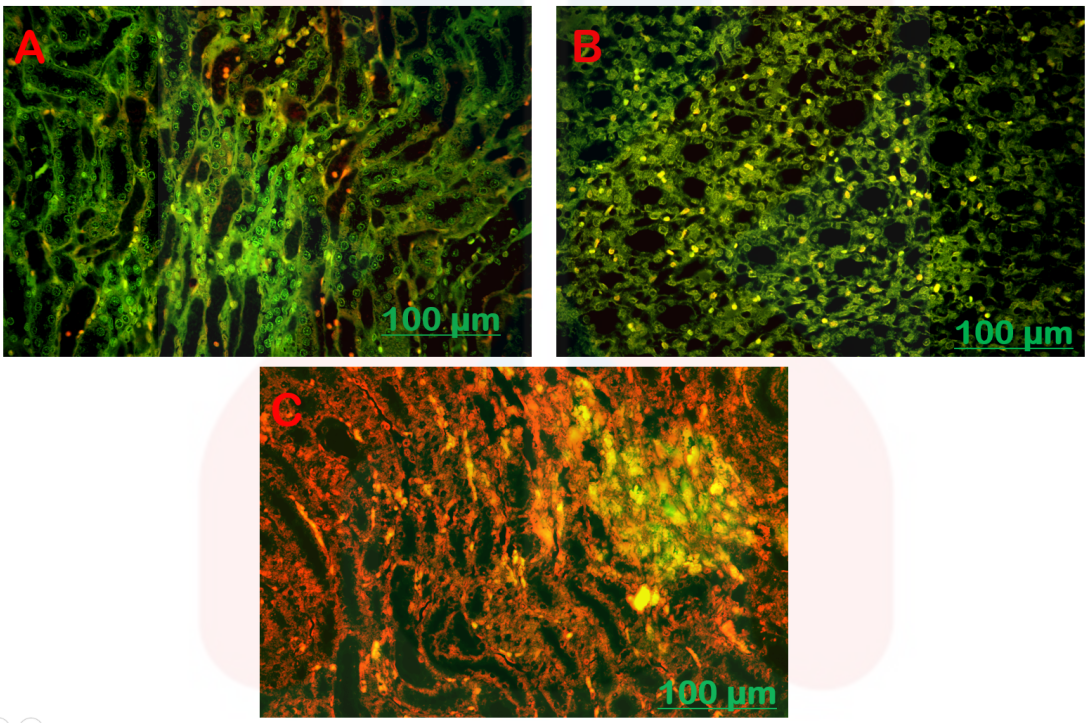

Figure 12. Representative fluorescence photomicrographs of kidney sections stained with Acridine Orange/Propidium Iodide (AO/PI) in the control (A), low-dose (B), and high-dose (C) groups. The control group (A) displays predominantly viable renal cells with strong green fluorescence and only occasional red/yellow fluorescent cells, indicating good baseline renal cell viability. The low-dose group (B) shows focal areas of orange/yellow fluorescence with minimal red-only cells, suggestive of early apoptosis or sub-lethal toxicity. The high-dose group (C) demonstrates substantial loss of viable cells with dominant apoptotic cells and bright red necrotic cells, consistent with severe renal toxicity. Scale bar = 100 µm.

CHAPTER 5

DISCUSSIONS

The results of this study provide mechanistic evidence that the consumption of EE–LNG bait induces germ-cell apoptosis in the testes of male rats. EE–LNG bait, originally formulated for female macaques, exerts significant anti-androgenic effects when consumed by male rats. Within the 14-day feeding period, we observed suppressed testosterone levels, degenerative testicular histology, and germ-cell apoptosis, particularly in the high-dose treatment group.

Our research demonstrates a dose-dependent reduction in serum testosterone levels in male rats subsequent to the ingestion of EE–LNG bait. The marked reduction in testosterone confirms that exogenous EE–LNG effectively downregulates gonadal androgen production, likely via HPG-axis feedback inhibition of gonadotropin release. Low testosterone results in the regression of androgen-dependent tissues, such as the testes and epididymis. Our findings agree with previous studies reporting where it was mentioned that short-term exposure to contraceptive hormone compounds can induce reductions in male reproductive organ weights. For example, Massawe et al. (2018) fed multimammate rats (Mastomys natalensis) bait laced with quinestrol and levonorgestrel (EP-1) for 7 days and subsequently measured testicular, epididymal, and seminal vesicle weights alongside sperm parameters, finding significantly lower reproductive organ masses and impaired sperm quality compared to controls. Similarly, Stuart et al. (2022) administered combinations of these hormones by bait and/or gavage to rice field rats (Rattus argentiventer) for 7 days and found significant reductions in epididymal and seminal vesicle weights and reduced sperm counts and motility.. In our study, the high-dose group showed a notably lower combined weight of testes and epididymis (1.50 ± 0.55 g) compared to the control (3.50 ± 1.05 g) and low-dose (2.33 ± 1.03 g) groups, indicating atrophy.

The significant reduction in serum testosterone observed in both low- and high-dose groups is consistent with the known negative feedback imposed by exogenous estrogen and progestins on the HPG axis (Chimento *et al*., 2014; Sengupta *et al*., 2019). Furthermore, suppression of LH release impairs Leydig cell function, resulting in diminished intratesticular testosterone (Lei *et al*., 2025; Ramaswamy & Weinbauer, 2014), which is essential for maintaining spermatogenesis and Sertoli–germ-cell adhesion (Grande *et al*., 2022; Li *et al*., 2024; Walker, 2011). The histopathological lesions noted in the seminiferous tubules—including germ-cell degeneration and apoptosis, as revealed by AO/PI—align with previous studies on endocrine disruption (Corpuz-Hilsabeck *et al.*, 2023; Campion *et al*., 2012).

Previous studies have reported estrogen-induced apoptosis in male reproductive tissues and levonorgestrel's role in accelerating sperm suppression has been documented in human trials (Lu *et al*., 2009). However, this study is the first, to our knowledge, to apply AO/PI staining in vivo to examine testicular apoptosis following exposure to a clinically relevant EE–LNG combination. Unlike EP-1 contraceptive formulations used in rodent control, this formulation reflects widely prescribed female hormonal contraceptives. Thus, our model may have broader implications for environmental or occupational exposure to synthetic estrogens and progestins in males.

The observed increase in apoptotic cells validates the hypothesis that programmed-cell-death mechanisms, at least partially, mediate germ-cell loss under EE–LNG exposure. While the AO/PI technique does not differentiate the exact apoptotic pathways, previous research suggests involvement in Fas-FasL signalling (Wang & Su, 2018; Xiong *et al.*, 2009), oxidative stress, and mitochondrial dysfunction (Huang *et al.*, 2019). Future studies

should confirm these pathways using molecular markers such as caspase-3 activation, Bax/Bcl-2 ratio, and direct quantification of reactive oxygen species.

Importantly, our findings suggest that EE-LNG bait consumption in male rats causes androgen deprivation sufficient to trigger widespread germ-cell apoptosis, leading to testicular dysfunction. These effects may mimic mechanisms underlying certain idiopathic infertility conditions where endocrine disruptors are implicated (Amir *et al*., 2021; Sikka & Wang 2008). Furthermore, this work offers a toxicological model for testing the effects of combined hormonal exposures on male reproductive health.

Moreover, the discernible apoptotic cells in the testes serve as cellular evidence of tissue regression. It appears that EE-LNG bait not only arrests sperm production but also actively induces the death of germ cells. This agrees with previous studies, which show that Quinestrol induces apoptosis of spermatogenic cells in mice (Liu *et al.*, 2014) and EE-LNG induces apoptosis in Macaque ovarian cells (Mikail *et al.*, 2024) and in ovarian tissues (unpublished).

It is essential to note that the EE-LNG bait in the low-dose group also causes testosterone suppression and slight histopathologic changes, suggesting that the effective dose for female macaques is also applicable to male rats. This indicates that the EE-LNG bait may serve as a universal and multispecies contraceptive. Furthermore, it suggests that a low dose of EE-LNG can inhibit the endocrine axis in male rats. However, the high dose had a pronounced effect on fertility parameters, which is consistent with dose-dependent findings from similar studies involving EP-1 (Stuart *et al*., 2022). In our studies, the 14-day bait consumption produced a pronounced effect, whereas the low dose allowed partial recovery or incomplete suppression.

The histopathological lesion observed indicates that prolonged consumption of the bait could lead to lasting and/or irreversible infertility in male rats. Nevertheless, further studies are

needed to determine the recovery of testosterone levels and spermatogenesis. Previous studies in humans revealed that fertility is reversible after cessation (Piotrowska *et al*., 2017). However, the high-dose treatment might carry some risk of permanent infertility, although in the context of pest control, it is desirable.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

This study shows that male rats exposed to EE-LNG contraceptive bait experience significant reproductive toxicity. It only required fourteen days of exposure to significantly lower testicular and epididymal weights, causing significant germ-cell apoptosis, and suppressed serum testosterone. Histopathological evidence of tissue degeneration in the testes and AO/PI and DAPI staining, which confirmed nuclear condensation and DNA fragmentation, consistently supported these findings. Strong mechanistic evidence that exogenous synthetic steroid hormones disrupt spermatogenesis by affecting endocrine regulation and stimulating apoptotic pathways is provided by the combined biochemical, cellular, and morphological findings.

Based on the findings, more investigation is recommended to improve knowledge of EE-LNG toxicity and assess its broader implications. To determine whether the observed changes are reversible, future research should evaluate longer exposure times, a wider range of dosages, and the potential for reversibility following treatment discontinuation for it to meet ideal contraceptive agent characteristics. This study is laboratory-based; therefore, further study is recommended to validate the reproductive efficacy of these baits. This validation can be achieved through functional fertility tests, such as the mating trials conducted in a laboratory setting or field studies trial design to observe an increase or decrease in litter size. Additionally, to identify the precise pathways involved, more molecular studies are encouraged, such as assessing hormone regulators and apoptotic markers. Lastly, research on non-target species, ecological risks, and bait safety is advised to support well-informed decisions regarding the potential use of EE-LNG bait in wildlife population management, given the potential environmental relevance of contraceptive hormone exposure.

APPENDIX

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FAKULTI PERUBATAN VETERINAR
Faculty of Veterinary Medicine

Ruj. Kami (*Our Ref.*) : UMK.A06.800-1/2/8 (1)
Tarikh (*Date*) : 4 JUNE 2025

DR. MUHAMMED MIKAIL
Principal Investigator
Faculty of Veterinary Medicine
University Malaysia Kelantan

Dear Dr.,

APPROVAL OF INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) TO CONDUCT RESEARCH INVOLVING ANIMALS

We are pleased to inform you that your application for approval to conduct research from Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, University Malaysia Kelantan has approved. Please refer the table below for approval code:

Approval Code	***UMK/FPV/ACUE/RES/001/2025***
Title	**Understanding the Effect of Combined Hormonal Bait on Male Reproductive Hormone and Tissue: A Rat Model**
Duration of Research	**June 2025 – May 2026 (1 Years)**
Number of Sample/Strain/Gender	**32/ Sprague Dowley Rat/ Male**

You are advised to always follow "3R" (REDUCE, REFINE, & REPLACE) and all animal ethics and animal welfare principles to reduce suffering in animal.

Thank you.

"BERKHIDMAT UNTUK NEGARA"

Yours sincerely,

(DR. MOHAMMED DAUDA GONI)
Chairman
Institutional Animal Care and Use Committee
Faculty of Veterinary Medicine
University Malaysia Kelantan

ENTREPRENEURSHIP IS OUR THRUST

Appendix A: IACUC Approval to Conduct Research

Appendix B: Individual cages with label to differentiate between groups

Appendix C: Drying of Bait A in incubator

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